Recent Advances in Pharmaceutical Sciences II

Editors
Diego Muñoz-Torrero
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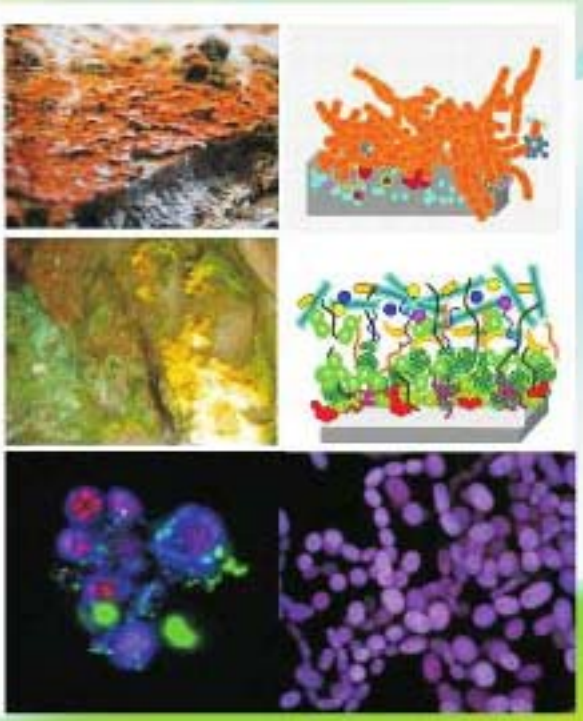
Joan Vallès



TRANSWORLD RESEARCH NETWORK

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Preface

Pharmaceutical sciences are more and more conducted in a multidisciplinary fashion, seeking to foster the integration of a broad range of areas of knowledge that focus on all facets of drugs and therapies, identification and control of organisms causing human disease, food sciences, as well as public and environmental health, with the ultimate goal of improving society's health and quality of life. To expedite the achievement of these benefits, pharmaceutical sciences have to confront current major economic and social challenges such as decreased resources for research, increased regulatory stringencies, overly costly and increasingly more complex drug development process, or ageing populations with chronic diseases, through the implementation of cutting-edge innovative research approaches.

This E-book describes some innovative integrated research spanning the entire spectrum of scientific disciplines of pharmaceutical sciences led by internationally recognized research groups of the Faculty of Pharmacy of the University of Barcelona, which continues to maintain a leadership position among the different faculties of the consistently ranked first research university of Spain. The E-book consists of 12 chapters, which encompass contributions in areas such as botany, pharmacology, plant physiology, nutrition and food science, preventive medicine and public health, soil science and agricultural chemistry, organic chemistry, physical chemistry, microbiology, parasitology, pharmacy and pharmaceutical technology (biopharmacy and pharmacokinetics), and physiology. Chapter 1 reports some examples of surface-associated microorganism assemblies, constituting the so-called biofilms, as well as their regulatory mechanisms and their role in the degradation of cultural heritage sites and some methods used in the study of their 3D structure to manage and prevent uncontrolled colonization. Chapter 2 deals with the involvement of c-Jun N-terminal kinases (JNKs) in apoptosis and presents this signaling pathway as a key target to prevent cell death for the efficient management of neurodegenerative diseases. Chapter 3 presents the cross-regulations among polyamines, stress hormone pathways and reactive oxygen species signaling that condition stress signaling in plants and the elucidation of the molecular mechanisms responsible for the protective effect of polyamines against stress in plants. In Chapter 4, nutritional genomics are highlighted as a novel nutritional approach that improves the knowledge of the interactions between diet nutrients and genome in both health and disease states, thereby showing promise as a tool to improve public health. Particularly, the molecular mechanisms that are behind the chemopreventive effects of food polyphenols in the context of cancer management are discussed. Chapter 5

reviews the epidemiology of community-acquired pneumonia (CAP) and assesses the effectiveness of the 23-valent pneumococcal polysaccharide vaccine in preventing CAP requiring hospitalization in the elderly to determine the need for vaccination programs. Chapter 6 reports on the environmental impact, ecotoxicity and potential biodegradability of ionic liquids as a new generation of chemicals with potential to enhance the greenness of chemical processes of interest for the pharmaceutical industry. In Chapter 7, organocatalysis and organocascade reactions are presented as emerging powerful approaches for the synthesis of enantiopure drugs through environmentally friendly processes in as much as they allow a reduction of reaction times, chemical waste and avoid the use of metals. Chapter 8 describes bicelles as novel highly versatile lipid nanostructures of interest for skin applications, as they may modify skin biophysical parameters thereby enhancing drug penetration or they can incorporate a number of drugs that can be carried through the skin layers. Chapter 9 reports on biosurfactant producing microorganisms as well as on their environmental and cost-effective pharmaceutical, biomedical, cosmetic and food industry applications. Chapter 10 presents environmental parasitology as an interdisciplinary field that involves knowledge from toxicology, environmental chemistry and parasitology, highlighting the role of parasites as potential indicators of environmental quality. Particularly, the use of intestinal parasites of different groups of vertebrates to assess heavy metal pollution in terrestrial non-urban habitats is therein discussed. Chapter 11 describes the pharmacokinetic modeling according to the population pharmacokinetic approach with the non-linear mixed effects models as a powerful tool to determine the relationships between the doses of the prodrug mycophenolate mofetil and the exposure of the active component mycophenolic acid, commonly used in immunosuppressant regimens after real transplantation. Chapter 12 discusses the effects of feeding a isomeric mixture of conjugated linoleic acid (CLA) during gestation, suckling and early infancy on the systemic and mucosal immune responses of Wistar rats at different life times, which support a modulatory effect of CLA on immune system development during early life and a long-term effect on the specific immune response in the adult age.

Thus, this E-book tries to address the considerable heterogenicity of pharmaceutical sciences through diverse approaches with a common final aim, the improvement of society's health and well-being. It is worth mentioning, in addition, that this multidisciplinary focus and reach of pharmaceutical sciences makes many of their contributions also valuable in other research fields, such as basic biological or chemical sciences.

Diego Muñoz-Torrero
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1. Biofilms on rocks

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Abstract. Microorganisms group themselves into assemblies known as communities or biofilms, which are associated with surfaces. A matrix of self-segregated polymeric substances enhances their attachment. Communication between bacterial cells involves the production and detection of diffusible signal molecules, known as quorum sensing, which is an important regulatory mechanism of biofilm strategies. Biofilms thrive everywhere; in subaerial surfaces they can be driven by sunlight, with photosynthesizing components. A special case is those which colonize works of art, forming patinas and becoming involved in the degradation of colonized substrata. Knowledge of three-dimensional structure of the biofilm and the distribution of species concerned is crucial for managing and preventing uncontrolled colonization and for preserving cultural heritage sites. This paper describes their role in this degradation, some examples of biofilms and their resilience mechanisms. The methods used in their study when growing in monuments and caves are also discussed.

Introduction

Most microorganisms live attached to a surface rather than as single, suspended cells [1]. The aggregation of microorganisms attached to a surface

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and enclosed in a matrix is called a biofilm [2,3] (Fig. 1). Biofilms constitute a protected mode of growth that allows microorganisms to survive in hostile environments [4]. Under suitable conditions, all unprotected natural and artificial substrata quickly become colonized by these communities. The characteristics of individual biofilms are extremely variable. Biofilms can have an effect on human health and are related to some diseases. They also play important roles in the context of energy and the environment.

Biofilm-forming microorganisms generate a matrix of hydrated extracellular polymeric substances (EPS), which form their immediate environment [5]. EPSs vary in their composition and thus in their chemical and physical properties. EPSs are mainly polysaccharides, proteins, nucleic acids and lipids. These substances provide the mechanical stability of biofilms, mediate their adhesion to surfaces and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells [6-8]. The relative amounts of different polysaccharides also depend on the physiological state of the biofilm and the availability of nutrients. Slow growth or nutrient shortage enhances the synthesis of EPSs [9], which are a key structuring component for nutrient absorption and protection against desiccation, and are part of the stress response. The coexistence of species in a biofilm depends on their capacity—and that of their competitors—to bind to the substrate.

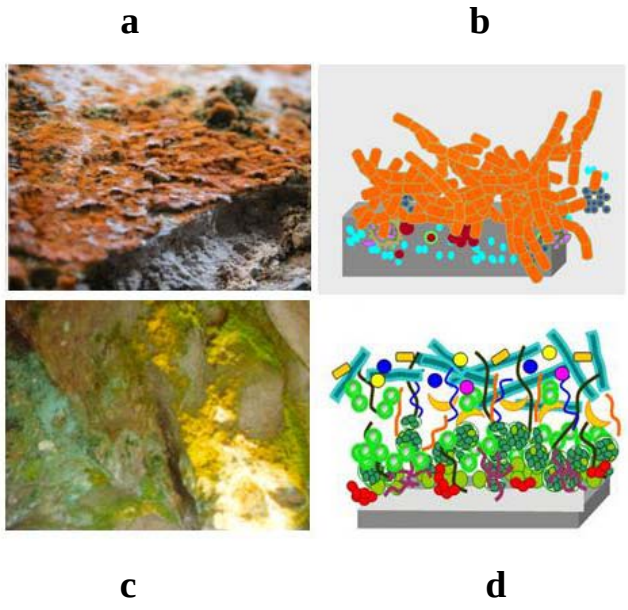

Figure 1. Detail of subaerial biofilms (a,c). a) Mayan monument (Chiapas, Mexico), mortar with tough green colonies of *N*. cf. *commune*, accompanied by *Trentepohlia aurea* (L.) Martius c) Collbató cave (Barcelona, Spain), speleothems with lampenflora, mainly *Scytonema julianum* (Kütz.) Meneg. and green microalgae. Schematic representation of biofilm structure and main species (b,d). b) from a. d) from c.

1. Biofilm development

Formation of a microbial biofilm is a complex multistep developmental process that consists of several overlapping stages. Most of our information comes from bacterial biofilms for which the formation of a biofilm has been described as a sequence of events [10,11]: i) the first event is the substratum conditioning with the absorption of water, organic matter or dissolved organic macromolecules; ii) the second event is the arrival or mass transport of microorganisms to the area; iii) the third event is the adhesion of the microorganisms, which is a prerequisite for the formation of biofilms on surfaces. Initial attachment to a variety of materials and early biofilm formation depend on the surface and type and degree of roughness of the substratum. Irregular surfaces are preferential starting points for attachment because they provide niches in which microorganisms are protected. After making contact with a surface, bacteria become attached in a process that was formerly considered reversible. This process is frequently mediated by the presence of extracellular materials but is also accompanied by physiological changes that end in irreversible surface binding [12]; iv) the fourth event is the expansion of the biofilm, which involves the aggregation of cells into microcolonies that then grow and mature. The microorganisms produce and release materials mediated by the microorganisms themselves. Changes in the gene expression and formation of exopolymeric material are regulated by cell-to-cell signals [13]; v) the fifth event is the return to temporary motility in response to nutritional cues, so that biofilm cells are released in order to repeat the process.

2. Quorum-sensing

Quorum-sensing (QS) regulates the communication, behavior and several cellular processes of the microorganisms, including biofilm formation. QS is a form of intercellular communication between single-cell organisms that allows them to act coordinately like multicellular organisms. Release of the QS signal compounds is dependent on the density of the population [14-15] and leads to changes in bacterial gene expression [2] and to significant changes in the phenotype [17]. QS signals synchronize with the growth stage of a culture and accumulate to a threshold level. This controls the switch from the behavior typical of single-cell organisms to that of cells within a colony or a biofilm [11, 18]. There are a number of different QS signaling systems employed by Gram-negative bacteria, such as oligopeptide autoinducers, furanones, triclosan or mixtures of fatty acids, but the most characterized is the component LuxR-N-acyl homoserine lactones (AHLs)

[19-20]. AHLs are produced by homologues of the AHL synthase LuxI from S-adenosyl methionine and the intracellular pool of acyl-carrier proteins [21]. AHLs differ in the length, degree of saturation and substitution of the acyl side chains. The autoinducer molecules bind to the appropriate transcription regulator(s) when the bacterial population reaches the quorum level (that is, the signal concentration reaches a threshold concentration high enough to facilitate binding to the receptor) [13]. Binding of the autoinducers is followed by activation or repression of target genes. Thus, quorum sensing allows bacteria to make a unified response. This benefits the population [22] because it enhances access to nutrients and more favorable environmental niches and improves action against competing bacteria and environmental stresses [11, 15] (Fig. 2). The bacterial species within a consortia respond to different QS signals [23,16]. There are few reports of AHL activity in cyanobacteria [24, 15], but this could be due to the lack of studies.

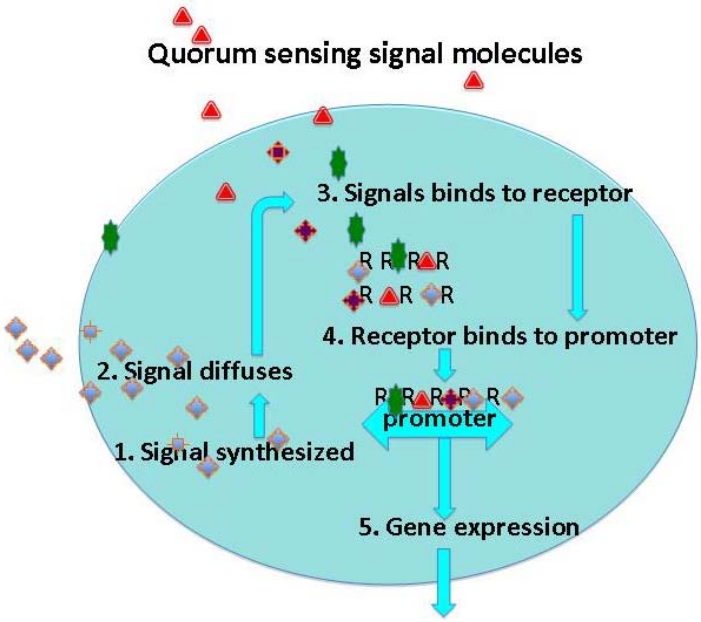

Figure 2. Communication between bacterial cells involves the production and detection of diffusible signal molecules known as quorum sensing (QS). Numbers in the flow chart represent different stages of the QS process. Arrows show the main direction of signal transport (based on Dobretsov et al. 2009).

3. Methods of study

A multistep approach involving molecular, chemical and microscopy techniques is used to assess the composition, 3D structure and distribution of the biofilms.

Nonculture-based molecular methods allow community compositions and activities to be characterized *in situ*. These methods include DGGE, clone library analysis, quantitative PCR, and stable isotope probing that can be used to obtain the phylogeny, relative abundance and genetic activity of individual members of a biofilm community [25, 26]. In particular, functional genomic approaches provide important clues about phototrophic biofilm biology [27].

A number of techniques for detecting and identifying quorum sensing molecules or for monitoring the activity of these compounds have been described [15, 13, 28]. Approaches used for detecting and identifying AHLs include cell-based assays using AHL-specific bacterial biosensors, thin-layer chromatography, gas chromatography/mass spectrometry, and liquid chromatography. Bacterial biosensors cannot synthesize AHLs, but they can express specific compounds when exogenous AHL [a common one is *Agrobacterium tumefaciens* (Smith & Townsend) Conn] is added.

Microscopy techniques include light microscopy (LM), scanning electron microscopy in back-scattered electron mode (SEM-BSE), energy dispersive X-ray microanalysis (EDX), X-ray diffraction (XRD), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), which enables non-disruptive observation of live cells.

Scanning electron microscopy (SEM) is useful for examining surface topology and the distribution of specimens as well as monitoring the interactions between biofilms and substrates (Fig. 3). However, conventional SEM requires samples to be imaged under vacuum, and biological material tends to be susceptible to dehydration. Sheaths and mucilaginous outer layers may become condensed or blur the surface of the specimens. Other types of SEM overcome some of these processing inconveniences. For example, environmental scanning electron microscopy (ESEM) allows hydrated samples to be observed without the need for coating them or further processing. Scanning microscopes can be coupled to backscattered electron imaging (BSE) (Figs. 3b and d) or energy dispersive spectroscopy (EDS). These two analytical techniques are mainly used to discriminate inorganic from biological substances or for the elemental analysis of a sample.

Confocal scanning laser microscopy (CLSM) is a tool for 3-D localization of fluorescent organisms or items dyed with fluorescent labels, externally or inside the substrata. The technique provides an efficient way of determining the presence, the viability, the functionality and the spatial organization of specific organisms. It makes non-invasive optical sectioning possible by subtracting out-of-focus planes of the image. The surface and the in-depth structure of the sample can be examined by CLSM with minimal preparation and without disturbing the structure [29, 30].

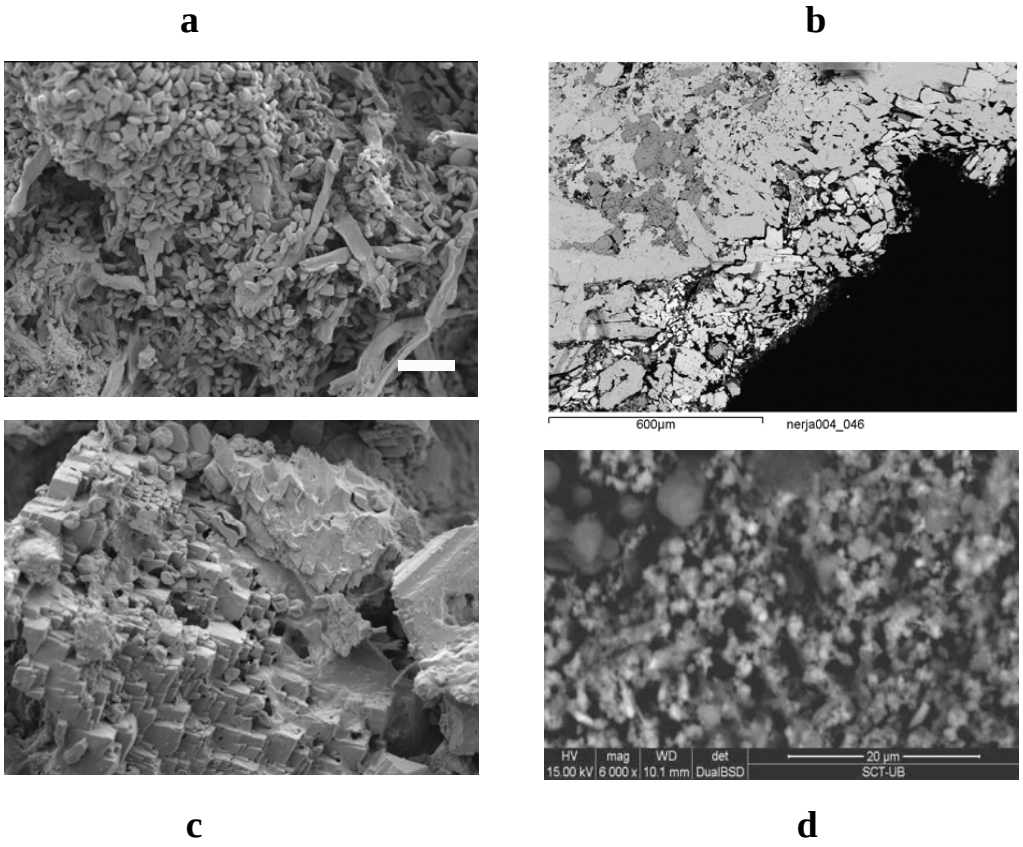

Figure 3. SEM-BSE images. Low magnification overview of biofilms colonizing the surface of subaerial biofilms. a) SEM image of a biofilm formed by diatoms (*Diadesmis gallica* W. Smith) and moss protonemata (Zuheros cave, Cordoba, Spain). b) SEM-BSE image of endolithic coccoid growth (*Chroococcidiopsis* sp.) through the intercrystalline porosity of a speleothem (Nerja cave, Malaga, Spain). c) SEM image of a biofilm formed by a single-celled chlorophyta (*Muriella* sp.) irregularly distributed on a dolomite surface (Zuheros cave, Córdoba, Spain). d) SEM-BSE image showing coccoid cyanobacteria (grey) entrapped with inorganic granules (in a bright shade of gray).

The above described microscopy techniques complement each other, providing an efficient method for determining the presence and viability of biofilms, which allow designing control strategies and biofilm monitoring.

4. Subaerial biofilms

Most earth-illuminated surfaces are covered by biofilms formed by subaerial or endolithic photosynthesis-based microbial ecosystems. Subaerial means at the surface, exposed to the air, and endolithic means into outer centimeters of rocks, close to the surface [31, 32]. Photosynthetic microorganisms only need light as a source of energy, and inorganic substances

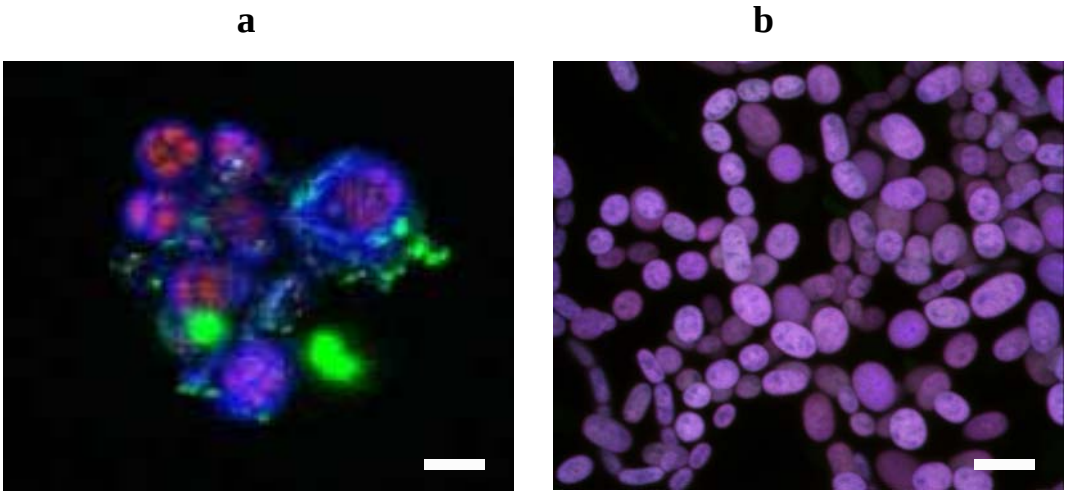

Figure 4. Confocal compound images of biofilm forming microorganisms from aerophytic biofilms: a) Field material of *Chroococcidiopsis* sp. b) General view of a cultured strain of *Nostoc* cf. *commune*. Color key: photosynthetic pigments (Chlorophyll *a* and/or phycobilins), magenta. EPS (labeled with ConA-Alexa 488), green. *Chroococcidiopsis* sp. sheaths, blue. Scale bar = 10 μm.

to grow. Heterotrophic organisms are integral parts of the communities and use organic matter both as a source of energy and as a substrate to synthesize their own components. The EPSs and organic matter produced by phototrophs are often exploited by non-photosynthetic microorganisms, such as fungi or bacteria, which subsequently flourish. Reported examples include proteobacteria and actinobacteria, mainly in epilithic communities, and acidobacteria, actinobacteria and low GC firmicutes, mainly in endolithic communities [33-36].

Subaerial biofilms are composed of photosynthetic cyanobacteria, algae, lichens and mosses, and several kinds of heterotrophic bacteria are companions [36-40]. Sequences of bacteria and archaea on monuments around the world are phylogenetically related to sequences found on different surfaces and at different geographical locations [41]. Striking similarities have been observed in subsurface (hypogean) environments, such as caves [42, 43], mainly associated with crystal formation. The main biomass observed with optical and electronic examination usually corresponds to cyanobacteria, also known as blue-green algae, so named because these organisms have the characteristics of bacteria and photosynthetic pigments like algae, which makes them blue-green or dark in color. Cyanobacteria show entangled filaments or heterogeneous aggregates with air spaces between them.

Cyanobacteria in subaerial biofilms resist environmental changes (e.g. extended droughts, high temperatures or prolonged solar exposure). They have

several well-known survival strategies related to desiccation [44, 45], which include using water retained within the substrata and the formation of protective, drought-resistant compounds [46]. Their persistence and success in terrestrial environments has been attributed to their ability to tolerate desiccation and to rapidly rehydrate and recover metabolic activity once favorable conditions have been reestablished [44, 47, 48]. Resilient species such as *Chroococcidiopsis* spp. (Fig. 4a) and *Nostoc* spp. (Fig. 4b) dominate certain communities in both hot and cold deserts due to their heat-tolerance and ability to recover after desiccation [48, 49, 50].

Here we report some examples of biofilms mainly made up of photosynthetic organisms.

Mexican Mayan monuments have low diversity of species due to extreme environmental conditions (Fig. 1a). In a habitat dictated by alternating wet and dry seasons, a *N.* cf. *commune* (Fig. 4b) survives by varying its developmental stages. Its life cycle comprises two seasonally-determined stages -growth during the wet season and dormancy during the dry season- and two transitional stages -preparation for the dry season, and rehydration and recovery-. To survive the driest conditions, the biofilm reduces the number of cells inside thick sheathed colonies and synthesizes the pigment phycoerythrin, which increases cell tolerance against the detrimental effects of strong light [50]. In addition, some photosynthetic microorganisms cope with high solar irradiance by synthesizing UVR screens [51, 52].

Subaerial biofilms can also thrive in dim environments. In cave habitats and catacombs the most common stress factor is light shortage, followed by humidity, a lack of nutrients, and to a far lesser extent, temperature [53, 54]. The amount of light varies depending on the cave type, and also within the cave according to gradients that go from the mouth to the interior [55, 56]. From the mouth of the cave to the sampling point furthest inside the cave, the organisms are organized in mosaics or belts according to light gradients, relative humidity and temperature. The diversity of microalgal and cyanobacterial species decreases with decreasing light [56]. Coccoid forms are more abundant in illuminated areas and dripping sites, and form biofilms marked by vertical stratification [39]. Filamentous forms tend to be more diverse in darker or more humid locations [55]; *Scytonema julianum* can withstand strong environmental fluctuations [38], while other filamentous cyanobacteria such as *Loriellopsis cavernicola* Hernández-Mariné & Canals require long-term stability [56, 57]. This heterogeneous distribution reflects the different adaptation strategies used by microorganisms [54], and provides certain advantages for some of the constituent microorganisms in the biofilm structure.

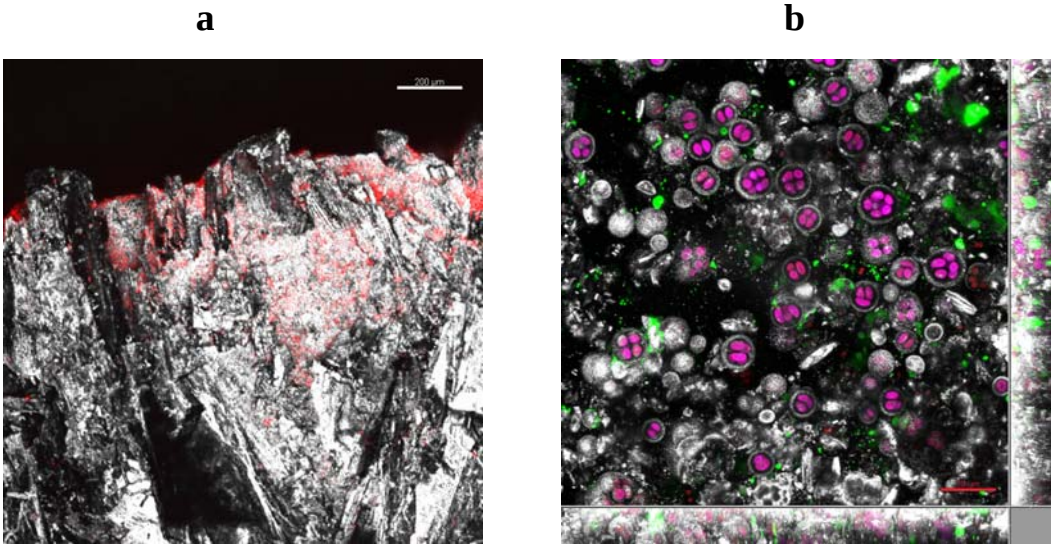

Figure 5. Confocal three-dimensional images of aerophytic biofilms: a) Projection showing inorganic calcareous material (white) (Nerja cave, Cordoba, Spain), plus spatial distribution of pigment autofluorescence (red) of single celled chlorophytes (*Muriella* sp.) distributed in a thin layer, penetrating the fissures and fractures. b) Extended projection of a biofilm dominated by *Asterocapsa divina* J. Komárek (Mayan monuments, Chiapas, México). Color key: photosynthetic pigments (Chlorophyll *a* and/or phycobilins), red. EPS (labeled with ConA-Alexa 488), green. Reflection from inorganic materials, white.

5. Biodeterioration

Cultural heritage sites made of natural or artificial materials (e.g. rocks or stones and concrete or plaster) are governed by similar principles to any other terrestrial system, and are therefore susceptible to weathering. Subaerial biofilms colonize both the stone surface and the porous interior. Biofilm colonization of buildings or artwork is considered damaging, basically due to their chemical and physical activity [33, 58, 59]. Moreover, detectable colored patinas are considered dirty [42, 60].

Biodeterioration of exposed stone is primarily dependent on the availability of water and nutrients. Specific parameters, like porosity, permeability and architectural conditions, exposure and environmental factors at the site will determine the intensity and rate of biocorrosive attacks [61]. In many cases, these processes have been found to deteriorate the stone. Alterations are associated with repeated wetting and drying cycles that lead the organisms, which are attached to the rocks by EPSs, to expand and contract. In addition, the action of organic acids or metabolic products can enhances the weathering reactions and decrease the integrity of the mechanical properties of the natural or artificial materials. In spite of this, it is

difficult to determine whether photosynthetic biofilms will have a biodeterioration or bioprotection effect on the surface they are attached to because biofilms can be both beneficial and detrimental depending on the substrate and microorganisms involved. Thin superficial biofilms can cause discoloration of stone surfaces and mechanical and biochemical deterioration, or alternatively, they can protect the surface from weathering processes [54, 62].

Figure 6. Optical micrographs: a) Discoloration on a roman fresco caused by a ubiquous development of actinobacteria and cyanobacteria forming a subaerial biofilm. b) Typical subaerial biofilm that develops in an area with run-out water (Vittoriosa, Malta). The black stain is mainly caused by actinobacteria, cyanobacteria and fungi.

6. Conclusion

Biofilms are the default mode-of-life for many bacterial species and present remarkable complexity aimed at the protection of constituent microorganisms. Understanding factors that control the microorganisms as well as the effects of environmental factors on biofilm formation and control strategies will require further studies.

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2. Inhibitors of the M2 channel of influenza A virus

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Abstract. Influenza is a highly contagious, major respiratory tract disease affecting millions of people each year. At present, two classes of antivirals are available: the neuraminidase inhibitors and the M2 proton channel blockers amantadine and rimantadine. However, rapid emergence of M2 blockers resistance makes imperative the development of new anti-influenza drugs. In the last few years several groups have synthesized and evaluated several analogs of amantadine. While several of them are active against wild-type M2 channel only a few are able to inhibit the mutant ion channels that lead to amantadine-resistance.

Introduction

Influenza is a worldwide epidemic that causes substantial morbidity and mortality. Of the three types of influenza viruses, A, B and C, influenza A and B

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cause seasonal epidemics. Moreover, influenza A viruses are responsible for sporadic pandemics that usually cause higher mortality rates than seasonal influenza epidemics. The most severe pandemic, the “Spanish flu”, occurred in 1918, is thought to have killed more individuals than any disease outbreak in history, resulting in approximately 40 million deaths worldwide [1]. More recent pandemics in 1957 (“Asian flu”, H2N2 strain) and 1968 (“Hong Kong flu”, H3N2 strain) were not as deadly, yet influenza remains a grave health hazard [2]. For example, in the United States, according to the Center for Disease Control and Prevention (CDC), influenza and its complications are currently the leading cause of death due to any infectious disease. In fact, in 2009, a new influenza virus (“swine flu”, H1N1 strain) originated a new pandemic that caused much concern, although, thankfully, was not as deadly as initially thought [3]. In addition, H5N1 viruses (“bird flu”), which are also currently worldwide circulating, are extremely virulent in humans but have not acquired the ability for efficient human-to-human transmission yet [4].

Influenza A viruses infect a wide range of avian and mammalian hosts, unlike influenza B viruses, which infect only humans. Influenza A and B viruses are enveloped negative-strand segmented RNA viruses. The envelope of influenza A viruses contains two different surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [5]. Influenza A viruses are categorized into antigenic HA and NA subtypes: 16 HA (H1–H16) and 9 NA (N1–N9) antigenic subtypes have been identified so far. For example, the 2009 “swine flu” is an H1N1 virus because it contains a H1 subtype HA and a N1 subtype NA.

The major influenza A subtypes that have infected humans during seasonal epidemics are H1N1, H2N2 and H3N2. Within a subtype, different strains arise as a result of point mutations in a process known as ‘genetic drift’. These new strains cause seasonal epidemics. A new pandemic can emergence by two different mechanisms: by direct transmission from animals, usually birds, to humans, as happened in 1918 with the “Spanish flu”; or through reassortment of an avian influenza virus with a human influenza virus, as occurred in 1957 with the “Asian flu” (H2N2) and, again, in 1968 with the “Hong Kong flu” (H3N2) [6]. The H1N1 virus of the 2009 “swine flu” is an apparent reassortment of four endemic strains of influenza: one from humans, one from birds, and two from pigs, a fact that further exemplifies the versatility of the influenza A virus [3]. When a new pandemic starts, the HA of the new strain differs substantially from recent HAs of seasonal influenza A viruses and, consequently, most of the human population lacks immunological protection against this virus, resulting in a pandemic.

There are two different strategies for combating influenza: vaccination and chemotherapy. The primary defense against influenza A has been

vaccination with inactivated or live-attenuated virus. However, vaccination effectiveness is limited due to the antigenic drifts and shifts that the influenza virus undergoes from year to year, and new influenza vaccines must be designed every year by predicting the genetic drift of seasonal influenza A. Antivirals have also been used for both prophylactic and therapeutic treatments during seasonal epidemics [5b]. Additionally, antivirals are particularly important at the beginning of a fast-spreading pandemic because the timely production of sufficient amounts of an effective vaccine is difficult. Current antivirals are directed against the M2 ion-channel protein of the influenza A virus (amantadine and rimantadine, Fig. 1) and the NA of the influenza A and B virus (zanamivir and oseltamivir) [8]. However, many influenza virus strains have developed resistance to adamantanes and/or oseltamivir (the only orally bioavailable NA inhibitor), highlighting a major health risk [9]. For example, after four decades of effective use of amantadine, resistance by influenza viruses of the A/H3N2 subtype currently exceeds 90% in the United States, and virus mutants are as fit as the wild-type (wt) virus. The situation is even worst with the new 2009 pandemic H1N1 influenza. In both strains, the basis for resistance is a single Ser to Asn amino acid replacement (S31N) in the matrix M2 ion channel, which interferes with the drug's ability to block M2 ion channel activity and viral replication [10].

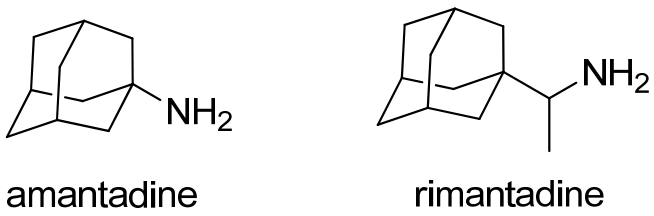


Figure 1. The structures of the anti-influenza A drugs amantadine and rimantadine.

Another important problem encountered in the administration of amantadine and rimantadine is related to the central nervous system side effects of both drugs. In fact, amantadine has been used in the treatment of Parkinson’s disease, although its antiparkinsonian effect is poorly understood [11]. The side effects of rimantadine are analogous, but somewhat less pronounced than those of amantadine [12].

The appearance of pandemic H1N1 and highly pathogenic avian influenza viruses of the H5N1 subtype being able to infect humans, the emergence of resistances, and the side effects of amantadine and rimantadine reveal the urgent need for the development of new antiviral drugs [13].

In this review, we will focus on the design of new amantadine analogs targeting the matrix M2 ion channel of influenza A virus.

1. The M2 protein

The influenza A virus M2 protein is a homotetrameric protein containing four parts: a short unstructured N-terminal extracellular domain, important for incorporation into the virion; a transmembrane helix that is necessary for tetramerization, proton conductance and drug-binding; a cytoplasmic amphiphilic helix, involved in cholesterol-binding, membrane localization, budding and scission; and a disordered tail that interacts with the matrix protein M1 [14].

The influenza virus enters its target cells by receptor-mediated endocytosis, which is followed by acid-induced fusion of the viral and endosomal membranes. This fusion event is mediated by a conformational change of the influenza HA proteins, triggered by the low pH in the endosome lumen [15]. The transmembrane region of the M2 protein forms a pH-activated channel that selectively conducts protons along a chain of water molecules and ionizable sidechains, including His37, playing an essential role for viral replication equilibrating the pH of the virus interior with that of the acidic endosome. When the endosome is acidified, His37 residues in the transmembrane region of M2 become protonated, leading to the opening of the M2 channel and to a proton influx from the endosome into the virus interior [16]. The acidification of the virus interior enables the release of the viral RNA into the host's cytoplasm after membrane fusion has taken place. In addition, it has been shown that, for some strains of influenza A virus, the M2 proton channel function is required for preventing a premature HA conformation transition when newly synthesized viral proteins are trafficked through the trans-Golgi network [17].

The replication of the influenza A virus can be stopped by inhibiting the activity of the M2 channel, using amantadine and rimantadine.

Although the role of the M2 protein as the target for amantadine and rimantadine has been known for more than twenty years [18], only very recent functional, structural [19] and computational [20] studies have revealed that the drugs inhibit proton conduction by binding to an aqueous cavity adjacent to M2's proton-selective filter, thereby blocking access of proton to the filter [14]. These recent works enable novel insights into the adamantanes-resistance and provide a solid basis for structure-based drug design.

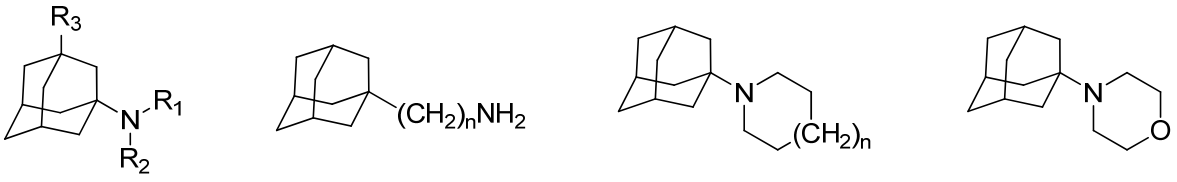
The number of drug-resistant variants of influenza A M2 channel is limited by the very conserved nature of the binding site within the channel. Thus, only a few amantadine-resistant mutations, namely V27A, L26F and

S31N, have been widely observed in transmissible strains of the virus in the past eight decades for which a genetic record is available [21], although other mutations can easily be observed *in vitro* [22]. The mutations that cause the greatest decrease in inhibition, S31N and V27A, increase the polarity of pore-lining residues.

2. 1-Substituted-adamantanes

Amantadine and rimantadine exhibit their inhibitory activity at micromolar concentrations. Rimantadine has a superior intrinsic antiviral activity compared to amantadine, but peak plasma levels of rimantadine are 2-3 fold lower than those achieved with amantadine when given at the same dose [23]. Amantadine was initially licensed in USA in 1966. Interestingly, for many years, amantadine was mainly used in western countries, while rimantadine was used in the former USSR and eastern European countries [24].

Both drugs are rather old, therefore it is not a great surprise that hundreds of derivatives have been synthesized and pharmacologically tested. In fact, soon after the publication of the antiviral activivity of amantadine by du Pont de Nemours' researchers [25], several amantadine derivatives were synthesized and evaluated as anti-influenza agents [26]. Most of these analogs were alkylaminoalkyl derivatives of adamantane (Fig. 2), although some derivatives featuring aditional polar groups, such as alcohols, amines, ethers, or derivatives lacking an amino group were also synthesized and tested (Fig. 3).



R_1, R_2 = H, methyl, ethyl, allyl, propargyl, propyl, butyl, etc.; R_3 = H, methyl, phenyl, etc.; n = 0, 1, 2

Figure 2. Alkylamantadines and related compounds.

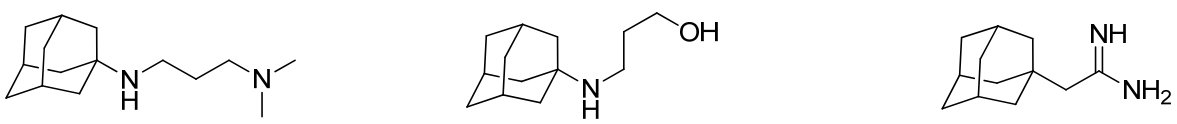


Figure 3. Amantadine analogs featuring polar groups.

Although several of these compounds displayed anti-viral activities similar to that of amantadine, they showed cross-resistance with amantadine and rimantadine, so their therapeutical interest is rather low.

Worthy of note, while amantadine is, for the most part, excreted without metabolism [27], rimantadine is extensively metabolized by hydroxylation before excretion in the urine [23c,28]. Manchand and coworkers reported the synthesis of three hydroxylated metabolites of rimantadine and showed that 2-hydroxyrimantadine was as active against several influenza A virus as amantadine, while the 3- and the 4-hydroxy derivatives showed only very modest inhibitory activity (Fig. 4). Unfortunately, rimantadine-resistant strains exhibited cross-resistance to the 2-hydroxyamantadine [29].

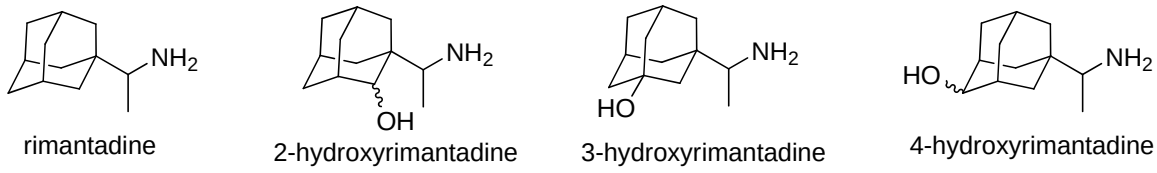


Figure 4. Rimantadine and its hydroxylated metabolites.

The antiviral activity of 2-hydroxyrimantadine was the first example of a trend that has also been observed in much more recent work using amantadine and other polycyclic systems, that is, the introduction of polar groups in the polycyclic scaffold is tolerated, but does not enhance the potency of amantadine and related aminopolycyclic derivatives. For example, in 2011, Wang *et al*. described that the aminoalcohol **3** showed an IC_{50} = 16 μM against the wt M2 channel from influenza A virus, exactly the same value than that reported for amantadine [30]. Surprisingly, they found that 1-adamantanol, **1**, and 2-methyl-2-adamantanol, **2**, showed IC_{50} values very close to that of amantadine, while the 3-amino-1-adamantanol, **4**, showed to be inactive (Fig. 5).

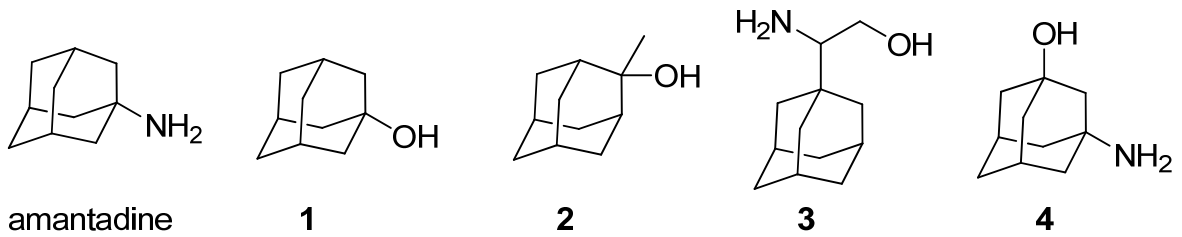


Figure 5. Several hydroxylated analogs of amantadine. IC_{50} values (against wt M2 channel): amantadine (16 μM), **1** (20 μM), **2** (14 μM), **3** (16 μM), **4** (not active).

Kolocouris' group has reported the synthesis and anti-influenza activity of a series of heterocyclic rimantadine analogs (Fig. 6) [31]. The aziridine and the azepine derivatives were much less active than amantadine, while azetidine **6** (R_1=H), pirrolidines **7a** (R_1=R_2=H) and **7b** (R_1=H, R_2=CH_3), and piperidine **8** (R_1=H) showed to be more potent than amantadine and rimantadine against the influenza A_2/Japan/305/57 (H2N2) strain. Compounds **6** and **7b** also showed good inhibitory activity against the influenza A/Hong Kong/68 (H3N2) strain. While amantadine displayed IC_{50} of 42 and 6 μM against A_2/Japan/305/57 and A/Hong Kong/68, respectively, the most potent compound within this series, **7b**, showed IC_{50} of 1.6 and 1.8 μM against A_2/Japan/305/57 and A/Hong Kong/68, respectively. The introduction of an additional alkyl group in the nitrogen atom caused a dramatic reduction in anti-influenza activity.

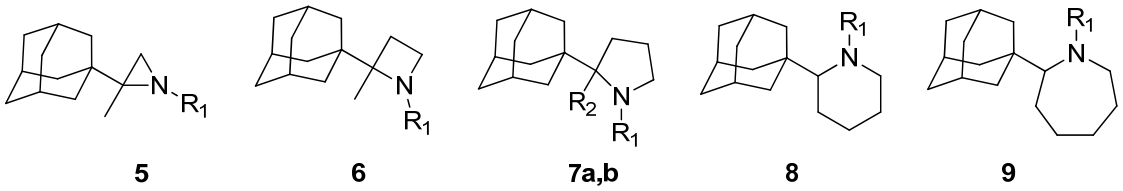


Figure 6. Heterocyclic rimantadine analogs.

Later on, the same group reported analogs of rimantadine featuring a second nitrogen atom, the aim of this modification being the incorporation of additional hydrogen bonding interactions with the M2 protein [32]. Although the presence of this second amino group was compatible with anti-influenza activity, the new analogs were not more potent than rimantadine. Thus, compounds **10** and **11** (Fig. 7) displayed EC_{50} of 18.3 and 24.1 μM, respectively, against A/Hong Kong/68 (H3N2) strain, very similar values to that of rimantadine (EC_{50} = 19.1 μM).

Figure 7. Rimantadine analogs featuring a second nitrogen atom.

As previously stated, amantadine interferes with the ion channel function of the M2 protein of influenza A virus at low micromolar concentrations. Interestingly, a second mechanism of action of amantadine, at least in some influenza A strains, is on the hemagglutinin, at concentrations around 100 times

higher. Theoretically, an amantadine derivative able to simultaneously interact with both targets at the same concentrations should have a reduced probability to develop resistance. In this case, two mutations, one in each target protein would be necessary at once. With this aim, Scholtissek and coworkers reported the synthesis and evaluation of forty adamantane derivatives and tested them against the influenza A/Singapore/1/57 (H2N2) strain [33]. They found several analogs active against this strain and also against A/Swine/1976/31 and A/Udorn/307/72, although all the products were inactive against A/WSN/33, which is amantadine-resistant. Most of the active compounds at low micromolar concentrations (e. g., **12-14**) interacted with the M2 protein; the corresponding escape mutants produced with them had amino acid replacements at positions 27, 30 or 31 of the M2 protein. Interestingly, they found two compounds, **15** and **16** (Fig. 8), able to interact with both the ion channel and the hemagglutinin at about the same concentration. It was expected that in order to become resistant the virus should mutate both proteins. However, the resistant mutants to these compounds showed mutations only in the HA protein [33].

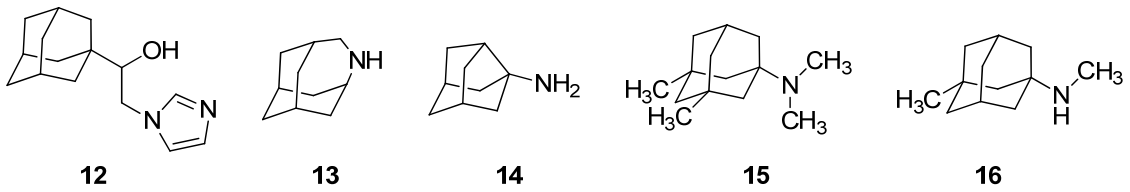


Figure 8. Some of the amantadine analogs reported by Scholtissek and coworkers.

Very recently, Zarubaev and coworkers have reported the synthesis and the anti-influenza activity of a series of di-, tri- and tetrazole derivatives of amantadine. Interestingly, several compounds were active against the amantadine-resistant influenza A/Puerto Rico/8/34 strain, which bears the S31N mutation in its M2 channel. Tetrazoles such as **17**, **18** and **19** (Fig. 9), showed micromolar values of EC_{50} and higher selectivity index (SI) than rimantadine [34]. It remains to be clarified if the target of these adamantane derivatives is the M2 channel of the influenza virus.

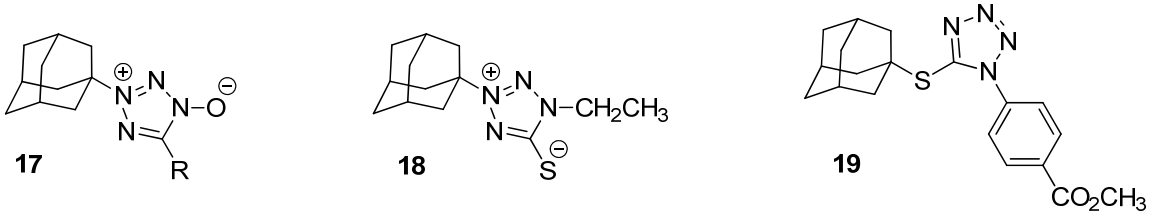
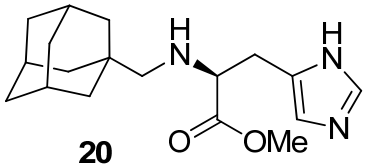


Figure 9. Tetrazolo-adamantanes with anti-influenza A virus activity.

Finally, Zhang *et al.*, have reported that an *L*-histidine derivative of adamantane, **20** (Fig. 10), was able to inhibit the wt, the S31N, and the double mutant S31N/L26I M2 channels of avian H5N1 influenza expressed in cell lines of transformed HEK 293. The IC_{50} of **20** against the wt, the S31N mutant and the double mutant S31N/L26I channels were 5.84, 10.96 and 9.77 μM, respectively [35]. However, these data were not confirmed with viral inhibition assays.

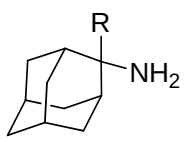


20

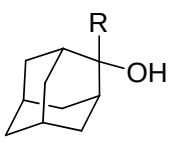
Figure 10. Structure of histidine derivative **20**.

3. 2-Substituted-adamantanes

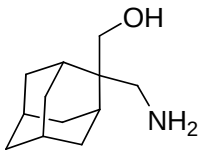
2-Amantadine is only moderately active against influenza virus. The anti-viral activity improved by the incorporation of a 2-ethyl or 2-*n*-propyl group, although the introduction of a methyl group in C-2 diminished the activity. Interestingly, 2-methyl-2-adamantanol, **2a**, showed an EC_{50} of 3 μM against influenza A/Japan/305/57 (H2N2) strain, very similar to the EC_{50} of amantadine against this strain (1.1 μM) [36]. As previously stated, **2a** inhibits the wt M2 channel of influenza A with an IC_{50} of 14 μM [30]. In 2010, Kolocouris' group reported that several adamantanaminoalcohols such as **23** and **24** (Fig. 11) had potent anti-influenza activity. For example, aminoalcohol **23**, displayed submicromolar activity against the influenza A /Hong Kong/7/87 (H3N2) strain [37].



21a-c



2a-c



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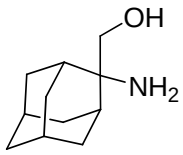


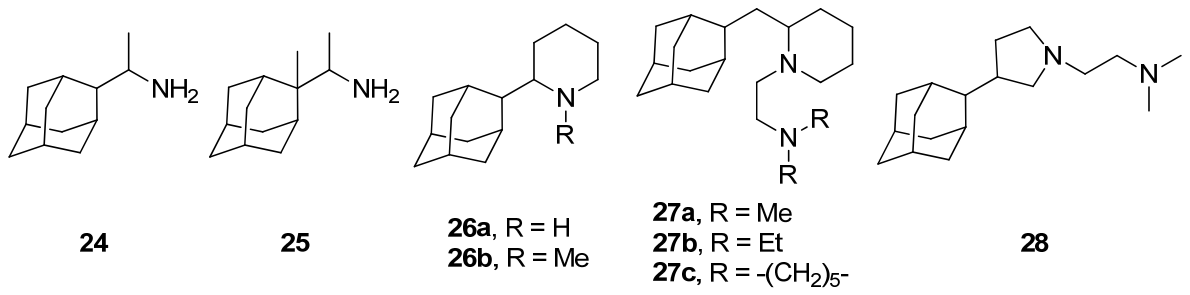
23

Figure 11. 2,2-Disubstituted adamantanes. a, R = methyl; b, R = ethyl; c, R = *n*-propyl.

Although 2-amantadine is only moderately active against influenza virus, the 2-isomer of rimantadine, **24** (Fig. 12), was found to be 4 times more potent than rimantadine against the influenza A_2/Japan/305/57 (H2N2) strain. This

finding led to Kolocouris' and De Clercq's groups to investigate the antiviral activity of several 2-alkyl and 2-cycloalkyl analogs of rimantadine [38]. They found that alkylation of the nitrogen atom reduced the anti-viral activity as did the introduction of a methyl group in the C-2 of the adamantane, as in **25**. Unfortunately, **24** was much less potent against X-31, a reassortant influenza A H3N2 strain (A/Hong Kong/1/68 with A/Puerto Rico/8/34) carrying the S31N mutation.

They also investigated the activity of 2-(2-adamantyl)piperidines, 2-(2-adamantylmethyl)piperidines and 3-(2-adamantyl)pyrrolidines. In these series they found that while the alkylation of the nitrogen atom reduced the activity, as in going from **26a** to **26b**, the introduction of a further nitrogen atom two carbon away from the heterocyclic ring, as in **27a-c** or **28** led to high anti-viral potency. For example, compounds **27a-c** showed EC_{50} between 3 and 7 μM, against the X-31 strain, much lower than amantadine (EC_{50} = 49 μM) or rimantadine (EC_{50} = 19 μM). Taking into account the size of the diamines, it seems like the M2 receptor site can accommodate cages much larger than the adamantane. Unfortunately, the selectivity index (SI) of these compounds was much lower than that of amantadine or rimantadine [39].



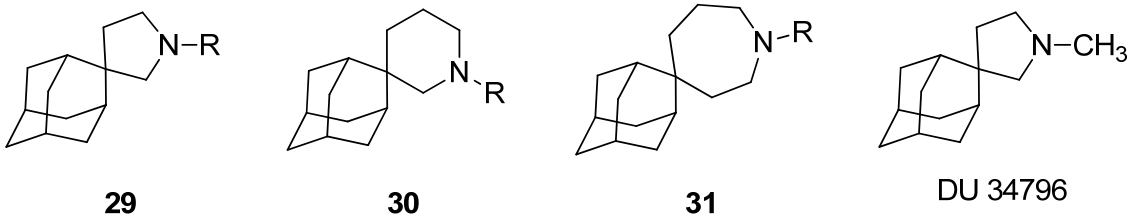
24 **25** **26a**, R = H; **26b**, R = Me **27a**, R = Me; **27b**, R = Et; **27c**, R = -(CH2)5- **28**

Figure 12. 2-Substituted analogs of rimantadine.

4. Azaspiroadamantanes

A unique kind of 2-substituted adamantanes is the group of the azaspiroadamantanes, because several of these derivatives have very potent anti-influenza activity.

Forty years ago, researchers at N. V. Philips-Duphar synthesized a series of azaspiroadamantanes (Fig. 13) [40]. Several of these amantadine analogs showed anti-influenza activity and, in fact, one of them, DU 34796, that had an antiviral spectrum *in vitro* wider than that of amantadine and was more potent than amantadine against mouse influenza, entered clinical trials, although finally the drug was not further developed [41]. The main problem of these compounds was, once again, the cross-resistance with amantadine and rimantadine.

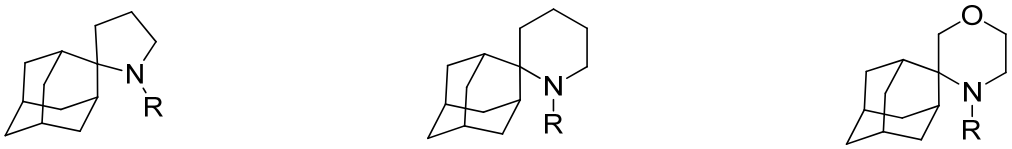


29 **30** **31** DU 34796

Figure 13. Spiro adamantane derivatives synthesized by N. V. Philips-Duphar. R is a lower alkyl group.

As 2-adamantanamine is only moderately active against influenza virus, the antiviral activity of the aforementioned derivatives points out that a carbon substituent in the vicinity of the 2-adamantyl carbon leads to a remarkable increase in antiviral activity. We will see further examples of this behaviour in the following paragraphs and also in different analogs that will be shown in the next sections.

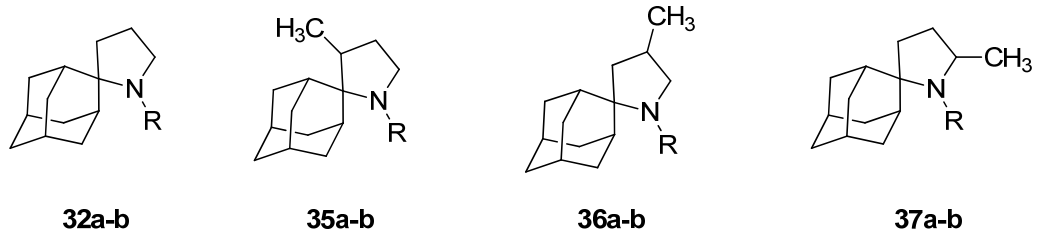
In the nineties, Kolocouris' group, successfully revisited the topic, synthesizing several azaspiro- and oxazaspiro-adamantanes, such as those shown in Fig. 14. The compounds were examined against several influenza A strains (H1N1, H2N2 and H3N2) by De Clercq's group. Interestingly, against a H2N2 strain, the compound **32b** was found to be up to 230 times more active than amantadine. Although **32b** showed a SI of 714 *in vitro*, unfortunately, it proved rather toxic *in vivo*. Worthy of note, the change of a methylene unit by an oxygen atom was compatible with anti-influenza activity, although these oxa-analogs were less active than amantadine [42].



32a-d **33a-c** **34a-c**

Figure 14. Azaspiro- and oxazaspiro-adamantane derivatives. a, R = H; b, R = methyl; c, R = ethyl; d, R = cyclopropylmethyl.

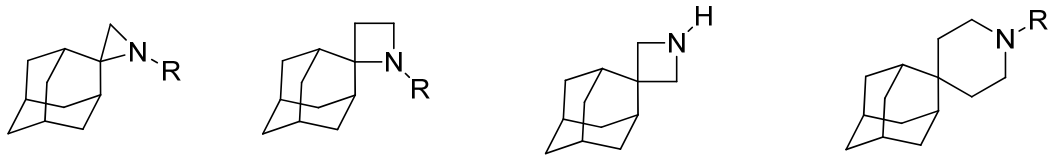
Later on, with the aim of improving the antiviral activity, Kolocouris's group explored the introduction of a methyl group in the pyrrolidine ring of **32a** and **32b** (Fig. 15). While the introduction of a methyl in either C-3 or C-4 of the pyrrolidine ring of **32a** and **32b** led to slightly less active compounds, introduction of a methyl in C-5 of the pyrrolidine was optimal for biological activity against H2N2 strain. Unfortunately, all pyrrolidines had lower SI than amantadine [43].



32a-b **35a-b** **36a-b** **37a-b**

Figure 15. *C*-methyl derivatives of azaspiroadamantanes **32a-b**. a, R = H; b, R = methyl.

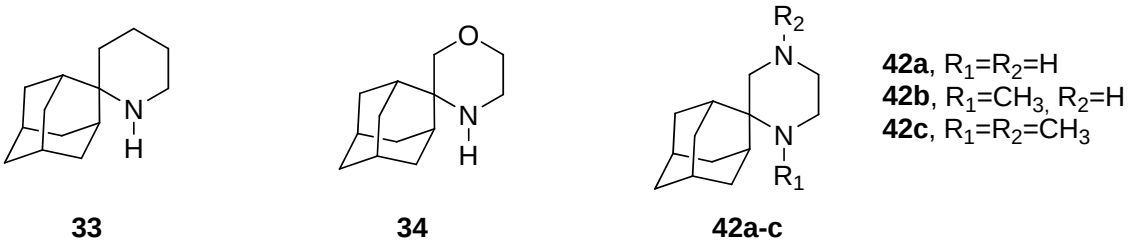
More recently, Kolocouris et al. have completed this series with the synthesis of ring-contracted and ring-expanded analogs of **32a** (Fig. 16) [44]. Azaspiro derivatives **38-41** were synthesized and tested against an H3N2 strain of influenza A. Whereas aziridine derivatives **38a,b** were less potent than amantadine, azetidines **39a,b** and **40**, and the piperidine derivatives **41a,b** were more potent than amantadine. Piperidine **41a**, the most potent of them, showed significant anti-influenza A virus activity, being 12-fold more active than amantadine and about 2-fold more active than rimantadine. Azetidine **36a**, while being slightly less potent than **41a** showed a better SI (694 vs 106). Methyl substitution at the nitrogen atom of all heterocycles caused reduction in anti-influenza virus A potency.



38a-b **39a-b** **40** **41a-b**

Figure 16. Ring-contracted and ring-expanded analogs of **32**. a, R = H; b, R = methyl.

Very recently, Kolocouris' group has reported the synthesis of several spiropiperazines of general structure **42** (Figure 17) [45]. These compounds can be regarded as analogs of **33** and **34** featuring an additional nitrogen atom. The main aim of this approach was to introduce a further group able to establish additional hydrogen bonds within the channel. However, piperazine derivative **42a** was three times less active than spiropiperidine **33** or amantadine. Moreover, *N*-methylation of **42a** to **42b** and **42c** further reduced the activity, probably by hampering the hydrogen bonding ability of the ligand, **42c** being inactive against influenza A/HongKong/68 (H3N2). No significant antiviral effect was observed against the amantadine resistant influenza A/WSN/33 (H1N1) strain. Notwithstanding the introduction of a second nitrogen atom was negative in this spiroadamantanes, other series of adamantane derivatives increased their potency with the introduction of a second amino group, as we have already seen in section 2.



33 **34** **42a-c**

Figure 17. Spiropiperidine 33 and analogs featuring an additional heteroatom.

Finally, it should be noted that, recently, Kolocouris and coworkers have reported the binding constants of some spiroadamantanes against the M2 channel of the influenza A/chicken/Germany/27 (H7N7, Weybridge strain), expressed in *E. coli* [36]. The binding affinity of spiropiperidine **33** was in the submicrolar range (K_d = 0.39 μM), very similar to that of amantadine (K_d = 0.32 μM), although much higher than that of rimantadine (K_d = 0.016 μM). Spiropirrolidines **32a**, **32b**, and **37b** displayed binding affinities in the micromolar range (1.16, 2.93, and 1.5 μM, respectively).

Unfortunately, sometimes it is difficult to compare the anti-viral activity of the different adamantane derivatives. This is, at least partly, a reflection of the time-span lasting more than four decades in which these compounds were synthesized and tested. For example, while spiropiperidines **30**, published in 1972 [40d], and **33a-c**, published in 1996 [42b], were tested against the influenza A_2/Japan, an H2N2 strain, **41a-b**, described in 2007 [44], were tested against the influenza A/HongKong/7/87, an H3N2 strain. As the activity of amantadine against these strains is different, it is difficult a quantitative comparison between the activity of all these compounds. Moreover, sometimes the description of the antiviral potency is not very accurate. For example, van Hes and coworkers, in describing the antiviral activity of **30**, only reported "*activity comparable to that of amantadine or better*" without stating a value for the IC_{50} [40d].

In order to investigate the SAR for their compounds, Kolocouris and coworkers have reported a conformational analysis study by a combination of NMR spectroscopy and theoretical calculations. They found that, in general, for the most active compounds the amine nitrogen atom lies in a distance of 1.5 to 2.5 Å away from the 2-adamantyl carbon [47].

5. Aminospiroadamantanes

As part of its monumental work in adamantane chemistry, Kolocouris' group has also reported the synthesis of several aminospiroadamantanes such

as those shown in Fig. 18. These compounds retain the pharmacophore group of rimantadine in the C-2 position of the adamantane ring. Compounds **43b** and **44a** showed to be more than 100 times more active than amantadine when tested against the influenza A_2/Japan/305/57 (H2N2) strain [42a] with SI of 83 and 24, respectively.

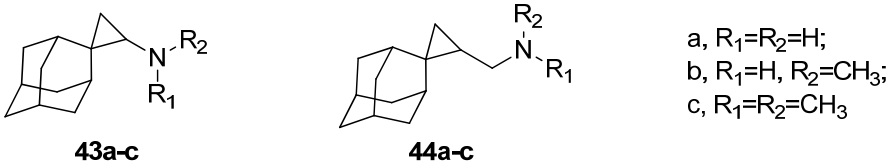


Figure 18. Aminospiroadamantanes **43** and **44**.

Analogs of **43** featuring a cyclobutyl or cyclopentyl ring have also been synthesized and tested against A_2/Japan/305/57 (H2N2) and X-31 (H3N2, with S31N in the M2 protein) strains [38]. Cyclobutyl derivatives **45** had similar potency against the H3N2 strain than rimantadine. Ring enlargement resulted in spirocyclopentane analogs **46** which were less potent than their cyclobutane analogs. When tested against the X-31 strain, all the new compounds showed to be less potent than rimantadine (Fig. 19).

Overall, in going from cyclopropyl analogs **43** to cyclopentyl derivatives **46**, it appears as if increasing the carbon crowding around the spiro carbon leads to compounds with reduced antiviral potency. However, it must be taken into account that Philips-Duphar researchers reported, in the 1970s, that the cyclopentyl derivative **47** had, against the A_2/Japan/305/57 (H2N2) strain, an antiviral activity of the same order of DU 34796, that, as we have already stated, entered clinical trials [40d]. Moreover, very recently, DeGrado's group disclosed in a patent the structure of the cyclohexyl derivative **48**, somehow related to spiropiperidine **41a**. Compound **48**, when tested against the wt M2 channel of influenza A virus expressed in oocytes of *Xenopus laevis*, showed an IC_{50} of 18.7 μM, very similar to that of amantadine (IC_{50} = 16 μM) and was slightly less active than rimantadine (IC_{50} = 10.8 μM). As rimantadine, **48** was inactive against the mutant S31N. However, compound **48** revealed to be a submicromolar inhibitor of the clinically important mutant V27A (IC_{50} = 0.31 μM) and also showed to be active against the mutant L26F (IC_{50} = 5.6 μM). To the best of our knowledge, **48** is the most potent compound ever reported against the mutant V27A [48]. Unfortunately, there is no data regarding the activity of compounds **43-47** against the V27A mutant M2 channel. Worthy of note, analog **49** has not been synthesized yet.

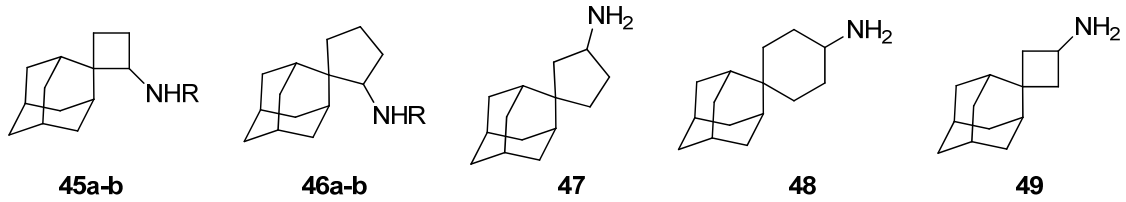


Figure 19. Aminospiroadamantanes **45**-**48** and unkown compound **49**. a, R = H; b, R = methyl.

Taken together the antiviral acitivity of compounds **43** to **48**, it seems that the distance and orientation between the nitrogen atom and the adamantyl cage is more important than the steric hindrance around the spiro carbon atom.

6. 1,2-Annulated adamantane derivatives

In the earlier 1970s, several patents by Squibb claimed anti-influenza activity for a series of 1,2-annulated adamantanopyrrolidines of general structure **50** (Fig. 20), although no much details regarding biological activity were given [49]. Nearly forty years later, Kolocouris's group synthesized several adamantanopyrrolidines **51**-**52**, the related compound **53** and 1,2-annulated adamantanopiperidines of general structures **54**-**56** and tested them against influenza A/Hong Kong/7/87 (H3N2) strain [50].

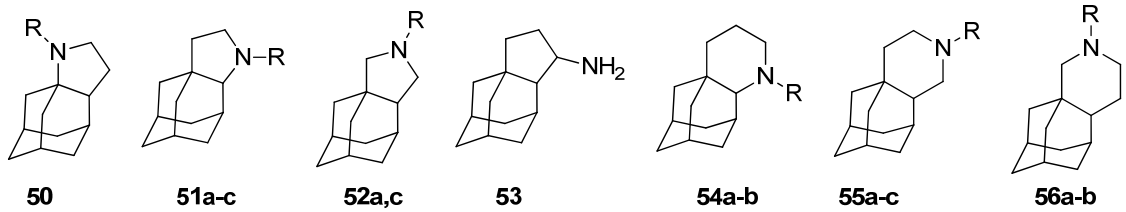


Figure 20. 1,2-Annulated adamantane derivatives **50**-**56**. a, R = H; b, R = methyl; c, R = ethyl.

The compounds **52a** and **56a** elicited submicromolar activities (IC_{50} of 0.5 and 0.6 μM, respectively) and a SI of 732 and 200, respectively, being equipotent to rimantadine (IC_{50} = 0.36 μM). Compounds **51** and **54**, with the nitrogen atom attached directly to the C-2 position of the adamantane ring, showed low micromolar activities (between 2 and 8 μM), similar to that of amantadine (2.0 μM).

As previously seen in other series, these results showed that a large lipophilic moiety in the vicinity of adamantane skeleton is compatible with good anti-viral activity, that moving the amine nitrogen atom away from the

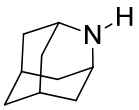
2-adamantyl carbon atom enhaces activity (compare **52a**, IC_{50} = 0.6 μM, with **51a**, IC_{50} = 2.2 μM), and that *N*-alkylation reduced the potency (compare **52a**, IC_{50} = 0.5 μM, with **52c**, IC_{50} = 2.4 μM, or **56a**, IC_{50} = 0.6 μM, with **56b**, IC_{50} > 500 μM).

In closing sections 2 to 5, it should be mentioned that in 2009, K.-C. Chou published a fragment-based quantitative structure-activity relationship (FB-QSAR) study with 34 substituted adamantanes. His main conclusion was that position 2 of the adamantane was more sensitive to substitution than position 1 [51].

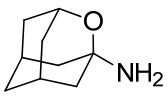
7. 2-Azaadamantanes and (2-oxaadamant-1-yl)amines

Geigy has claimed that 2-azaadamantane, **57** (Fig. 21), first described in 1964 by Stetter et al. [52], displayed antiviral activity against three different influenza A H2N2 strains: A/Bethesda/10/63, A/Taiwan/1/62 and A/Singapore/1/57, but not further progress was published later [53].

More recently, we have found that replacement of the methylene unit of C-2 in amantadine by an oxygen atom to obtain (2-oxaadamant-1-yl)amine, **58**, reduced the antiviral activity [54].



57



58

Figure 21. 2-azaadamantane and (2-oxaadamant-1-yl)amines.

8. BL-1743 and related compounds

In 1995, Bristol-Myers Squibb's researchers carried out a high-throughput screen based on the ability of inhibitors to reverse the toxicity associated with M2 channels expressed in the yeast *Saccharomyces cerevisiae* membranes. They found an azaspiro[5.5]undecane derivative, BL-1743, able to efficiently inhibit the activity of wt influenza A M2 channels (Fig. 22) [55]. The mechanism of action of BL-1743 was further characterized by electrophysiological methods. BL-1743 was also able to inhibit the AM2 channel expressed in *Xenopus* oocytes, as determined using the two-electrode voltage clamp (TEV) technique. It was found that the majority of M2 sequences isolated from influenza viruses resistant to amantadine were also resistant to BL-1743, which suggests that BL-1743 binds competitively with

amantadine. Interestingly, the kinetics of channel inhibition by BL-1743 were more rapid, showing a fast onset of inhibition as well as a reasonably rapid reversal of inhibition following removal of the compound [56]. This behavior contrasts with that of amantadine, whose second-order rate constant for the onset of inhibition is much slower than the diffusion-controlled rate, and whose off-rate is essentially irreversible on the minute to hour time scale of the experiment. The Hill coefficient for inhibition was 1.0, which is consistent with the binding ratio of one BL-1743 per AM2 tetramer [56].

It should be noted that twenty years before the discovery of BL-1743, A. H. Robins Company Inc., in a US patent [57], claimed anti-influenza activity for a series of aminospiranes that were already known from older literature [58]. Compounds **59-61** (Fig. 22) protected chicken embryos against influenza A/Taiwan/1/64 (H2N2) strain better or similarly than amantadine. No information about the activity of these compounds against amantadine-resistant strains was disclosed.

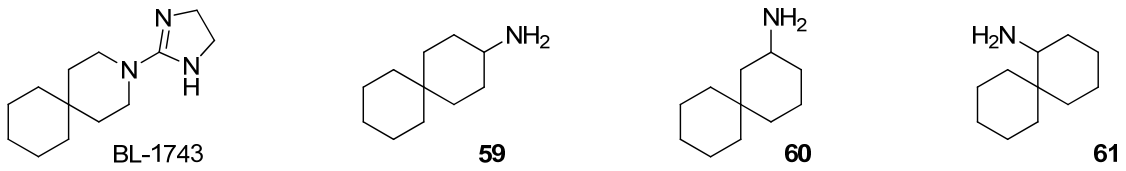


Figure 22. BL-1743 and related aminospiro[5.5]undecanes.

Taking into account the recent determination of the 3-D structure of the M2 ion channel of influenza A virus [19], and the structural difference between BL-1743 and the amantadine class of compounds, in 2008 Pinto's and DeGrado's groups started a SAR study of this scaffold with the aim of discovering new inhibitors of amantadine-resistant mutants [59].

Interestingly, spiropiperidine **62a** (Fig. 23), an analog of BL-1743 lacking the imidazoline group, had an IC_{50} of 0.9 μM against the influenza A wt M2 channel expressed in the *Xenopus* oocytes membrane, which is more than one order of magnitude more potent than amantadine (IC_{50} = 16 μM) and represents a more than 45-fold increase in potency relative to BL-1743 (IC_{50} = 45.3 μM). Alkylation of **62a** with a methyl group to **62b** reduced the potency (IC_{50} = 20.6 μM), and alkylation with larger groups as in **62c** led to inactive compounds. Several *N*-heteroarylmethyl derivatives of **62** were also inactive. Worthy of note, solid-state NMR data indicated that **62a** interacts with influenza A M2 channel differently from amantadine, affecting a longer stretch of the transmembrane helix and immobilizing the G34-I35 region. Ring-contracted analogs **63** and **64** were also active (IC_{50} = 8.1 and 12.0 μM, respectively)

although were less potent than **62a**. Dithiene **65** was moderately active (IC_{50} = 37.6 μM), while ketal **66** was inactive [59a]. Finally, it should be noted that **62a** can be seen as a simplified analog of Kolocouris' spiroadamantane **41a** (Figure 16), a compound that, when tested against influenza A/Hong Kong/7/87 (H3N2) strain, was found to be 12-fold more active than amantadine [44].

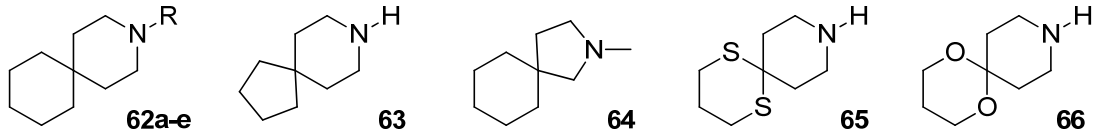


Figure 23. Spiropiperidine **62** and related compounds. a, R = H; b, R = methyl; c, R = methylcyclopropyl; d, R = methyl-2-pyridyl; e, R = methyl-2-imidazolyl.

Moving the nitrogen atom out of the spiro-ring led to the aforementioned amine **59**. DeGrado's group found that **59** had an IC_{50} of 12.6 μM, very similar to amantadine. Analogs **67** and **68** were also active (IC_{50} = 15.7 and 14.6 μM, respectively), while more complex derivatives, such as **69** were inactive (Fig. 24) [59b]. Interestingly, while **59** was less potent than **62a** against the wt channel of influenza A, **59** was active against the amantadine-resistant L26F and V27A mutants (IC_{50} = 30.6 and 84.9 μM, respectively) and also inhibited replication of recombinant mutant viruses bearing these mutations in plaque reduction assays. However, **59** was inactive against the S31N mutant. It is interesting to compare the structure and the activity of **59** with the spiroadamantane **48** (Fig. 19). While **59** and **48** displayed very similar activities against the wt channel (IC_{50} = 12.6 and 18.7 μM, respectively), **48** is much more potent against the amantadine-resistant mutants (V27A, IC_{50} = 0.3 μM; L26F, IC_{50} = 5.6 μM) [48].

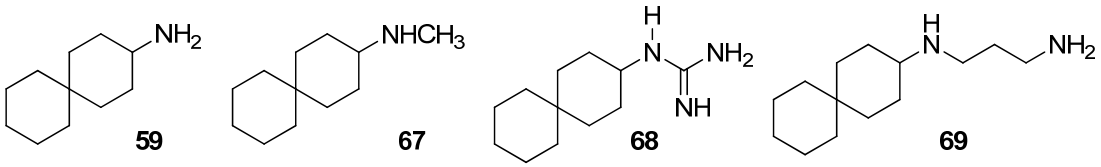


Figure 24. Spiro[5.5]undecan-3-amine **59** and related compounds.

9. Ring-contracted adamantane analogs

For the wt M2 protein, the diameter of the hole made from four Ser31 of separate trans-membrane chains is about 8 Å. However, after the mutation of residue 31 from Ser to Asn, the diameter of this hole was reduced to 6.32 Å [20i]. As the X-ray structure of the M2-amantadine complex shows that

amantadine is located in the hole between Ser31 and Ala34, the mutation of Ser to Asn leaves less space for amantadine entering or being stabilized [19d].

Taking into account this reduction in the space available for binding, we synthesized a series of ring-contracted amantadine and rimantadine analogs, featuring noradamantane and bisnoradamantane scaffolds (Fig. 25). Several derivatives showed low micromolar inhibitory activities of the wt M2 channel ranging from IC_{50} = 2.4 μM for guanidine **72** to IC_{50} = 17 μM for **71** and **75**. The activity was confirmed by plaque reduction assays with influenza A/Udorn/72 (H3N2) strain, carrying wt M2 protein and, for **70** it was also confirmed in an assay of inhibitory effect on virus replication using influenza A/Hong Kong/7/87 (H3N2) strain [60]. However, only bisnoradamantane derivative **74** showed to be moderately active against the S31N channel (IC_{50} = 252 μM), being less potent than amantadine (IC_{50} = 200 μM) [60b]. Several bisnoradamantanes carrying additional rings were also studied and some of them showed to be slightly less potent than amantadine. For example, pyrrolidine derivative **76**, had IC_{50} = 24 μM against the wt channel of influenza A.

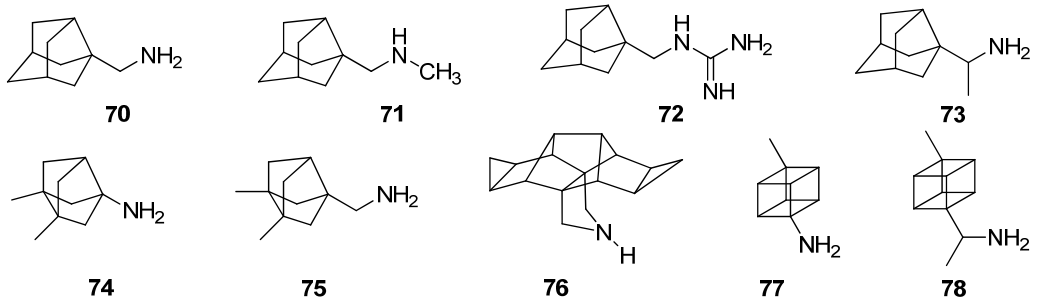


Figure 25. Ring-contracted analogs of amantadine and rimantadine.

Cubylamines also can be regarded as ring-contracted analogs of amantadine and rimantadine. In 1971, Du Pont de Nemours, claimed in a patent anti-influenza activity for several cubane derivatives, such as 4-methylcubane-1-amine, **77**, and α,4-dimethylcubane-1-methylamine, **78** (Fig. 25). When mice infected with the influenza A/Ann Arbor/2/60 (H2N2) strain were treated with the rimantadine analog **78**, there was a 70% survival rate as compared with 20% survivors in the infected, non-treated control animals [61].

10. Aminobicyclo[2.2.1]heptanes, aminobicyclo[2.2.2]octanes and related compounds

As early as in 1969, Smith Kline & French disclosed that bicyclo[2.2.1]heptanes **79-81** (Fig. 26) had anti-influenza activity. They

reported that compound **80**, at oral and subcutaneous doses of 25-100 mg/kg, caused a 35-80% and a 30-75% increase in survival of mice infected with the influenza A/Ann Arbor/2/60 (H2N2) strain, and with a swine strain of influenza A, respectively [62]. Although no further details have been published in the western literature related to the antiviral activity of **79** or **80**, later, Russian researchers found that an isomeric mixture of **81** and **82** effectively inhibited replication of influenza viruses and this mixture, as its hydrochloride, known as deitiforin, has been in used as antiviral in the former USSR for several years [63]. As an anti-influenza drug, deitiforin is equal to rimantadine from the standpoint of the protective effect in the treatment of influenza infection, and it can not only efficiently supress virus-specific growth, but can also selectively act on virus-infected cells. It has been found that influenza A/Victoria/35/72 (H3N2) strain resistant to deitiforin mutated the M2 protein in 3 amino acids: Met14Leu, Ala30Val and Met59Leu [64]. Interestingly, compound ICI 130685, which can be regarded as a derivative of **81** with further rings, advanced into clinical trials, but was not approved for clinical use [65].

García Martínez and coworkers have reported that several 1-norbornylamines were also endowed with potent anti-influenza activity. Secondary amines **83** and **84** were more potent than amantadine and showed very high SI [66].

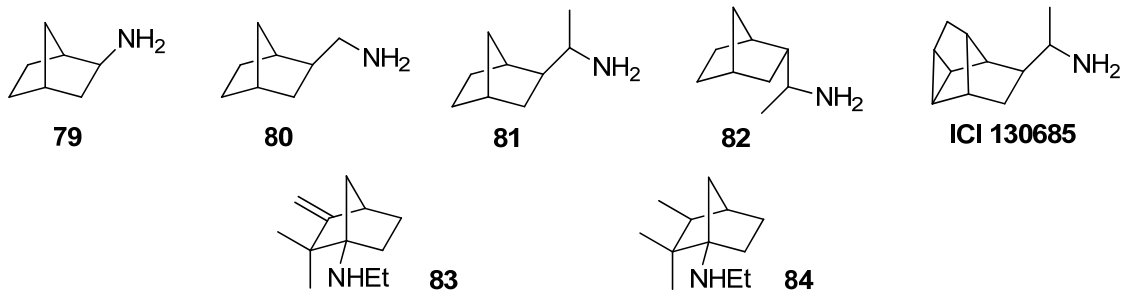
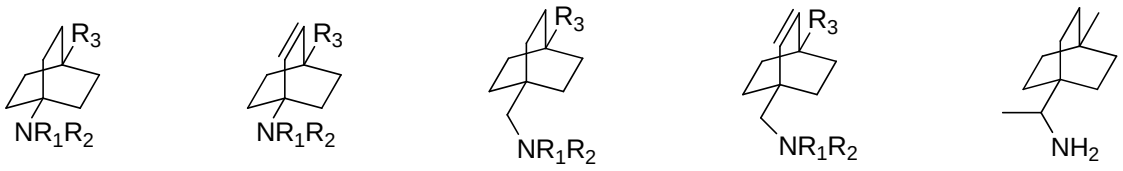


Figure 26. Bicyclo[2.2.1]heptanes with anti-influenza activity.

In 1969, DuPont de Nemours & Co, also claimed anti-influenza activity for a series of bicyclo[2.2.2]octan-1-amines, **85**, bicyclo[2.2.2]oct-2-en-1-amines, **86**, bicyclo[2.2.2]octane-1-methylamines, **87**, and bicyclo[2.2.2]oct-2-ene-1-methylamines, **88** [67]. They tested the compounds in mice using the influenza A/swine/S-15 strain and found that the unsaturated cage amines were similar in antiviral activity to their saturated counterparts. As seen in other families of polycyclic amines, substitution on the amino group with alkyl groups decreased the anti-viral activity. The addition of a methyl group in C-4 of the bicyclo[2.2.2]octane was optimal but inclusion of a larger group

reduced the activity. Finally, the presence of α-alkyl groups in the bicyclo[2.2.2]octane-1-methylamine series enhances antiviral activity. Overall, rimantadine analog **89** was the most active compound.



85 **86** **87** **88** **89**

Figure 27. Bicyclo[2.2.2]octan-1-amine, **85**, and related compounds.

Interestingly, Inamoto and co-workers reported the synthesis of several tricyclo[5.3.1.$0^{3,8}$]undecane (4-homoisotwistane) derivatives, such as amines **90** and **91**. 4-Homoisotwistanes can be seen as bicyclo[2.2.2]octane derivatives carrying an additional ring. Amines **90** and **91** were quite active against the Newcastle disease virus, which is sensitive to amantadine and is the causal agent of a bird disease that, when infecting humans, causes influenza-like symptoms. However, they did not test these compounds against influenza virus [68].

90 **91**

Figure 28. Tricyclo[5.3.1.$0^{3,8}$]undecane derivatives.

11. Other polycycloalkanes with anti-influenza activity

Finally, in this section we will discuss several unrelated polycyclic structures that have shown anti-influenza activity. For example, there are several amines derived from the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane that have been biologically tested. In the 1970s, Smith, Kline & French, reported that amine **92** (Fig. 29) showed marginal activity against influenza A/Ann Arbor, while its isomer **93** was inactive [69]. Very recently, DeGrado and coworkers have found that amine **94** inhibited the activity of the wt M2 channel of influenza A expressed in oocytes of *Xenopus laevis*, with an IC_{50} = 8 μM, lower than that of amantadine (IC_{50} = 16 μM) and rimantadine (IC_{50} = 10.8 μM). As seen in other polycyclic derivatives, the addition of a

hydroxyl group, as in **95**, was compatible with inhibitory activity (IC_{50} = 24 μM), but not increased the potency [30].

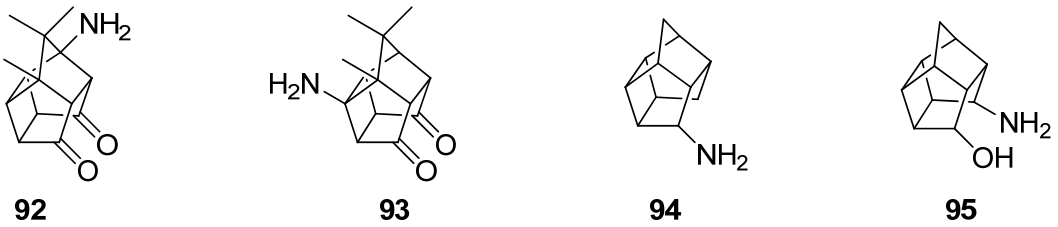


Figure 29. Derivatives of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane.

In 2010, Hu et al. carried out the screening of a small primary amine library as M2 protein inhibitors. They reported that linear alkyl amines, aromatic amines and unsubstituted monocyclic amines were inactive. However, they found five compounds, **14**, previously studied by Scholtissek [33], and **96-99** (Fig. 30), with similar activities to that of amantadine. Isopinocamphenylamine **99**, the most potent inhibitor, was three times more active than amantadine (IC_{50} = 1.4 μM vs IC_{50} = 6.0 μM) for viral inhibition of the influenza A/Hong Kong/8/68 (H3N2) strain [70]. Encouraged by these results, the same group has very recently published a small library of derivatives of **99** obtained by keeping the scaffold constant and modifying the amino functionality. The compounds were evaluated for viral inhibiton against influenza A/WS/33 (H1N1), amantadine resistant, and influenza A/Hong Kong/8/68 (H3N2), amantadine sensitive. Although there was no inhibition of the amantadine resistant strain, most of the compounds exhibited antiviral inhibition as good as amantadine against the amantadine sensitive strain. Compound **100** (IC_{50} = 0.09 μM) was nearly 240-fold more potent than amantadine against wt influenza A virus [71].

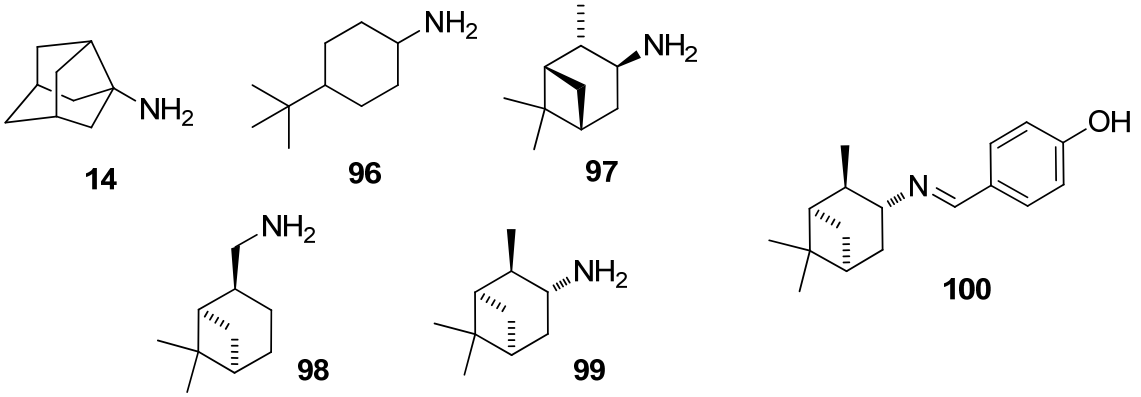


Figure 30. Primary amine inhibitors of M2 channel and derivative **100**.

Finally, DeGrado and coworkers tested the inhibitory activity against wt M2 channel of a series of branched and polycyclic amines (Fig. 21) [30]. Surprisingly, branched alkyl amine **101** was nearly as active as amantadine (IC_{50} = 21 μM vs IC_{50} = 16 μM), tricyclic amine **102**, showed higher activity than amantadine (IC_{50} = 9 μM) and four homoadamantane derivatives, **103**-**105** and **13** showed similar activity as amantadine, suggesting that the M2 channel can accommodate a wide range of structural diversity and that is insensitive to minor scaffold modifications, so long as the shape of the molecule conforms to the M2 cavity. All these compounds were found to be less potent or inactive against V27A and/or S31N mutant channels, probably as a consequence of the higher polarity of the mutant channels [30].

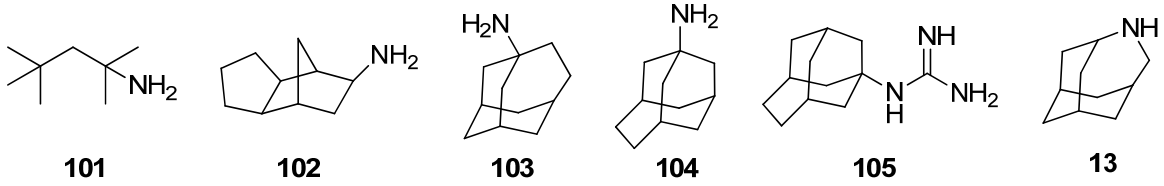


Figure 31. Several inhibitors of M2 channel.

12. Conclusion

Although amantadine and rimantadine have been in clinical use for many years and hundreds of analogs have been tested as anti-influenza agents, the results obtained so far are a bit disappointing. While several active compounds have been found, occasionally having more potency than amantadine and rimantadine, cross-resistance with both drugs is still an unresolved issue. The above notwithstanding, the recent structural, functional, and computational studies carried out with M2 protein have opened the door to the rational design of new inhibitors [72], and, very recently, some derivatives have shown promising activity against the V27A amantadine-resistant mutant [30,48,59]. The S31N mutant is still even a major challenge.

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3. Polyamine metabolism and signaling in plant abiotic stress protection

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Abstract. Polyamines (PAs) are small polycationic compounds present in all living organisms. Compelling evidences indicate a role for PAs in plant protection against stress. During the recent years, genetic, molecular and 'omic' approaches have been undertaken to unravel the role of PAs in stress signaling. Overall, results point to intricate relationships between PAs, stress hormone pathways and ROS signaling. Such cross-regulations condition stress signaling through the modulation of abscisic acid (ABA) and ROS amplification-loops. In this chapter we compile our recent findings which elucidate molecular mechanisms and signaling pathways by which PAs contribute to stress protection in plants.

1. Introduction

1.1. The importance of crop protection against abiotic stress

Abiotic stresses such as cold/freezing, salinity, heat and drought represent serious threats to agriculture. About 70% of yield losses among crops are attributed to abiotic stresses. Climatic change is predicted to increase global temperature, alter precipitation patterns, and intensify drought, increasing the need to grow crops in saline soil [1,2]. Within the

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European Union, the area affected by drought has doubled in 1991-2006, causing an estimated loss of € 8.7 billion in 2003, and up to 25% of yield losses in 2006. The specific threat of drought was acknowledged by an EU impact assessment calling for a multi-faceted policy including the 'use of more drought resistant crops'. Drought damages cannot be however viewed in isolation as they are often accompanied in the field by other stresses, such as heat, high light and increasing ozone concentrations. According to environmental predictions, during the 21st century global effects of desertification, salinization and atmospheric pollutants will produce severe limitations in arable lands with dramatic consequences in crop productivity.

In response to abiotic stress, plants initiate a plethora of biochemical and physiological changes. Importantly, a remarkable natural diversity exists in the ability of plants to cope with various stresses, ranging from highly sensitive plants to more tolerant ones. Thus, there is a genetic potential for plants to adapt to these stresses, preserving growth and/or high yield, but this potential has not been the main selective criteria in the domestication process, which yielded many crop cultivars that poorly cope with stress conditions. The past decade of international research characterized about 40 to 50% of gene functions conserved in the model plants Arabidopsis and rice, and sorted them into specific pathways. These efforts have created network models of stress and hormone regulatory pathways, as well as the definition of frameworks of co-regulated target genes of abiotic stress response pathways e.g. [3]. Except for few regulatory genes, transgenic approaches with individual stress-regulated candidate genes made so far little impact in breeding [4]. By contrast, regulation of the metabolism of compatible osmolytes, proline and polyamines (PAs) in particular, has emerged as more promising approach to practical applications. Elevated levels of PAs are one of the most remarkable changes that occur in plants in response to abiotic stress conditions [5]. The PA pathway interacts with metabolic routes of several signaling molecules (i.e. ethylene, NO, hydrogen peroxide) involved in abiotic stress responses [5]. Transcriptomic studies revealed differential regulation of genes in PA metabolism in response to different types of abiotic stress indicating that PAs are key regulatory molecules in abiotic stress signaling [6]. Here we focus on recent advances about the role of PAs in drought stress performed in the model species *Arabidopsis thaliana*, and discuss future perspectives and potential applications in crop protection.

1.2. Polyamine biosynthesis, catabolism and conjugation

Polyamines (PAs) are small polycationic compounds of low molecular weight which are present in all living organisms [5]. Most abundant

polyamines are the diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm) (Fig. 1). Presence of amine groups in their chemical structure provide positive charges under physiological pH, which allows the binding of PAs to negatively charged macromolecules such as DNA, proteins and phospholids [5,7]. In all living organisms, the first PA synthesized is Put by decarboxylation of ornithine through an enzymatic reaction catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17; Fig. 1). Plants and bacteria contain an alternative route to Put production by decarboxylation of arginine by arginine decarboxylase (ADC, EC. 4.1.1.19; Fig. 1). The product of ADC activity is agmatine, which is converted into Put in two enzymatic steps catalyzed by agmatine iminohydrolase (AIH, EC 3.4.3.12) and N-carbamoyl putrescine amidohydrolase (CPA, EC 3.5.1.53) (Fig. 1). Higher molecular weight PAs are produced by sequential addition of aminopropyl moieties to the Put skeleton through enzymatic reactions catalyzed by spermidine and spermine synthases (SPDS, EC 2.5.1.16 and SPMS, EC 2.5.1.22; Fig. 1). Donor of aminopropyl groups is decarboxylated S-adenosyl methionine (dcSAM), which is synthesized from decarboxylation of S-adenosyl methionine (SAM) by SAM decarboxylases (SAMDC, EC 4.1.1.50; Fig. 1).

The levels of free PAs depend on their biosynthesis, but also catabolism, transport and conjugation [5]. Polyamines are catabolized through diamine oxidases (DAO, EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAOs catalyze the oxidation of Put producing 4-aminobutanal, H_2O_2 and ammonia. DAOs are present in monocots and dicots, but genes encoding these enzymes have been documented in few species [8]. PAOs bear a non-covalently bound molecule of flavin adenine dinucleotide (FAD) and are present at high levels in monocots [9]. PAOs are involved either in catabolism or back-conversion of PAs [5]. From the first group of PAOs, the maize PAO (ZmPAO) is the best characterized. ZmPAO is involved in the terminal catabolism of Spd and Spm producing 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (Dap) and H_2O_2 [8], Fig. 1. The second group of plant PAOs resembles mammalian Spm oxidases (SMO, EC 1.5.3.3) that catalyze the back-conversion of Spm to Spd with concomitant production of 3-aminopropanal and H_2O_2 [10].

As anticipated, regulation of free PA contents is also achieved through their conjugation to hydroxycinnamic acids. So far, caffeoylputrescine, coumaroylputrescine, feruloylputrescine, coumaroylagmatine, dicoumaroyls-permidine, diferuloylspermidine, diferuloylspermine and feruloyltyramine have been identified in different plant species [11]. The ratios between free and conjugated PAs vary between plant species, being the conjugated forms especially abundant in Solanaceae [5].

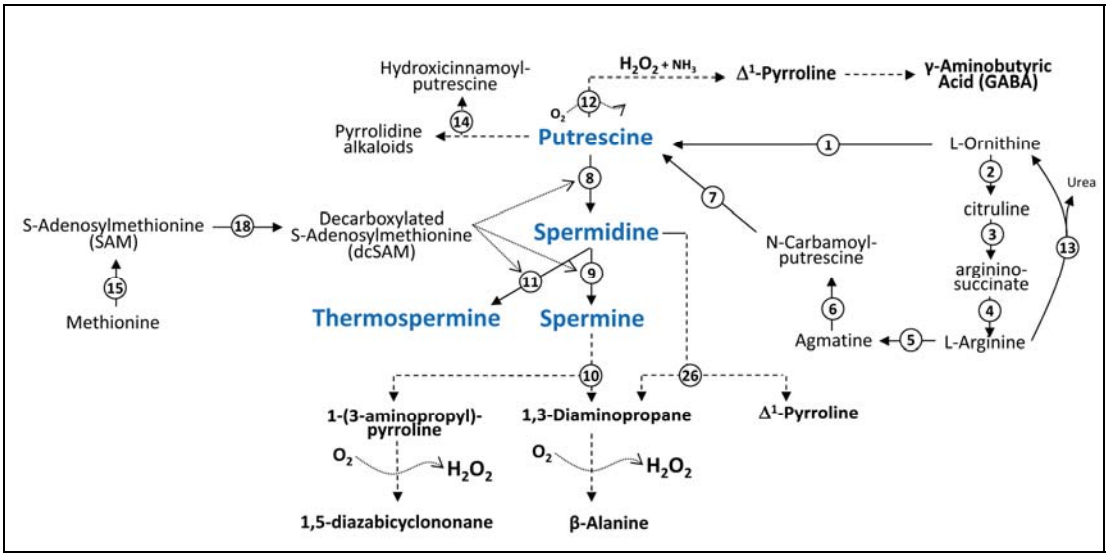

Figure 1. Polyamine metabolic pathway. Numbers refer to the following enzymes: 1, ornithine decarboxylase; 2, ornithine-carbamoyl transferase; 3, argininosuccinate synthase; 4, argininosuccinate lyase; 5, arginine decarboxylase; 6, agmatine iminohydrolase; 7, N-carbamoylputrescine amidohydrolase; 8, spermidine synthase; 9, spermine synthase; 10, polyamine oxidase (also involved in back-conversion to Spd and Spm); thermospermine synthase ACL5; 12, diamine oxidase; 13, arginase; 14, putrescine hydroxycinnamoyl transferase; 15, SAM synthetase; 18, SAM decarboxylase; 26, polyamine oxidase.

1.3. *Arabidopsis thaliana*: A model for polyamine research

Arabidopsis thaliana is a wild species distributed all over the world, mainly in the northern hemisphere. Its reduced size, easy transformation, high natural genetic variation and large number of molecular and genetic tools have made this species a model for molecular and genetic analyses. Also, knowledge derived from *A. thaliana* has successfully been applied to crop species.

The sequencing of the *A. thaliana* genome revealed the absence of the ODC pathway in this plant species [12]. Remarkably, ODC encoding genes are also absent in the genome of *A. lyrata*, ancestor of *A. thaliana*, thus suggesting that loss of *ODC* pathway occurred before the split between *A. thaliana* and *A. lyrata* lineages. *AIH* and *CPA* are found as single copy genes in *A. thaliana* [13,14]. Mutations in either *AIH* or *CPA* that disrupt their enzymatic activities lead to embryo lethality, thus evidencing the importance of keeping a minimum pool of PAs for plant survival (Alcázar *et al.*, unpublished results). Whereas *AIH* and *CPA* are found as single copy genes, *ADC* and *SPDS* encoding genes are found as duplicates (*ADC1*, *ADC2*, *SPDS1* and *SPDS2*) [7]. The finding of gene duplicates in *A. thaliana*

is frequent, due to the occurrence of large genome duplications in this species. However, the different gene paralogs may have evolved different *cis* elements in their promoters that provide differential transcriptional responses under stress [7]. This is the case for *ADC1* and *ADC2*. Whereas the expression of *ADC1* is highly up-regulated in response to cold [15], *ADC2* is responsive to drought, oxidative stress, salinity and biotic stress [7]. In addition, *ADC2* mRNA levels under non-stressed conditions are much lower than *ADC1*, which shows a more constitutive expression [16]. SPDS are also encoded by two genes namely *SPDS1* and *SPDS2*, whereas only one gene is found to encode SPMS [7]. Before its functional characterization, the *ACL5* gene was annotated as a spermine synthase [17]. This gene was identified in screens for mutants showing reduced stem size [17]. However *acl5* mutants do not show obvious reductions in Spm content (Alcázar *et al.*, unpublished observations). In the recent years it has been demonstrated that *ACL5* does not code for a spermine synthase, but for thermospermine synthase [18,19], thus evidencing that Spm biosynthesis also depends on a single gene (*SPMS*). Whereas depletion of Put and Spd levels in *A. thaliana* lead to loss of viability [20,21], the double *acl5/spms* mutant is still viable, thus evidencing that Spm and tSpm are not required for cell survival [22]. Nonetheless, *acl5/spms* mutants are more sensitive to stress conditions [23], although it remains to be clarified if this is due to pleiotropic effects of *acl5* mutation (Alcázar *et al.*, unpublished observations).

For an efficient metabolic canalization, some enzymatic pathways are assembled in macromolecular complexes called metabolomes. In the recent years our group has reported the first metabolon in plants involving aminopropyl transferases SPDS and SPMS [24]. Through a yeast two hybrid screen using one SPDS as bait, our group identified SPDS and SPMS interacting proteins [24]. Remarkably, ACL5 did not interact with SPDS or SPMS [24]. Through gel fractionation experiments from plant cell protein extracts, SPDS-SPMS protein assemblies were found associated to higher molecular weight complexes for which the molecular partners have not yet been identified [24]. The association between SPDS and SPMS in *A. thaliana* would provide an efficient canalization of the PA Put to Spm, something that has recently been observed in different species [25]. It remains to be studied at the proteomic level if the other components of the PA biosynthetic pathway (e.g. AIH, CPA, ADC and SAMDC) belong to the same macromolecular complex. Efforts are currently underway.

Arabidopsis thaliana contains five genes encoding putative PAOs [7]. PAO1 and PAO4 catalyze the same reaction as SMO [26,27], while PAO3 acts in the back-conversion pathway, converting Spm to Spd and Spd to Put [10]. The third class of plant PAO-domain proteins are relatives of the human

lysine-specific demethylase 1 (LSD1) that possesses an amine oxidase domain similar to that of FAD-dependent PAOs [28]. LSD1 acts as a histone demethylase, representing an important regulator of chromatin structure and gene expression [29]. *Arabidopsis* has four LSD1-related genes, some of which participate in the repression of *FLC*, a negative regulator of flowering time [30,31].

In the recent years, increasing interest has been shown to characterize the function of PAs in abiotic stress tolerance. In the following sections, we summarize where indications are found that PAs are key molecules in abiotic stress signaling and protection. Due to space limitations, we have focused on the role of PAs in drought stress, although our research laboratory is also interested in cold, salinity and we plan to get insight into biotic stress as well.

2. Polyamines in drought stress

The perception of water stress is rapidly sensed by plants and translated into a molecular signal which involves activation of mitogen-activated protein (MAP) kinase cascades, protein phosphatases, phospholipid signaling and multiple posttranslational modifications [32]. These signals induce transcriptional reprogramming of drought responsive genes, which are required to survive dehydration [32]. Eventually, stress signaling pathway activation leads to the accumulation of different osmolytes to cope with dehydration conditions. PAs accumulate to high levels in response to drought, consistent with their role on drought protection. In the recent years, molecular and genetic studies have revealed specific mechanisms of PAs in drought protection and signaling pathways involved. We describe in the following sections recent findings performed by our group evidencing PA cross-regulations with stress hormone pathways and metabolic canalizations of PAs in response to dehydration, in line with the PA-metabolon previously described [24].

2.1. ABA regulates PA-responsiveness to drought

The phytohormone abscisic acid (ABA) plays a key role in drought signaling and protection. Many drought-inducible genes are ABA-responsive, but also ABA-independent pathways are activated in response to drought conditions [33]. In order to determine the involvement of ABA in the transcriptional regulation of the PA biosynthetic pathway, Alcázar *et al.* [34] analyzed the expression of PA biosynthetic genes *ADC1, ADC2, AIH, CPA, SPDS1, SPDS2, SPMS, ACL5, SAMDC1* and *SAMDC2* in *A. thaliana* wild type plants and mutants impaired in ABA biosynthesis (*aba2-3*) or signaling (*abi1-1*). The ABA-deficient *aba2* mutants are blocked in the conversion of

xanthoxin to ABA-aldehyde and contain reduced levels of ABA in seeds and leaves [35]. These mutants also show reduced accumulation of ABA in response to drought conditions [35]. The *abi1* mutation affects ABA sensitivity in vegetative tissues and several ABA-mediated stress responses [36].

Wild type plants, *aba2-3* and *abi1-1* mutants where exposed to severe dehydration conditions during 24 h, and the expression of PA biosynthetic genes analyzed by quantitative RT-PCR after 0, 1, 2, 4, 8 and 24 h of treatment. *ADC2*, *SPDS1* and *SPMS* genes were among the most responsive to drought treatment under the imposed drought conditions (Fig. 2). Indeed, *ADC2* and *SPDS1* expression increased up to 32- and 25-fold respectively, whereas *SPMS* expression increased 75-fold after 24 h of treatment [34]. These observations suggested a key role of *ADC2, SPDS1* and *SPMS* conferring drought tolerance. Interestingly, whereas *ADC2* and *SPDS1* expression increased several fold after drought treatment, the expression of their gene paralogs *ADC1* and *SPDS2* did not change substantially [34]. These observations are consistent with the acquisition of certain stress-specificity probably due to divergent evolution of *cis* regulatory elements in their promoters. Indeed, different *cis* regulatory elements are found in the promoters of PA biosynthetic genes (Fig. 3). ABA-responsive elements (ABRE) or ABRE-related motifs are also found in the promoters of *ADC2, SPDS1* and *SPMS* [7], highly up-regulated in response to drought stress (Fig. 3).

The analysis in *aba2-3* and *abi1-1* mutants exposed to drought conditions showed much more moderate increases in *ADC2*, *SPDS1* and *SPMS* expression (Fig. 2) [34]. Hence, *ADC2* increased to a maximum of 7.5-fold after 24 h of treatment, which represented a reduction of 78% in fold induction compared to the stressed wild type [34]. *SDPS1* only increased up to 3.2-fold after 8 h (87% less increase than wild type) and *SPMS* increased 3.2-fold after 24 h (96% less than wild type). These results evidence that transcriptional up-regulation of *ADC2*, *SDPS1* and *SPMS* imposed by drought stress is mediated by ABA. Hence, ABA is an upstream regulator of PA biosynthesis in response to drought (Fig. 4).

To determine the effect of the transcriptional regulation of PA biosynthetic genes on PA levels, we analyzed the content of Put, Spd and Spm levels in response to drought. Wild type plants showed a progressive accumulation of Put in response to drought conditions, whereas this accumulation was absent in *aba2-3* and *abi1* mutants (Fig. 5) [34]. Hence, the ABA-dependent up-regulation in *ADC2* expression observed under drought conditions leads to an effective Put accumulation. An interesting finding from these results was the progressive reduction is Spm levels observed during dehydration, which was identified later on as a Put to Spm metabolic canalization coupled to back-conversion which serves as reactive oxygen species (ROS) amplification signal [25].

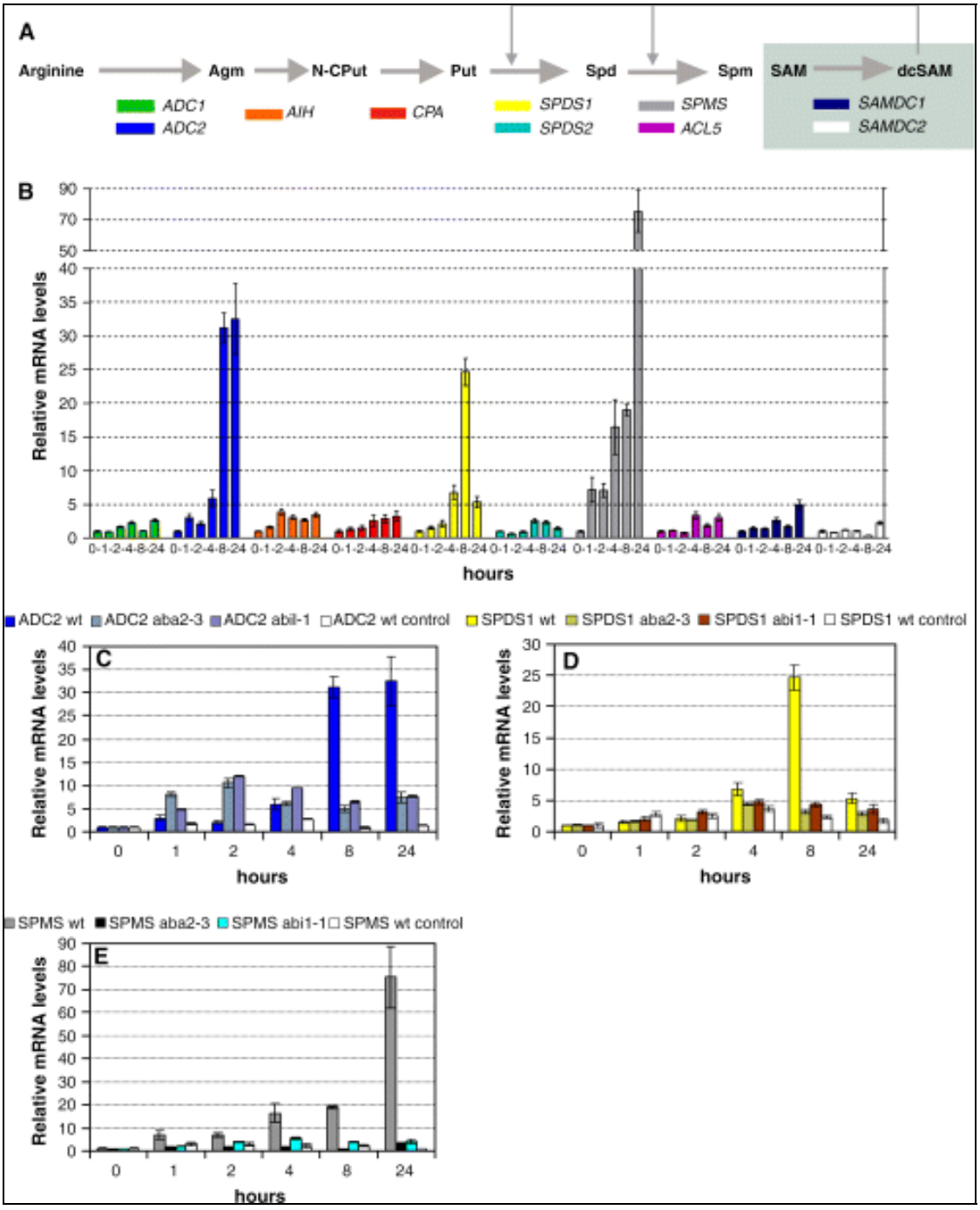

Figure 2. (A) Polyamine (PA)-biosynthetic pathway in *Arabidopsis thaliana*. **(B)** Relative transcript levels of PA-biosynthetic genes encoding arginine decarboxylase (*ADC1*, *ADC2*), agmatine iminohydrolase (*AIH*), N-carbamoylputrescine amidohydrolase (*CPA*), spermidine synthase (*SPDS1, SPDS2*), spermine synthase (*SPMS, ACL5*), S-adenosylmethionine decarboxylase (*SAMDC1*, *SAMDC2*) in wild-type (wt) plants exposed to water stress after 0, 1, 2, 4, 8 and 24 h of treatment. **(C)** Relative transcript levels of *ADC2*, **(D)** *SPDS1* and **(E)** *SPMS* in wt, *aba2-3* and *abi1-1* plants subjected to water stress, and non-stressed wt. These results were published by Alcázar *et al*. [34].

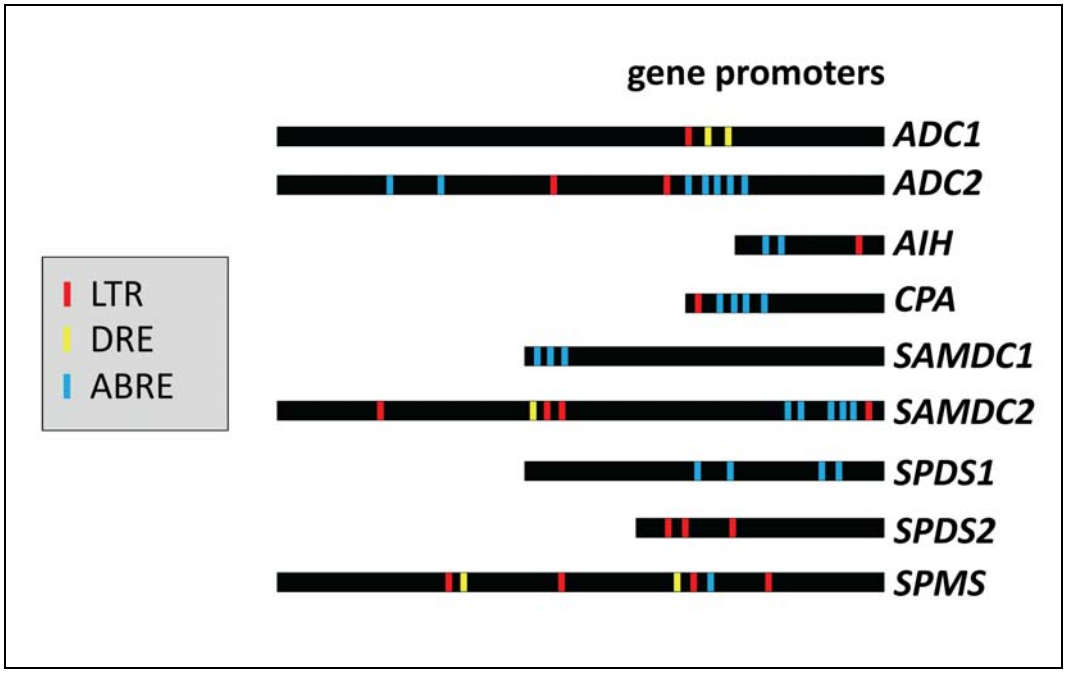

Figure 3. Cis-regulatory elements found in the promoters of PA biosynthetic genes. LTR, low temperature response element; DRE, dehydration responsive element; ABRE, ABA-responsive element. Picture adapted from Alcázar *et al.* [7].

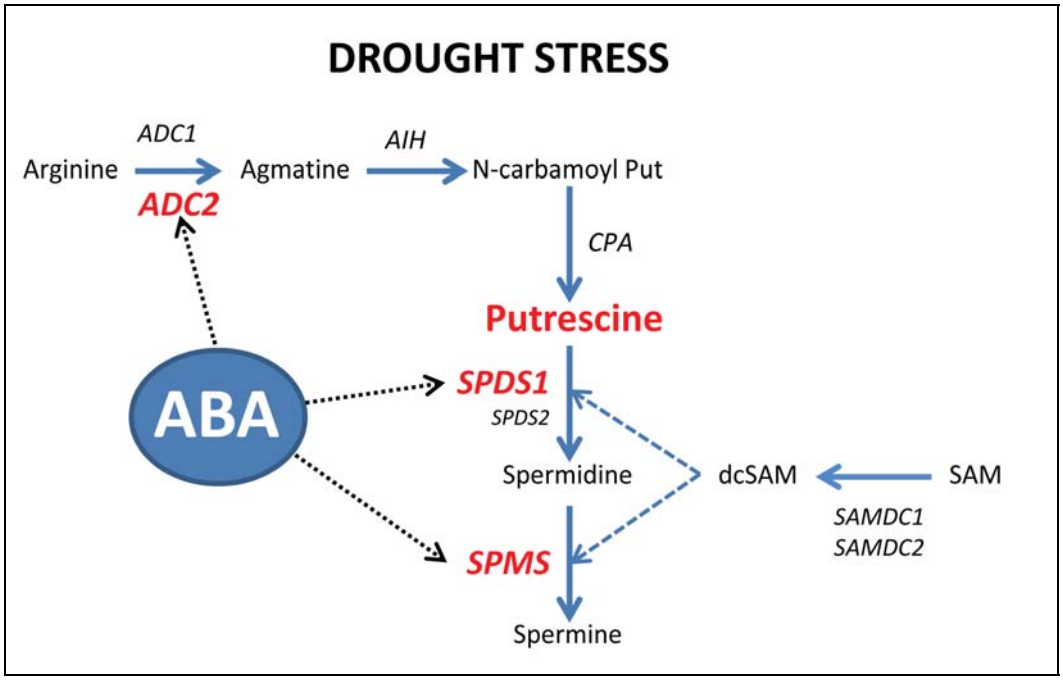

Figure 4. Scheme of the transcriptional regulation of PA biosynthesis by ABA. Drought stress leads to an increase in ABA levels which enhances the expression of ABA-responsive *ADC2*, *SPDS1* and *SPMS* genes. The increase in *ADC2* expression leads to Put accumulation, whereas increases in the expression of *SPDS1* and *SPMS* do not lead to accumulation of Spd or Spm.

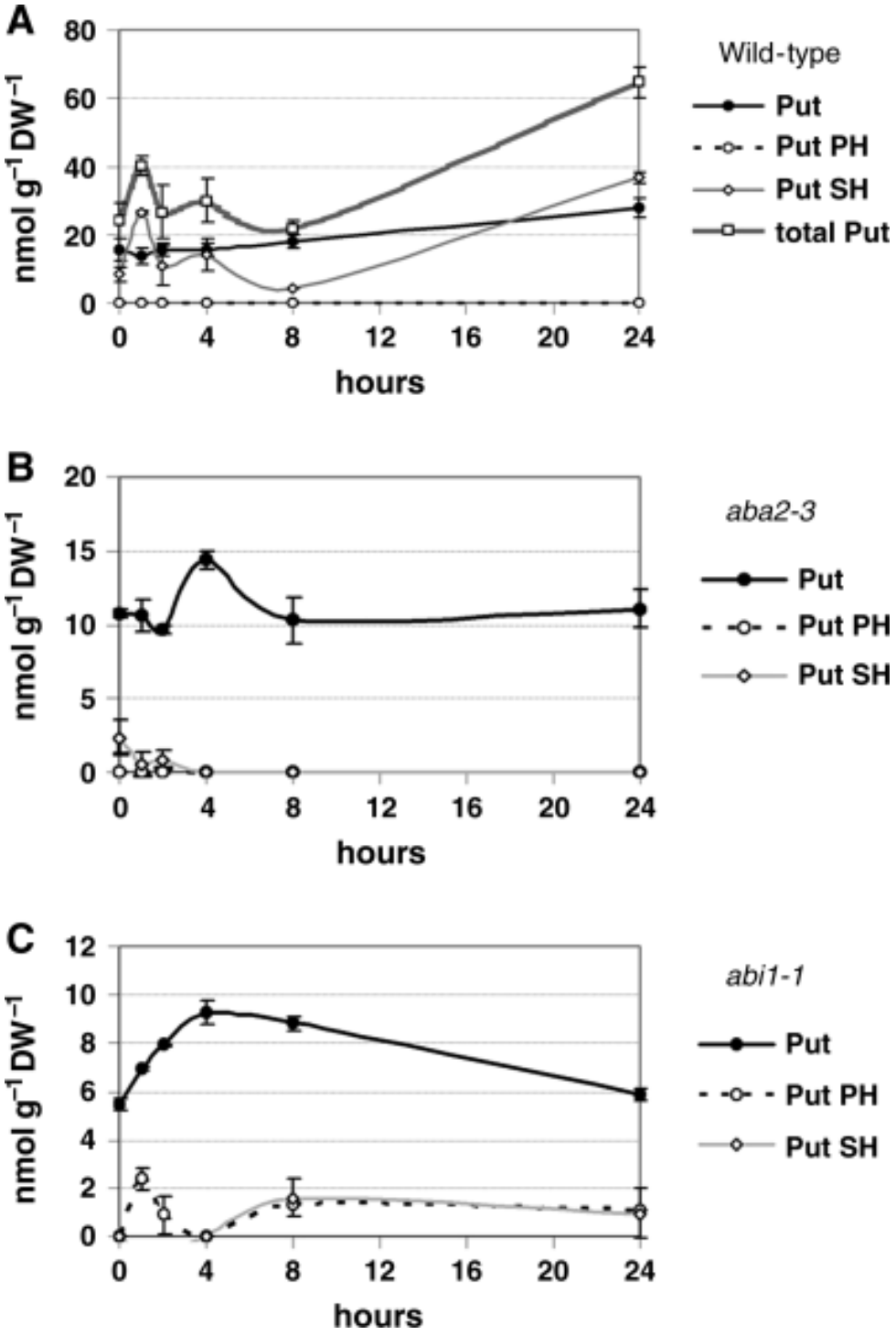

Figure 5. (A) Levels of putrescine (Put) from different polyamine fractions in wild-type, (B) *aba2-3* and (C) *abi1-1* plants exposed to water stress. Leaves from at least five plants per point of analysis were sampled after 0, 1, 2, 4, 8 and 24 h of stress treatment. Free, insoluble conjugated and soluble conjugated Put levels are referred as nmol g−1 dried weight (DW). Total levels of Put are also shown for wild-type plants. Values are mean ± standard error of three replicates in each one from three independent experiments. Published by Alcázar *et al.* [34].

2.2. Putrescine to spermine canalization in response to drought in *A. thaliana* and resurrection plants

A number of drought tolerant species have been used to study the molecular basis of desiccation tolerance. Among them, the most well characterized example is the South African resurrection plant *Craterostigma plantagineum*.

In a recent article by Alcázar *et al.* [25] we have compared the PA profiles and transcriptional responses of *A. thaliana* wild type plants and PA levels of *adc1-3*, *adc2-3*, *spds1-2*, *spds2-3* and *spms-2* mutants under a gradual drought acclimation response to the PA profiles of the resurrection plant *C. plantagineum* exposed to desiccation treatment. This is the first report on PA levels in a resurrection plant that provided clue on the differential regulation of the PA biosynthetic pathway between drought tolerant (*C. plantagineum*) and drought sensitive (*A. thaliana*) species.

In this work, we exposed plants to a progressive drought acclimation response by withholding water for 16 days and analyzed PA levels after 0, 2, 4, 6, 8, 10, 12, 14 and 16 days of treatment. The levels of Put accumulated to 1.8-fold after 2 days of treatment in wild type plants, which was in agreement with previous observations [34]. Interestingly, higher Put accumulation was observed in *spds1-2* mutant compared to wild type after 2 and 6 days (Fig. 6) [25]. As described in the introduction, *SPDS1* encodes one of the two *SPDS* gene paralogs that catalyze the conversion of Put to Spd. The accumulation of the precursor (Put) in *spds1-2* mutant under drought stress indicates that SPDS1 enzyme is involved in the Put to Spd conversion in response to dehydration [25]. Remarkably, peaks for Put accumulation correlate with higher *ADC2* expression [25]. On the other hand, mutations in *SPDS2* in the *spds2-3* mutant did not lead to evident increases in Put content in response to drought compared to wild type plants (Fig. 6) [25]. These observations evidence that *SPDS1* and not *SPDS2* is involved in the conversion from Put to Spd under drought stress [25,34].

An interesting finding in this work [25] was the absence of Spd accumulation (Fig. 6) even though a Put to Spd conversion was detected and mediated by *SPDS1*, thus suggesting that conversion to higher molecular weight polyamines (Spm) or degradation of Spd by PAO activity may contribute to Spd homeostasis. Indeed, the expression of deoxyhypusine synthase (*DHS*) followed similar kinetics to *SPDS1* and other ABA-inducible genes (e.g. *RD29A* and *RD22*) [25].

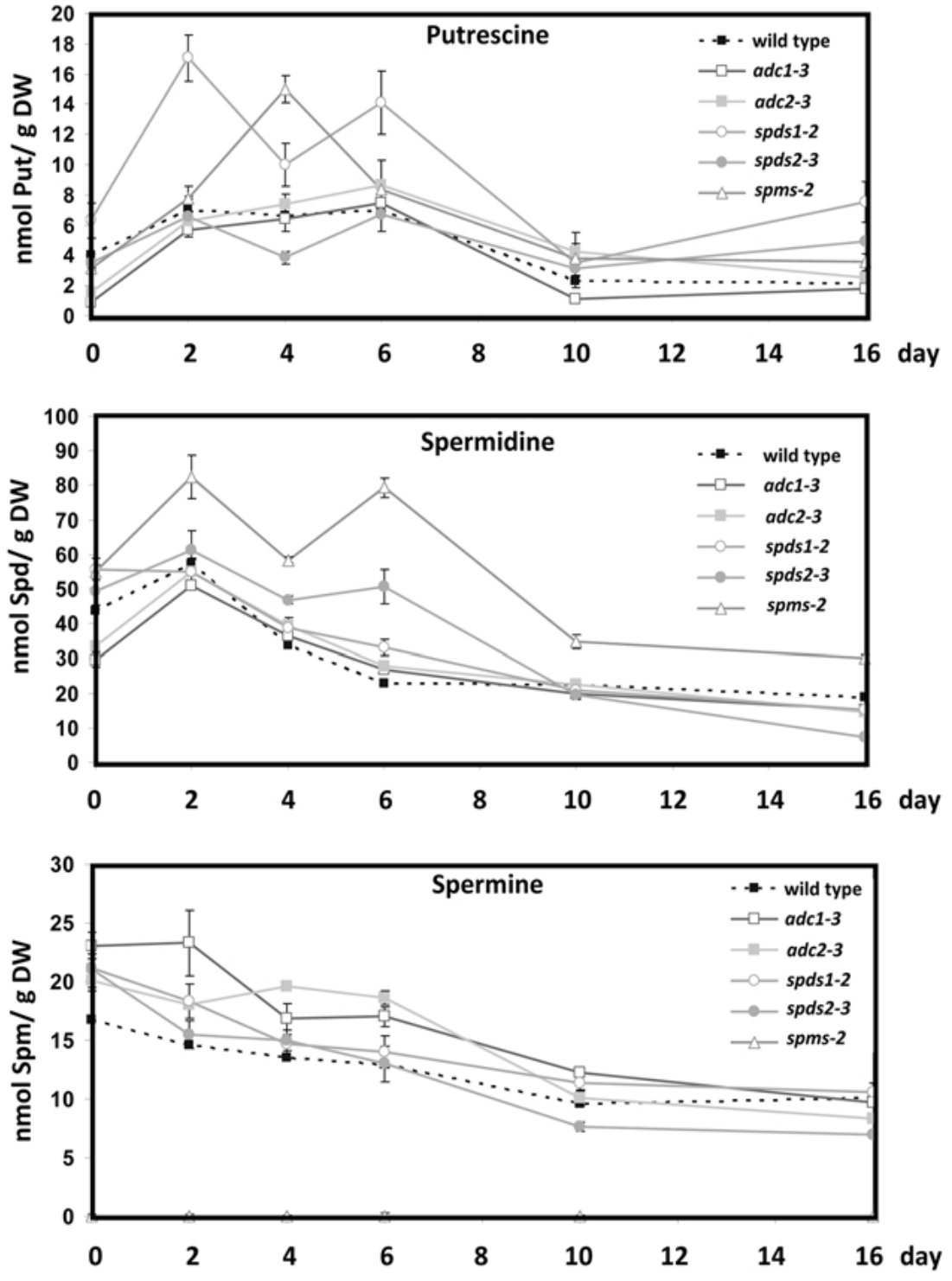

Figure 6. Polyamine (PA) profiles under drought stress in PA-biosynthetic mutants and wild-type *Arabidopsis thaliana* plants. Free putrescine, spermidine and spermine levels were analyzed in wild-type, *adc1–3*, *adc2–3*, *spds1–2*, *spds2–3* and *spms-2* mutants after 0, 2, 4, 6, 10 and 16 days of drought treatment. Values are the mean from three biological replicates ±standard deviation (SD). DW, dry weight. Results published by Alcázar *et al.* [25].

The deoxyhypusine synthase enzyme catalyzes the NAD-dependent formation of deoxyhypusine in the eukaryotic translation initiation factor 5A (eIF-5A), which requires Spd as substrate [37]. However, whether DHS activity significantly contributes to Spd homeostasis requires further analysis.

The conversion of Spd to Spm under drought stress was not observable in wild type plants (Fig. 6), which instead of accumulating Spm showed a progressive reduction is Spm levels [25]. However, loss of function of *SPMS*, involved in the enzymatic conversion of Spd and Spm, lead to evident increases in Spd and Put precursor in response to drought [25]. These observations were consistent with a Put to Spm canalization in response to drought that did not lead to the accumulation of the higher molecular weight polyamine Spm. To further determine a possible role of PAO in the oxidative deamination of Spm and depletion of Spm pools in response to drought, we measured Spm oxidase activity by detection of radiolabelled 1,5-diazabicyclononane in protein extracts supplemented with [^{14}C] Spm (Figure 7) [25]. SMO activity was detectable in wild type protein extracts, but did not increase in response to the imposed drought conditions [25].

These observations indicated that depletion of Spm pools was not due to Spm degradation. The reason of why Spm did not accumulate regardless of a strong Put to Spm canalization and absence of Spm degradation could involve the back-conversion pathway (Fig. 8). In the recent years, PAO involved in the back-conversion of Spm to Spd and Put have been characterized [5]. In *A. thaliana*, PAOs *AtPAO2* and *AtPAO3* are involved in the back-conversion of Spm to Put via Spd [10]. Interestingly, the expression of these two *PAO* is induced by ABA [10], which suggests a possible role in

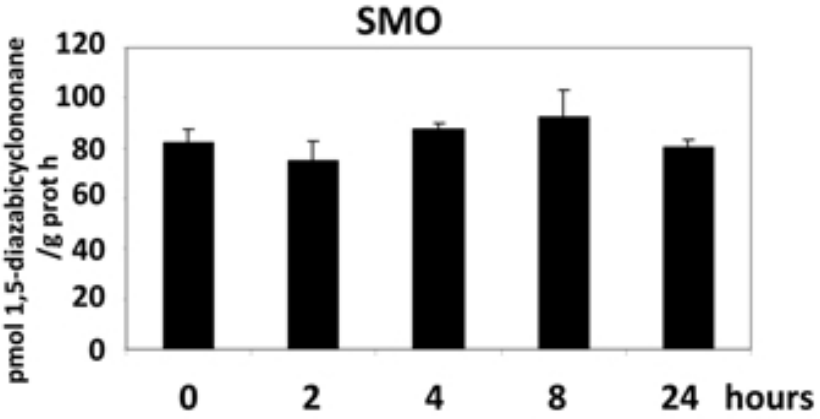

Figure 7. SMO enzymatic activities under drought. Wild type Arabidopsis plants exposed to drought stress were used for the analysis of spermine oxidase (SMO) activity at different time points of 24 h drought treatment. Values are the mean from three biological replicates ±SD. Results published by Alcázar *et al.* [25].

drought stress. Indeed, an increased expression of *AtPAO2* in response to drought was observed in wild type plants and followed similar expression kinetics as ABA-inducible genes *RD29A* and *RD22* [25]. Therefore, our results pointed to an active participation of the back-conversion pathway in the depletion of Spd and Spm pools during drought stress (Fig. 8).

A possible scenario for the contribution of PA back-conversion pathway under drought stress is the occurrence of a PA recycling loop that would serve as ROS signaling amplification by recurrent generation of hydrogen peroxide. Indeed, ROS signaling mediates many abiotic and biotic stress responses and is involved in activation of mitogen activated protein (MAP) kinase cascades [38].

In this work [25], we also analyzed the PA levels in response to drought in the resurrection plant *C. plantagineum*. During the course of the dehydration treatment, the levels of Spd and Spm in *C. plantagineum* progressively increase up to 3-fold and 8-fold, respectively, during 96 h of treatment (Fig. 9) [25]. Accumulation of Spd and Spm and consumption of the Put precursor correlated with enhanced drought tolerance. Hence, it is likely that Put to Spm canalization is an evolutionary conserved response between species, whereas the capability to accumulate high Spd and Spm levels discerns between drought tolerant or intolerant plants. These observations open a gate to manipulate PA levels for the development of plants with enhanced drought resistance. The modification of PA levels in plants can be achieved through transgenic manipulation or by exploitation of the natural variation in PA levels already present in nature.

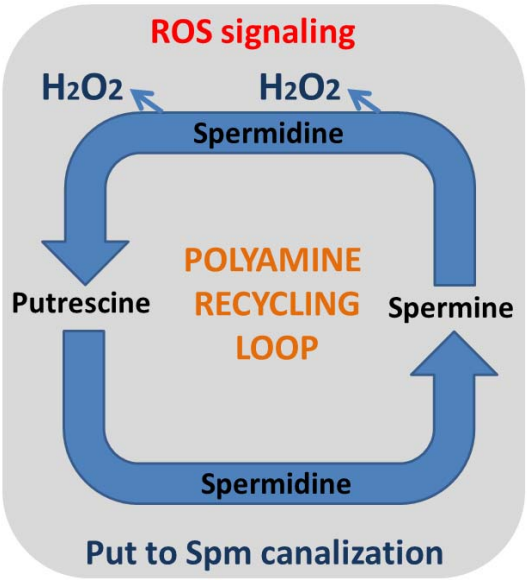

Figure 8. Polyamine recycling loop. Drought treatment leads to a Put to Spm canalization which is coupled with a Spm to Put back-conversion, releasing hydrogen peroxide that would serve as amplification of ROS signaling.

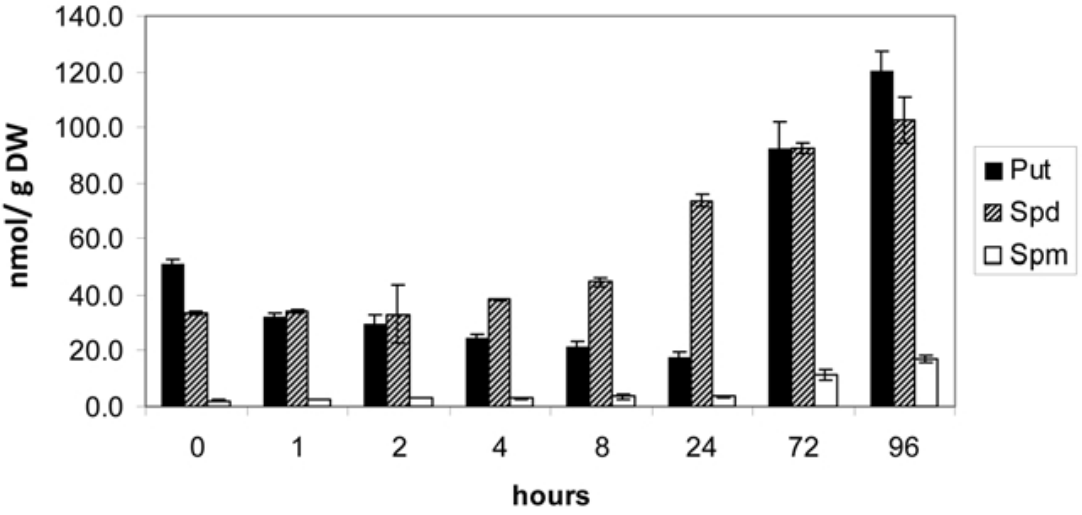

Figure 9. Free putrescine (Put), spermidine (Spd) and spermine (Spm) levels in *Craterostigma plantagineum* plants exposed to drought stress conditions for 0, 1, 2, 4, 8, 24, 72 and 96 hours. Values are the mean from three biological replicates ±SD. DW, dried weight. Results were published by Alcázar *et al.* [25].

2.3. Drought tolerance by genetic engineering of PA levels

The expression of *ADC2* is highly up-regulated in response to drought, and translated in an accumulation of Put. To determine a potential role of *ADC2* in conferring drought tolerance, we transformed *A. thaliana* plants with the homologous *ADC2* gene under the constitutive CaMV 35s promoter. The different lines analyzed (2.1, 3.6, 7.2) showed contrasting degrees of *ADC2* expression and Put accumulation [16]. Total Put content was between 12- and 2-fold higher than wild type depending on the transgenic line (Put content line 2.1 > 3.6 > 7.2 > wild type) [16,39]. These lines and wild type were exposed to drought stress conditions by withholding watering for 14 days, and their survival rates determined (Fig. 10) [39]. The extent of tolerance was scored by counting the number of plants that resumed growth after 7 days of recovery after re-watering. Interestingly, plants which accumulated higher levels of Put were more resistant to drought stress (Fig. 10) [39]. Hence, the line 2.1 which accumulated 12-fold more Put showed a survival rate of 75% compared to the wild type (12%) (Fig. 9) [39]. The enhanced drought tolerance correlated with a reduced stomata aperture and transpiration rate [39]. These observations are consistent with a role of PAs in the regulation of stomata aperture through modulation of ROS and NO signaling [5]. Our observations indicate that enhanced drought tolerance in plants can be achieved by manipulation of the PA pathway.

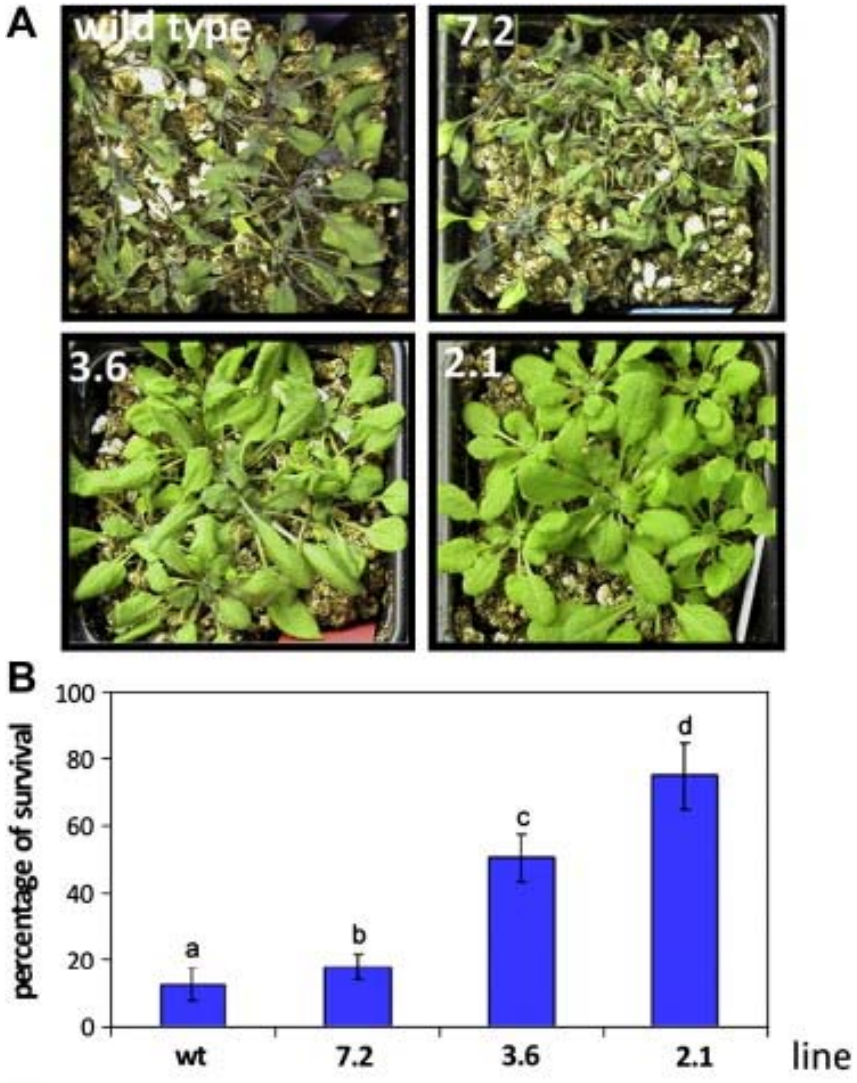

Figure 10. Drought resistance phenotypes of 35s *ADC2* lines 2.1, 3.6, 7.2 compared to wild-type. **(A)** Phenotype of 4-weeks-old plants dehydrated during 14 days. Wild-type, 35s *ADC2* lines 7.2, 3.6 and 2.1 have increased Put content which correlates with enhanced drought tolerance. **(B)** Survival percentages from 35s *ADC2* and wild-type plants after rehydration. Results were published by Alcázar *et al.* [39].

3. Genetics of natural variation for polyamine content

3.1. QTL analyses for PA levels using recombinant inbred line populations (RILs)

In addition to genetic engineering for PA content manipulation, the study of natural variation for PA content provides an alternative to the achievement of plants with enhanced PA levels and associated stress tolerance. In this regard, the study of natural variation for PA content in the model species *A. thaliana* is a good starting point. In the recent months, we have undertaken

some experiments to determine the feasibility of cloning QTLs for PA content in *A. thaliana*.

A preliminary analysis for PA content at 16ºC was performed using a recombinant inbred line (RIL) population of 164 lines derived from the cross between the European accession Landsberg *erecta* (L*er*) and the central Asian accession Kashmir-2 (Kas-2) [40]. These lines show a high transgression in different phenotypes including growth, flowering time and pathogen resistance [40]. We measured the Put, Spd and Spm levels after 5 weeks of growth at 16ºC and analyzed QTLs for Put, Spd and Spm contents. QTL detection was performed using the software MapQTL5. We could detect QTLs for Put, Spd and Spm which genetically explained significant variation of PA levels (Alcázar *et al.* unpublished results). These analyses evidenced that (i) QTLs for PA content are detectable in *A. thaliana*, (ii) part of the phenotypic variation for PA levels can be explained genetically and (iii) the mapping of QTLs for Put, Spd and Spm levels should identify novel genes contributing to PA homeostasis.

The identification of genes and natural alleles contributing to the modulation for PA contents may reveal geographical patterns of adaptation thus pointing to a close relationship between PA levels and local environments. These studies open a new gate to implement PA content regulation to breeding programs dedicated to pursue enhanced drought stress tolerance.

4. Concluding remarks and future perspectives

The genetic manipulation of PA levels enhances drought tolerance in different plant species. Nowadays, the mechanisms of action by which PAs confer enhanced drought resistance are beginning to be unraveled. Evidences point to the involvement of ROS signaling, possibly through PA-recycling loops involving PA back-conversion, as well as cross-talks with key stress hormone ABA. We anticipate that gaining insight into PA functions and exploiting natural variation for PA content regulation will provide new perspectives for crop protection against environmental change.

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4. Nutritional genomics. A new approach in nutrition research

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Abstract. There is an increasing evidence that nutritional genomics represents a promise to improve public health. This goal will be reached by highlighting the mechanisms through which diet can reduce the risk of common polygenic diseases. Nutritional genomics applies high throughput functional genomic technologies and molecular tools in nutrition research, allowing a more precise and accurate knowledge of nutrient-genome interactions in both health and disease. Understanding the inter-relationships among genes, genes products, and dietary habits is fundamental to identify those who will benefit the most or be placed at risk by nutritional interventions. This chapter provides an overview of this novel nutritional approach, including the most relevant results of our recent research on the nutrigenomic effects of food polyphenols on cancer cells. Those studies would highlight the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Introduction

Until recently, nutrition research concentrated on nutrient deficiencies and impairment of health. The importance of diet to sustain health,

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prevention and treatment of diseases has been known for a long time. The advent of genomics –high-throughput technologies for the generation, processing, and application of scientific information about the composition and functions of genomes – has created unprecedented opportunities for increasing our understanding of how nutrients modulate gene and protein expression influencing cellular and organismal metabolism and thus, ultimately impacting human health and well-being. Notably, the knowledge of the human genome has dramatically broadened the scope of studies in nutrition science [1-4].

Nutritional genomics is a relatively new and very fast-moving field of research and combines molecular biology, genetics, and nutrition [3, 5]. It provides a genetic understanding for how diet, nutrients or other food components affect the balance between health and disease by altering the expression and/or structure of an individual's genetic makeup. The conceptual basis for this new branch of genomic research is built on the following premises [1,6]:

- Diet and dietary components can alter the risk of disease development by modulating multiple processes involved with the onset, incidence, progression, and/or severity;
- Diet and dietary components can act on the human genome, either directly or indirectly, to alter the expression of genes and gene products.
- Diet and dietary components could potentially compensate for or accentuate effects of genetic polymorphisms.

The term nutritional genomics is frequently used as an umbrella term for two research specialties: **nutrigenomics** and **nutrigenetics**. However, it is important to note the difference between the terms nutrigenomics and nutrigenetics because although these terms are closely related they are not interchangeable. Nutrigenomics focuses on the effects of nutrients on genes, proteins, and metabolic processes, whereas nutrigenetics involves determining the effect of individual genetic variation on the interaction between diet and disease [2,7]. Thus, those working in nutrigenomics investigate the role of nutrients in gene expression, and those working in nutrigenetics determine how genetic polymorphisms (mutations) affect responses to nutrients [7,8]. Moreover, when reviewing scientific literature, other terms appear, such as epigenetics, transcriptomics, proteomics or metabolomics. All of them describe processes, new tools or situations of this emerging field of nutrition (Table 1). The key challenge is to determine

whether it is possible to utilize this information meaningfully to provide reliable and predictable personalized dietary recommendations for specific health outcomes.

Nutrigenetics and nutrigenomics hold much promise for providing better nutritional advice to the general public, genetic subgroups and individuals [11]. In the future, the integration of nutrition and genomics may lead to the enhanced use of personalized diets to prevent or delay the onset of disease and to optimize and maintain human health. The objectives of this chapter are to provide an overview of this novel nutritional approach. Moreover, we will also include the most relevant results of our research on the nutrigenomic effects of food polyphenols on cancer cells. In addition to the essential nutrients, such as calcium, zinc, selenium or vitamins, there are a variety of classes of nonessential nutrients and bioactive components, such as polyphenols, that seem to significantly influence health. Those bioactive components are known to modify a number of cellular processes associated with health and disease prevention, including carcinogen metabolism, hormonal balance, cell signaling, cell cycle control, apoptosis, and angiogenesis. Our studies are focused in highlighting the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Table 1. Definitions of terms used in nutritional genomics [9,10].

Term	Definition
Nutrigenomics	Investigates the effects of nutrients and other food components on genes, proteins, and metabolic processes. Transcriptomics, proteomics and metabolomics are used in nutrigenomics research
Nutrigenetics	Investigates the effect of individual genetic variation on the interaction between diet and disease. Genomics are often used in nutrigenetics studies
Epigenetics	Investigates the genome modifications that are copied from a generation to another but not implying changes on DNA sequence
Transcriptomics	Investigates gene expression changes at the mRNA level in response to different stimuli. Utilizes variety of technologies, most commonly microarrays and next-generation sequencing
Proteomics	Analyses all the proteins in a biological system, their interactions and their functional states although effectively, usually only the most abundant subset of 300 or so proteins is relatively easily analyzed
Metabolomics	Investigates the metabolome that consists of all of non-proteinaceous, small molecules present in a biological system. Changes in the metabolome content reflect the biological responses to external stimuli (nutrients among others), which involves altered gene expression and protein production/ activity associated with metabolic pathways

1. Nutrigenetics

Nutrigenetics focuses on the effects that genetic variations have on the binomial diet/disease or on the nutritional requirements and recommended intakes for individuals and populations. To achieve its objectives, the methodology used in nutrigenetics includes the identification and characterization of genetic variants that are associated with, or are the responsible for a different response to certain nutrients or food components [6,11]. These variations generically designated as polymorphisms, including the polymorphisms of a single nucleotide (SNP, single-nucleotide polypmorphisms), differences in the number of copies, inserts, deletions, duplications and rearrangements or reorganizations. Undoubtedly, SNPs are the most frequent as they appear every 1,000 base pairs [12].

These differences may determine the susceptibility of an individual to have a disease related to diet or to one or some diet components, as well as to influence in the individual's response to diet changes. There is certain parallelism between nutrigenetics and phamacogenetics, although in the field of nutrition is more difficult to draw conclusions, since there are important differences between drugs and food components, such as chemical purity, number of therapeutic targets and duration of the exposure, among others [3, 9, 11].

One of the best-described examples of the effect of SNPs is the relationship between folate and the gene encoding for MTHFR (5,10-methylenetetrahydrofolate reductase) [13]. MTHFR has a role in supplying 5-methylenetetrahydrofolate, which is necessary for the re-methylation of homocysteine to form methionine. Methionine is essential to many metabolic pathways including production of neurotransmitters and regulation of gene expression. Folate is essential to the efficient functioning of this MTHFR. There is a common polymorphism in the gene for MTHFR that leads to two forms of protein: the wild type (C), which functions normally, and the thermal-labile version (T), which has a significantly reduced activity. People with two copies of the wild-type gene (CC) or one copy of each (CT) appear to have normal folate metabolism. Those with two copies of the unstable version (TT) and low folate accumulate homocysteine and have less methionine, which increases their risk of vascular disease and premature cognitive decline [14].

Thus, in people with low folic acid intake, higher serum homocysteine levels would be detected in TT homozygotes compared with other genotypes, which would lead them to an increased risk of cardiovascular disease (Figure 1). However, when the intake of folic acid in diet is higher, this increased

amount would compensate the DNA defect in people with the TT polymorphism, and homocysteine serum concentrations would not reach such high values and consequently not show hyperhomocysteinemia. According to this example of gene-diet interaction, a practical application for cardiovascular disease prevention would be to recommend a higher daily consumption of folic acid-rich food to those people with the TT genotype, since these individuals have higher folic acid requirements than the general population due to their genetic susceptibility.

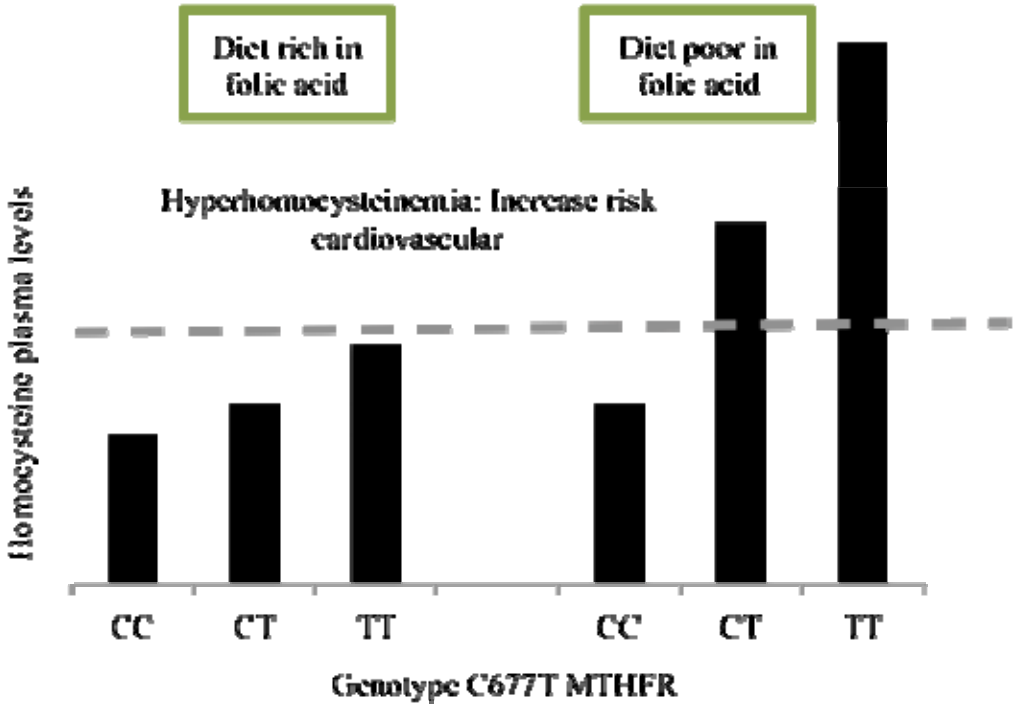

Figure 1. Gene-diet interaction. Folic acid intake may modulate the genetic risk of hyperhomocysteinemia conferred by the C677T polymorphism in the MTHFR gene. Hyperhomocysteinemia only would happen when the mutation occurs with a low folate intake [Adapted from 15].

Another of the genes on which a very active research has been developed is the one that encodes for the synthesis of the lipoprotein APOA1 [16]. APOA1 is the main component of plasmatic HDL and seems to play an important role in the transport of cholesterol. It has been reported that a polymorphism in the gene promoter the -75 A/G (substitution of guanine by adenine), has an influence on the individual's response to polyunsaturated fatty acids (PUFA) intake. Thus, women with the A/A genotype showed higher HDL-cholesterol levels in plasma after ingestion of PUFA, whereas those with genotypes A/G and G/G (wild type) did not show HDL-cholesterol

changes or even a certain decrease in response to the PUFA from diet (Figure 2). Therefore, for the individuals with the genotype A/A the ingestion of PUFA could be a good diet recommendation since it increases HDL. Those results illustrate the complexity of polymorphism-phenotype associations and underscore the importance of accounting for interactions between genes and environmental factors in population genetic studies.

The examples cited here and many others that can be found in the literature published until now [10,11,17-20] illustrate perfectly why nutrigenetics is also termed personalized nutrition, since its major goal is to identify and characterize genes, and nucleotide variants within these, that are associated (or account for) the differential responses to nutrients. In addition to providing a more rational basis for giving personalized dietary advice, the knowledge gained by applying genomic information to nutrition research will also improve the quality of evidence used for making population-based dietary recommendations. The sequencing of an individual's genome has fueled interest in the field of personalized medicine [21,22], but replicating and validating nutrigenetic studies need to remain a priority before personalized nutrition can be considered a worthwhile approach to improve human health [23].

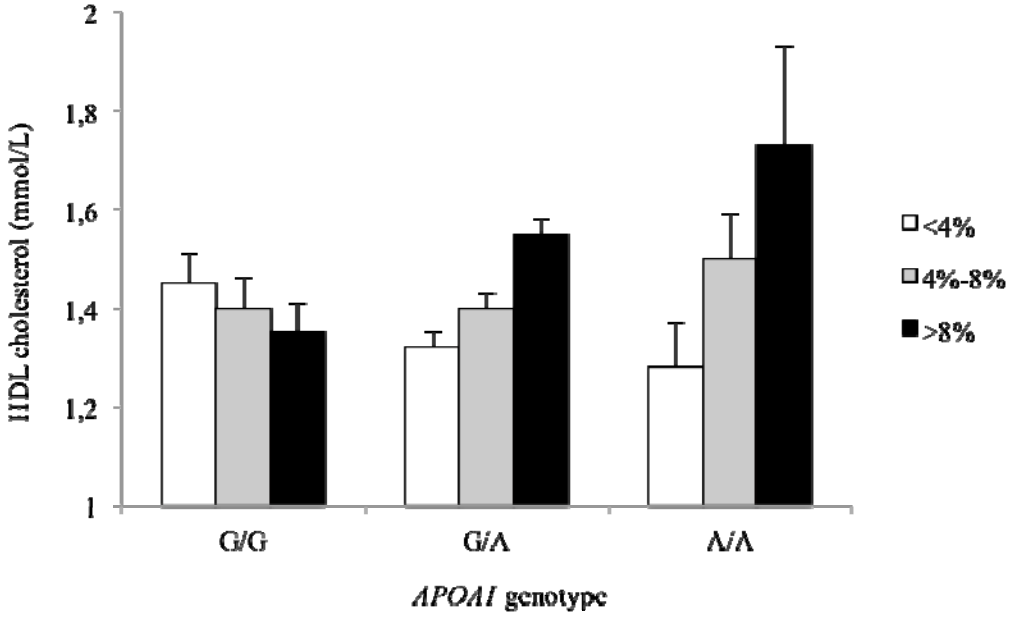

Figure 2. Effect of polyunsaturated fatty acid intake (>4%, 4-8% and >8% of energy) on high-density lipoprotein (HDL) cholesterol blood levels in women. Means were adjusted for age, body mass index, alcohol consumption, tobacco smoking, and intakes of energy, saturated fatty acids, monounsaturated fatty acids, and PUFAs [Adapted from 16].

2. Nutrigenomics

The term nutrigenomics was coined ten years ago to describe a branch of nutrition and food research that applies new profiling techniques for transcripts, proteins and metabolites to better understand the interplay of the genome with its nutritional environment. In this respect, nutrigenomics is still in its infancy and it will need time until it really delivers what was originally hoped [3,6,9].

The field of nutrigenomics harnesses multiple disciplines and includes dietary effects on genome stability (DNA damage at the molecular and chromosome level), epigenome alterations (DNA methylation), RNA and micro-RNA expression (transcriptomics), protein expression (proteomics) and metabolite changes (metabolomics), all of which can be studied independently or in an integrated manner [11, 24]. In this approach, nutrients, other food components, and even whole diets, are considered as "dietary signals" that are detected by "cellular sensors". These sensors, that are part of cellular signaling cascades, can affect, in turn, all the processes involved in cell function. Therefore, they influence the transcription, translation and protein expression and different metabolic pathways, which ultimately form the phenotype [25, 26].

Using the current genomic tools that include transcriptomics, proteomics and metabolomics, there are two approaches in nutrigenomic research. The first would identify genes, proteins or metabolites that are affected by the diet (nutrients or bioactive compounds) and determine which are the mechanisms involved in this interaction and, consequently, figure out the regulation pathways through which the diet induces these changes. In the second approach, early biomarkers are sought (genes, proteins or metabolites) that are linked with certain dietary compounds or to the whole diet [1,24]. Those biomarkers could act as a "warning signals" about changes in the homeostasis with could have implications for the health [10,11,24].

There are numerous examples [9,11,27,28] that illustrate the interaction between food components and the genome, from mammalian cells in culture to human studies. However, most applications are still of descriptive nature. As an example of a typical nutrigenomic approach research, we will explain our research which its main goal is to study mechanisms underlying the potential chemopreventive effects of a certain type of well-known food compounds called polyphenols.

Polyphenols are the most abundant antioxidants in the diet. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, cocoa, chocolate, and dry legumes also contribute to the total polyphenol intake. Their total dietary intake could

be as high as 1g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants [29]. Despite their wide distribution in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus [30]. However, our knowledge still appears too limited to formulate recommendations for the general population or for particular populations at risk of specific diseases.

For many years, polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mechanism of action [31,32]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [33]. Both antioxidant and prooxidant effects of polyphenols have been described, with contrasting effects on the cell's physiologic processes. As antioxidants, polyphenols may improve cell survival; as prooxidants, they may induce apoptosis and prevent tumor growth [30, 32]. However, the biological effects of polyphenols may extend well beyond the modulation of oxidative stress. One of the best-known examples involves the interaction of soy isoflavones with estrogen receptors and the effects of these compounds on endocrine function. These effects could explain the prevention by isoflavones of bone resorption among postmenopausal women [30]. A detailed understanding of the molecular events underlying these various biological effects is essential for the evaluation of the overall impact on disease risk and progression.

2.1. Coffee polyphenols and breast cancer: A transcriptomics approach

Coffee is one of the most popular and widely consumed beverages throughout the world. Recent meta-analyses demonstrate inverse associations between coffee intake and the risk of colon, liver, breast and endometrial cancer [34-37]. In prospective population-based cohort studies, the inverse association between coffee consumption and risk of cancer has also been showed. The group of Naganuma et al. [38] found that the consumption of at least one cup of coffee per day was associated with a 49% lower risk of upper gastrointestinal cancer in a Japanese population, while Wilson et al. [39] found that men who regularly drink coffee appeared to have a lower risk of developing a lethal form of prostate cancer. The lower risk was evident when consuming either regular or decaffeinated coffee.

It has been proposed that the inverse association between coffee intake and colon cancer could be explained, at least in part, by the presence of phenolic compounds in coffee [40]. Among the different phenolic compounds in coffee, the most abundant are hydroxycinnamic acids, which exist mainly in the esterified form. The best example is chlorogenic acid (5-caffeoylquinic acid). In fact, coffee is the major source of chlorogenic acid in the human diet; the daily intake in coffee drinkers ranges from 0.5 to 1 g, whereas coffee abstainers will usually ingest <100 mg/day. Studies have showed that approximately the 33% of ingested chlorogenic acid and 95% of caffeic acid are absorbed intestinally [41]. Thus, about two-thirds of ingested chlorogenic acid reaches the colon where it is probably metabolized to caffeic acid [42]. Bioavailability data suggest that the biological effects of chlorogenic acid would become apparent after its metabolism to caffeic acid, and hence studying the effects of this acid is necessary.

As mentioned before, there is enough evidence from epidemiological data supporting the theory that coffee seems to reduce the risk of certain types of cancer; however, the molecular mechanisms underlying the chemopreventive effects of coffee remain unknown. Using a transcriptomics approach, the effect at the molecular level of the main phenolic compound in coffee, caffeic acid, at concentrations equivalent to one cup of coffee on human colon cancer cells (HT29) was studied. Furthermore, the effect of coffee polyphenols was also evaluated in breast cancer cells.

Colon adenocarcinoma HT29 cells were incubated with caffeic acid at a concentration equivalent to one cup of coffee for 24 hours. It was previously determined that this concentration did not cause any cytotoxic effect in the cell incubations. Then, gene expression was analysed by hybridization to the GeneChip Human Genome U133A plus 2.0 microarrays from Affymetrix, containing 47,000 transcripts and variants. Quantification was carried out with GeneSpring GX v.11.5.1 software (Agilent Technologies), which allows multi-filter comparisons using data from different experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes.

A list of differentially expressed genes by 1.3-fold with a p-value cut-off of <0.05 was generated. Upon incubation with caffeic acid, 12 genes were overexpressed whereas 32 genes were underexpressed. Among the overexpressed genes, 33% belonged to the Transcription factors category, 25% to Cell cycle, and 17% to Biosynthetic processes or Immune response. Within the underexpressed genes, again the category corresponding to Cell cycle was the most affected (30% of the genes) followed by Biosynthetic processes (15%) and Transcription factors (12%). Using these data, a Biological Association Network (BAN) was constructed using the Pathway

Analysis within the GeneSpring v.11.5.1, as described in Selga et al. [43]. Signal transducer and activator of transcription 5B (STAT5B) and Activating transcription factor 2 (ATF-2) appeared as highly interconnected nodes (Figure 3). In fact, STAT5B was overexpressed with respect to the control by 33,4% in cells treated with caffeic acid, whereas ATF-2 was found underexpressed in HT29 incubated with caffeic acid (26% decrease compared to the control).

The changes in mRNA expression of these two main (STAT5B and ATF-2) nodes were confirmed by RT-PCR and at protein level by Western blot analysis (Figure 4). The key function of STAT5B is to mediate

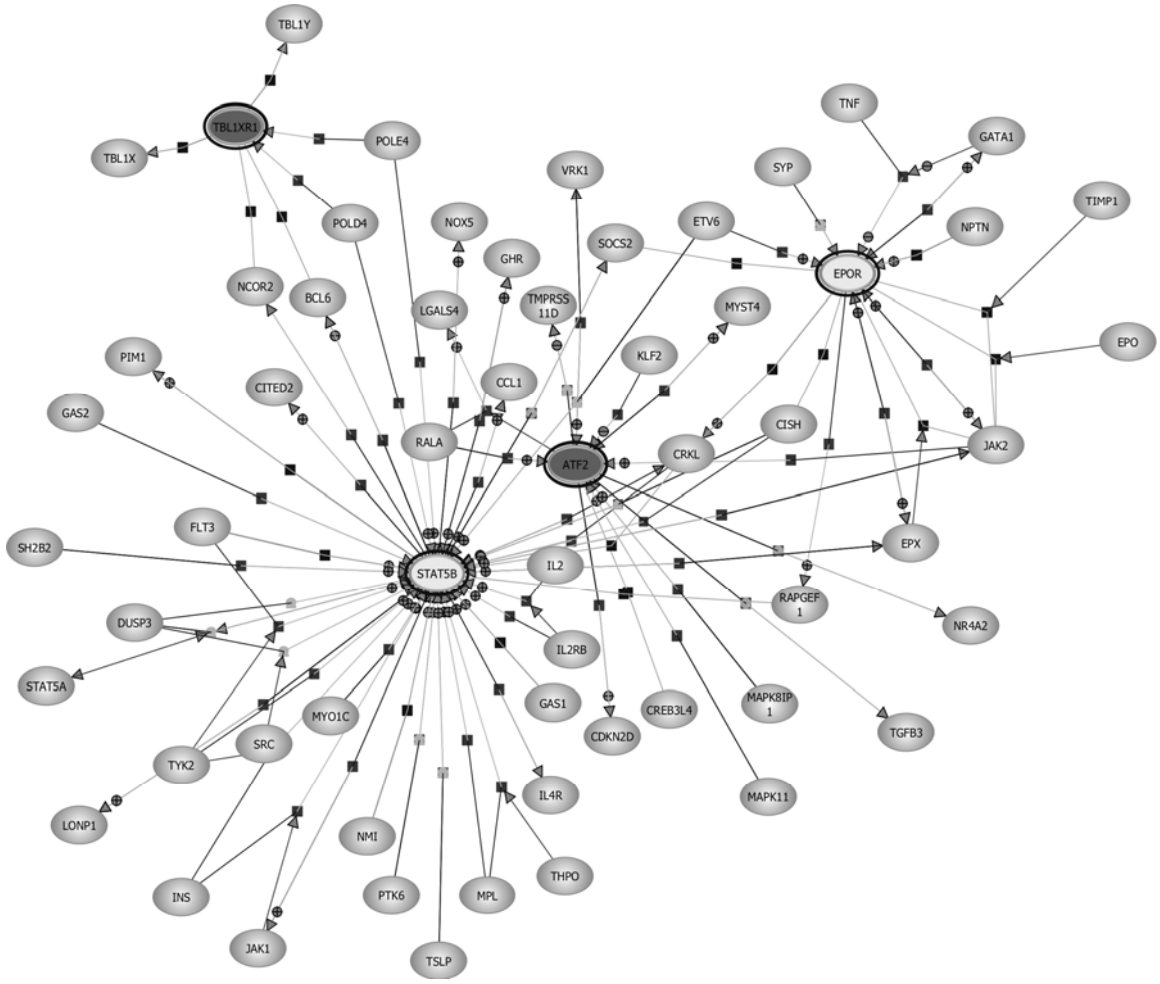

Figure 3. Biological Association Network (BAN) of differentially expressed genes under caffeic treatment. The BAN was constructed with the Pathway Analysis software within GeneSpring v11.5.1. An expanded network was constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation. Only proteins are represented. The BAN shows the node genes STAT5B and ATF-2 that were further studied.

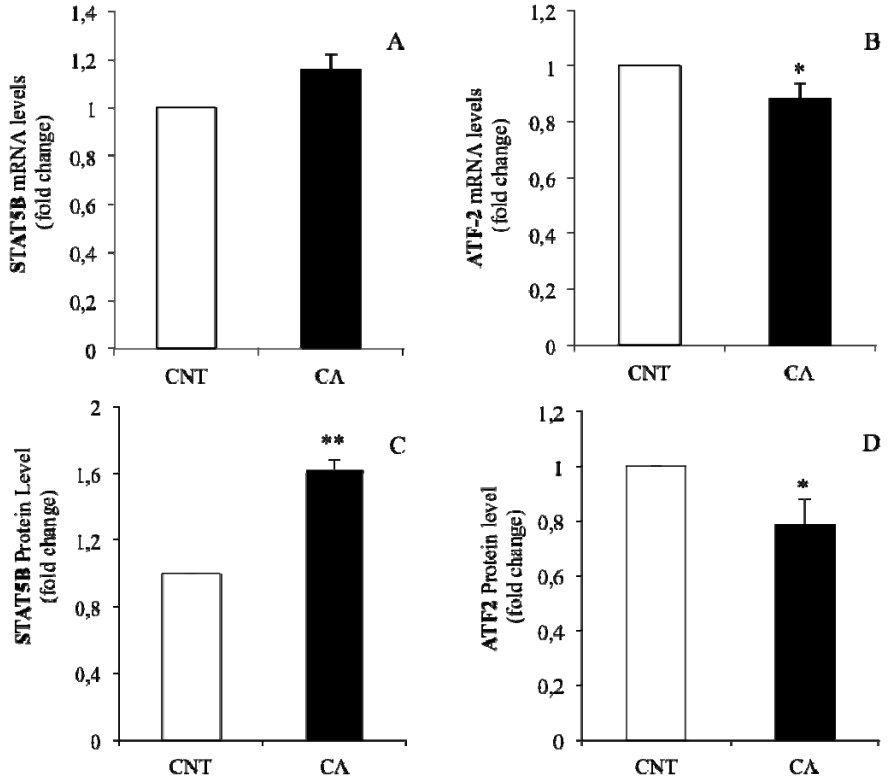

Figure 4. Quantification of mRNA and protein levels for STAT5B and ATF-2 in HT29 cells. The mRNA levels of STATB5 (A) and ATF-2 (B) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by RT-Real Time. Results are expressed in fold-changes compared to the control, and are the mean + SE of 3 different experiments. *$p<0.05$ compared with the corresponding control. The protein levels of STAT5B (C) and ATF-2 (D) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by Western blot. Blots were reprobed with an antibody against β-actin or tubulin to normalize the results. Results represent the mean ± SE of 3 different experiments. *$p<0.05$ and **$p<0.01$ compared with the corresponding control.

the effects of the Growth Hormone, as STAT5B-null mice failed to respond effectively to this hormone [44]. Modulation of STAT5 levels or transcriptional activity has already been described in cells treated with natural compounds such as nobiletin, acitrus flavonoid [45] thea flavins [46] and silibinin, a natural polyphenolic flavonoid which is a major bioactive component of silymarin isolated from *Silybum marianum* [47]. Activation of STAT5A/B in human breast cancer has been shown to positively correlate with the differentiation status of the tumour. STAT5 have been also shown to transcriptionally regulate E2-sensitive proliferative genes such as cyclin D1 and c-Myc [48] suggesting that STAT5 may play a role in E2-stimulated breast cancer growth. STAT5 activation has also been linked to regulating the expression of the cell cycle control protein cyclin D1 both directly and indirectly [48-50].

On the other hand, ATF-2 is a member of the ATF-cAMP response element-binding protein (CREB) family of transcription factors that can bind to the cAMP response element (CRE) found in many mammalian gene promoters [51]. ATF-2 exhibits both oncogenic and tumor suppressor functions [52)] CREs are found in several genes involved in the control of the cell cycle, e.g., the cyclin D1 gene and ATF-2 binding to this sequence stimulates the transcription of cyclin D1 [53]. ATF-2 has been correlated with proliferation, invasion, migration, and resistance to DNA-damaging agents in breast cancer cell lines.

Therefore, the two main nodes identified in our work regulate cyclin D1 transcription. Cyclin D1 is an important regulator of G1-S phase transition, and its expression in breast cancer cells is sensitive to estrogens and antiestrogens [54]. Cyclin D1 is over expressed at the mRNA and protein level in over 50% of the breast cancers either in the presence or absence of gene amplification and it is one of the most commonly over expressed proteins in breast cancer [55]. In order to know the influence that caffeic acid could have overcyclin D1 levels, since the expression of STATB5 and ATF-2 is modified by this phenolic compound, cyclin D1 levels in MCF-7 cells were analyzed upon incubation with caffeic acid by Western Blot. As shown in Figure 5, incubation of MCF-7 cells with caffeic acid led to a drastic decrease in the levels of cyclin D1 protein, together with an increase in the levels of STAT5B, but there was no decrease in the levels of ATF-2.

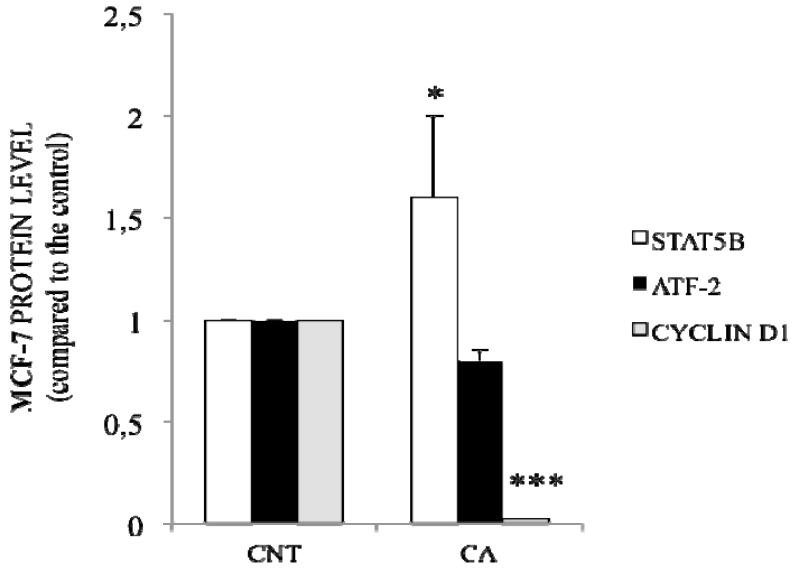

Figure 5. Expression of cyclin D1 upon incubation with caffeic acid in MCF-7 cells. The protein levels were determined in control MCF-7 cells (CNT) and in cells treated with caffeic acid (CA) by Western blot. Blots were reprobed with an antibody against β-actin to normalize the results. Results represent the mean ± SE of 3 different experiments. *$p<0.05$ and ***$p<0.001$ compared with the corresponding control.

It is believed that compounds that modulate cyclin D1 expression could have a role in the prevention and treatment of human neoplasia. For instance, flavopiridol, a synthetic flavonoid based on an extract from an Indian plant used for the potential treatment of cancer, induces a rapid decline in cyclin D1 steady-state protein levels [54]. Taking all these results together, inhibition of cyclin D1 expression appears to be a good approach for cancer treatment. In this direction our observation that coffee and caffeic acid are able to drastically reduce the expression of cyclin D1 in breast cancer cells could suggest that some coffee components could be used as a coadjuvant therapeutic tool in the treatment of breast cancer.

2.2. Cocoa polyphenols and changes in the CYP1A1 gene expression

Cocoa is rich in polyphenols. In fact, cocoa has the highest flavanol contents of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids [56]. The main subclasses of flavonoids found in cocoa are flavanols, particularly the flavanol monomers catechin and epicatechin, and their oligomers, also known as procyanidins [57]. Many examples of the health benefits of cocoa consumption can be found in the literature [58].

Epidemiologic studies of cocoa intake and cancer risk are few, and those assessing overall mortality provide only weak support of the benefits of cocoa. However, human intervention trials indicate that cocoa favours intermediary factors in cancer progression—specifically, markers of antioxidant status [59]. Moreover, there is growing evidence that polyphenols may play a role in regulating apoptosis [60]. Apoptosis may be triggered intrinsically, through the mitochondrial pathway or extrinsically by death ligands and receptors. It is the external pathway that may potentially be modulated by bioactive food components. Flavanols found in cocoa have exhibited pro-apoptotic effects. Proanthocyanadins inhibited growth of human lung cancer cells in vitro and in vivo [61], and epicatechin synergistically enhanced apoptosis in lung cancer cells treated with epigallocatechin-3-gallate (EGCG) [62]. Cocoa polyphenols have also been found to inhibit the mutagenic activity of heterocyclic amines *in vitro* and *ex vivo* [63].

It has been reported that catechins from green tea could be effective in modulating estrogen-induced breast carcinogenesis, either interfering with receptor mediated pathways or reducing the production of genotoxic estrogen metabolites [64,65]. In our functional genomic study, we sought to evaluate the effect of cocoa flavonoids in a type of breast cancer cells (MCF-7), that are estrogen-receptor (ER)- dependent [66]. Estrogens are implicated in the initiation and promotion stages of breast cancer, and lifetime estrogen

exposure is a major risk factor for breast cancer [67]. Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and independent mechanisms [68]. Most human breast cancers are initially positive for ER, and their growth can be stimulated by estrogens and inhibited by antiestrogens such as tamoxifen.

For that purpose, MCF-7 cells were incubated for 24h with a purified polyphenol cocoa extract (PCE). PCE was used as representative of the wide flavonoid spectrum (monomers and oligomers) present in cocoa and the concentrations used were not toxic. The differential gene expression analysis was done using PCR arrays. In particular, the expression profile of the 84 genes included in the Stress & Toxicity PathwayFinder™ PCR Array was analyzed in MCF-7 cells both control and treated with a PCE. It was observed that the exposure to PCE decreased the expression of serpine 1 and up-regulated the expression of the CYP1A1, GADD45A, GDF15, GPX1, RAD23A, TP53, and XRCC2 genes (Table 2).

Among those genes, CYP1A1 was chosen for further validation since: (a) it was one of the most overexpressed gene upon incubation with PCE, (b) its overexpression in response to polyphenols had already been described, and (c) it plays an important role in the oxidative metabolism of estrogens. CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in the metabolism of xenobiotics or carcinogens as well as in the oxidative metabolism of estrogens [2004]. CYP1A1 encodes aryl hydrocarbon hydroxylase (AHH) which catalyzes a

Table 2. List of under- and overexpressed genes in MCF-7 cells upon incubation with PCE for 24hours[1].

MCF-7 Gene symbol	Fold-up or down-regulation Test sample / control sample	p-value
CYP1A1	17.60	0.0001
GADD45A	4.20	0.0264
GDF15	2.60	0.0001
GPX1	4.25	0.0183
RAD23A	13.90	0.0394
SERPINE1	- 49.90	0.0216
TP53	2.26	0.0470
XRCC2	17.50	0.0356

[1]The expression of each gene was reported as the fold change obtained after each treatment relative to control after normalization of the data. A cut-off of 2-fold was chosen since small changes in gene expression may represent important changes downstream those differentially expressed genes. Lists of differentially expressed genes, with a p-value<0.05, were generated from three independent experiments.

hydroxylation reaction in Phase I metabolism as a first step to increase the polarity of different molecules. Some of these metabolites can be more active than the initial molecules and behave as electrophilic compounds, thus initiating or promoting tumorigenic processes. Additionally, other metabolites may behave as chemoprotectors, such as the result of 2-hydroxylation in E1 and E2 metabolism [70].

Therefore, the differential expression of CYP1A1 mRNA in control versus treated cells was validated by RT- Real Time PCR (Figure 6A). Next, we investigated whether the changes at the RNA level were translated into protein. PCE treatment for 24 h led to a very modest increase in CYP1A1 protein levels (1.2-fold). A time course incubation during 24, 48, 72 and 96 h led to an increase in CYP1A1 protein in MCF-7 cells of 3.9-fold after 48 h (Figure 6B). The difference between mRNA levels and the corresponding protein levels may indicate that many of the mRNA molecules do not reach

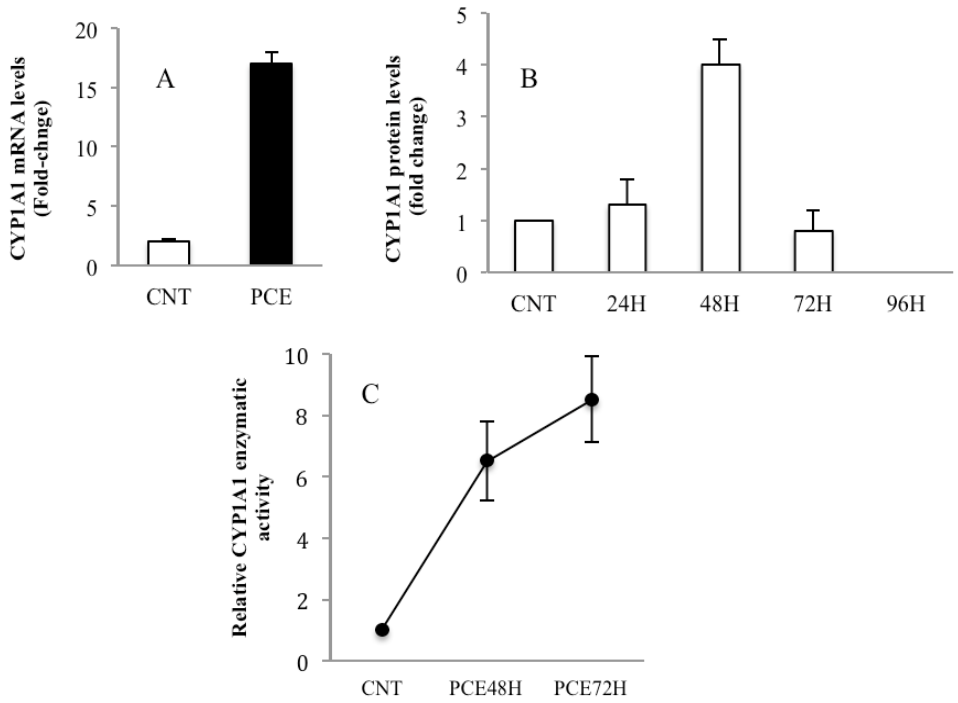

Figure 6. CYP1A1 overexpression in MCF-7 cells treated with PCE. (A) Determination of CYP1A1 mRNA levels. Results are expressed in fold changes compared to MCF-7 control and are the mean ± SE of 3 different experiments. (B) Determination of CYP1A1 protein levels. Results represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA plus post hoc Bonferroni comparison. (C) Determination of CYP1A1 activity in MCF-7 treated cells. Results are expressed relative to the activity of the control and represent the mean ± SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA, plus post hoc Bonferroni comparison.

the translational machinery, probably because the translation mechanism is saturated in these conditions. Finally, CYP1A1 activity was determined upon incubation with PCE. An increase in CYP1A1 activity in good correlation with the observed increased in CYP1A1 protein levels was determined for both cell lines (Figure 6C).

The changes in CYP1A1 expression upon incubation with PCE could explain the antioxidant effect of flavonoids at the molecular level since this gene is involved in different oxidative pathways. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells. The increase in CYP1A1 activity may shift estrogen metabolism toward the production of 2-OHE2 (2-hydroxyoestradiol), a relatively non-genotoxic metabolite [71].

Finally, we wanted to test whether cocoa polyphenols would exert a synergistic effect in combination with Tamofixen (TAM) since it has been previously described in breast cancer cells. Thus, MCF-7 cells were incubated with increasing concentrations of TAM (10^{-6}–10^{-3}M) either alone or in combination with PCE (250 ng/µL). Then, cell viability was determined after 48 h. The presence of PCE, which did not cause significant cell death by itself, increased the cytotoxic effect of TAM in MCF-7 cells (Figure 7).

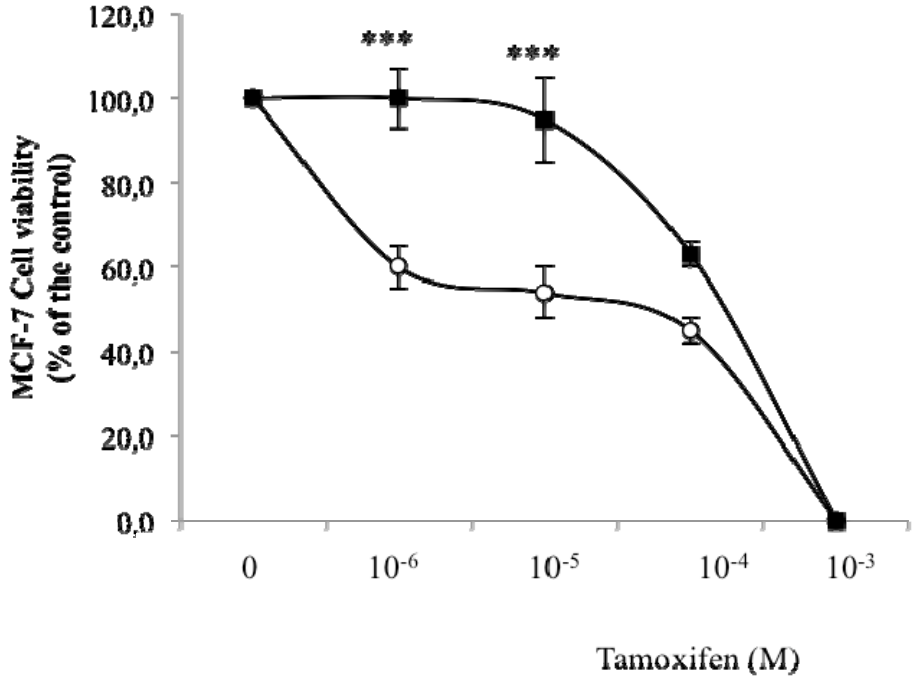

Figure 7. Effect of tamoxifen plus PCE on MCF-7 viability. Tamoxifen (TAM) either alone (filled squares) or in combination with PCE (250 ng/µL for 24H, empty circles). Results are expressed as % of living cells compared to the control only with DMSO (0.22%) and represent the mean ± SE of 3 different experiments. ***$p<0.001$.

The reduction in cell viability reached an increase of 44% when combined with 10^{-6}M TAM. Thus, in our conditions, the cytotoxic effect of TAM was enhanced by the combination with PCE in MCF-7 cells. The presence of PCE caused a synergistic effect, confirmed by the Chou-Talay method, which led to a decrease in cell viability of up to 40% in MCF-7 cells at tamoxifen concentrations that did not affect cell viability by themselves. A plausible explanation of the synergistic effect observed could be that the increase in estrogen metabolism, induced by the PCE on CYP1A1, could lead to the reduction in the levels of estrogens in mammary tumours, thus contributing to the cytotoxic effect of tamoxifen. Nevertheless, further *in vivo* studies are necessary to analyse the synergism between tamoxifen and cocoa and to establish the possible benefits of cocoa polyphenol consumption during breast cancer therapy.

3. Conclusions

Current global trends in food consumption may have an impact on disease progressions observed worldwide. The impact may occur because of gene regulation caused by nutrients, or by other unclear means that are yet to be discovered. The "omics" and associated technology will surely provide a greater understanding of the environmental and behavioral factors that influence phenotype and its relationship to health and wellness. It is highly likely that during the next decade the nutritional supplement and functional food industries will experience robust growth in response to advances in nutritional genomics research and its applications.

Parallel to this growth will be impressive progress in understanding the specific influence of certain food components on metabolic pathways and their role in health and disease. It will become increasingly less expensive to generate genetic information about individual persons, and such data are likely to redefine the current concept of preventive medicine. Moreover, through nutrigenomic research, new nutritional regulation of gene expression will hopefully come to light. If specific gene regulation by nutrients is identified in genes closely related to disease onset and progression, new arenas for disease prevention and potential for treatment will come to the foreground of nutrition and preventive medicine. Discoveries made in the field of nutrigenomics and nutrigenetics should translate into more effective dietary strategies to improve overall health by identifying unique targets for prevention.

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5. Is the 23-valent pneumococcal polysaccharide vaccine useful in preventing community-acquired pneumonia?

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Abstract. Although bacteremic pneumococcal pneumonia is the most severe form of pneumonia, non-bacteremic forms are much more frequent. Laboratory methods for the diagnosis of non-bacteremic pneumococcal pneumonia have a low sensitivity and specificity, and therefore all-cause pneumonia has been proposed as a suitable outcome to evaluate vaccination effectiveness.

This work reviews the epidemiology of community-acquired pneumonia (CAP) and evaluates the effectiveness of the 23-valent pneumococcal polysaccharide vaccine (PPV-23) in preventing CAP requiring hospitalization in people aged ≥65 years.

We performed a case-control study in patients aged ≥65 years admitted through the emergency department who presented with clinical signs and symptoms compatible with pneumonia. We included 489 cases and 1,467 controls and it was obtained a vaccine efectiveness of 23.6 (0.9-41.0). Our results suggest that PPV-23 vaccination is effective and reduces hospital admissions due to pneumonia in the elderly, strengthening the rationale for vaccination programmes in this age group.

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Introduction

Pneumonia is an inflammation of the lung parenchyma located below the terminal bronchioles (respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli) that leads to impaired gas exchange. The inflammatory reaction causes water density in chest X-rays, the hallmark of pulmonary consolidation. Pneumonia acquired outside the hospital is known as community-acquired pneumonia (CAP), in contrast to nosocomial pneumonia, which is acquired after 48 hours of admission to the hospital or the first week after discharge [1].

Pneumonia together with influenza is the seventh leading cause of death in the United States of America (USA) with a high estimated monthly mortality rate of 19.4/100,000 inhabitants [2].

On the basis of a study it was estimated roughly 915,900 cases of CAP could occur annually among seniors in the USA and that approximately 1 of every 20 persons aged ≥85 years could have a new episode of CAP each year [3]. Other studies estimated a number of cases of CAP requiring hospitalization in USA of 485,000 patients annually, and 43,000 of these persons died [4].

Currently, an etiologic diagnosis is achieved in between 29 and 60% of cases of CAP requiring hospital admission, depending on the number of samples and techniques used [5-16]. In some studies, the rate exceeds 70% [17-19], although the percentage of etiologic diagnoses may be significantly lower in hospitals without systematic diagnostic protocols or when patients have received prior antibiotic treatment, with some studies finding rates of 14% [20] (Tables 1 and 2).

CAP is caused by a wide variety of bacterial species, including, in order of frequency, *Streptococcus pneumoniae*, *Haemophilus influenzae, Chlamydophila pneumoniae, Mycoplasma pneumoniae, Staphylococcus aureus, Legionella pneumophila* and some enterobacteria [22]. Viral infections result in 10-25% of CAP, but the relative frequency of the different microorganisms varies according to factors such as age, underlying disease and the diagnostic methods used [23].

Various studies suggest that between 30 and 50% of cases of CAP requiring hospitalization are caused by *S. pneumoniae* [6, 8, 24, 25].

Some authors have suggested that the number of cases caused by *S. pneumoniae* is underestimated. A Spanish study found that many patients hospitalized with CAP in whom no etiologic diagnosis is obtained using routine techniques are diagnosed with pneumococcal pneumonia when more-sensitive detection techniques are used and samples are obtained by non-routine procedures such as transthoracic puncture [18, 26]. Ruiz-González *et al.*

Table 1. Percentage of etiologic diagnostic in several studies about CAP in Spain.

Author, year	Place, period	Hospitalized patient (%)	N	Etiologic diagnostic (%)	*Streptococcus pneumoniae* (%)
Blanquer, 1991 [13]	Valencia, 1985-1986	91	510	55.1	14.5
Gómez, 1996 [11]	Murcia, 1991-1994	-----	342	29	12.6
Santos de U, 1998 [21]	Mallorca, 1992-1994	26	91	52.7	9.8
Ruiz-González, 1999 [18]	Lleida, 1993-1994	71	109	50 83*	17 30*
Ruiz, 1999 [7]	Barcelona, 1996-1997	100	395	46	16.5
Sopena, 1999 [6]	Badalona, 1994-1996	88	392	58	23.9
Rosón, 2001 [8]	Hospitalet de Llobregat, 1995-1997	100	533	53.1	25.3

N=number of CAP studied.
*Percentage obtained after tests on samples of lung tissue.

found that 33% of cases of pneumonia labelled as of unknown etiology using standard methods (blood culture, sputum culture and serology) were finally diagnosed as pneumococcal pneumonia after tests on samples of lung tissue obtained by transthoracic puncture [18].

Studies show that the incidence of CAP varies according to several factors: the country where the study is carried out, the age groups included, whether the study includes all patients with CAP or only those requiring hospitalization, and sex [27, 28].

In Spain, the incidence of CAP in adults ranges between 2 and 10 cases per 1,000 persons/year, and from 2.3 to 35 per 1,000 persons/year in people aged ≥70 years [21, 27, 29, 30]. The incidence of CAP is always greater in the elderly and persons with underlying diseases, which, together with the ensuing mortality, is a concern for health authorities [25]. Tables 3 and 4 show figures of incidence in diferents studies in Spain and other countries.

Table 2. Percentage of etiologic diagnostic in several studies about CAP in other countries.

Author, year	Place, period	Hospitalitzed patient (%)	N	Etiologic diagnostic (%)	*Streptococcus pneumoniae* (%)
Bohte, 1995 [16]	Leiden (Holand), 1991-1993	100	334	62	27
Porath, 1997 [17]	Israel, 1991-1992	100	346	80.6	42.8
Jokinen, 2001 [24]	Kuopio (Finland), 1991-1982	44	345	53.4	41
Bochud, 2001 [15]	Switzerland, 4 years	8.2	170	54.1	20
Lim, 2001 [19]	Nottingham (U K), 1998-1999	100	267	75	48

N=number of CAP studied.

Table 3. Incidence of CAP in Spain.

Author, year	Place, period	Population	Age	N cases	Global Incidence*	Incidence in specific groups**
Almirall, 1993 [31]	Maresme, 1990-1991	39,793	≥14	105	2.6	-----
Aguirre, 1993 [32]	Andoain, 1991-1992	8,862	≥14	97	8.8	-----
Santos de U, 1998 [21]	Mallorca, 1992-1994	60,450	>14	91	1.8	≥65: 2.3
Almirall, 2000 [27]	Maresme, 1993-1995	74,368	≥14	241	1.6	≥65: 3.2 M: 5 W: 2
Gutiérrez, 2006 [28]	Vinalopó (Alicante), 1999-2001	200,430	>14	493	1.2	65-74: 2.4 ≥75: 5.3 M: 8.7 W: 3.0
Ochoa, 2008 [29]	Tarragona, 2002-2005	11,240	≥65	946	14	65-74: 9.9 ≥85: 29.4

*cases/1000 persons/year. **cases/1000 persons/year in specific groups: age (years) and gender. M: men; W: women

Table 4. Incidence of CAP in other countries.

Author, year	Place, period	Population	Age	N cases	Global Incidence*	Incidence in specific groups**
Woodhead, 1987 [33]	Nottingham (United Kingdom), 1984-1985	53,137	15-79	251	4.7	-----
Jokinen, 1993 [34]	Koupio (Finland), 1981-1982	46,974	>1month	546	11.6 M:13.9 W:9.4	<5: 36.0 5-14:16.2 15-19: 6.0 60-74: 15.4 ≥75: 34.2
Myles, 2009 [35]	United Kingdom, 1991-2003	-----	All	56,332	2.3	<5: 1.9 ≥65: 6.7
Marston, 1997 [4]	Ohio (USA), 1991	1,014,000	≥18	2,776	2.7	18-44:0.9 45-65:2.8 ≥65:10.1

*cases/1000 persons/year. **cases/1000 persons/year in specific groups: age (years) and gender. M: men; W: women

Although the criteria for hospitalization are not homogeneous, it is estimated that between 12 and 50% of patients with pneumonia require hospital admission [30-32, 36]. The figures increase with age and can reach between 67% and 75% in cases of CAP in people aged ≥65 years [29, 34], although some studies have reported figures of 73.2% for all ages [28].

The case-fatality rate of CAP is related to the severity of the disease and is lower in series that include patients treated as outpatients [27, 28, 31, 33, 34, 37] in which the rate is ≤5%, with some studies finding no fatalities [21, 32] (Table 5).

The case-fatality rate is higher in patients requiring hospitalization. In Spain, several series have reported a rate of around 7% in patients of all ages hospitalized for pneumonia [28, 38] with age-related increases being observed.

The case-fatality rate is much higher in cases of CAP requiring intensive care unit admission [37, 39], with a mean of 36.5% [37] and a range of 20-53% according to the study [40].

Taken together, these data show that the incidence and case-fatality rate of CAP are high and, therefore, strategies aimed at their reduction should be sought.

The 23-valent pneumococcal polysaccharide vaccine (PPV-23) has been available in the USA for 25 years and is currently available in most developed countries.

Table 5. Hospitalization and case-fatality rate in CAP.

Author, Year	Place, periode	Age	N	Hospitalization (%)	Case-fatality rate (%)
Woodhead, 1987 [33]	Nottingham (United Kingdom), 1984-1985	15-79	251	22	3 0.5 (outpatients) 11 (hospitalized)
Almirall, 1993 [31]	Maresme, 1990-1991	≥14	105	50.4	1
Aguirre, 1993 [32]	Andoaín, 1991-1992	≥14	97	12.4	0
Jokinen, 1993 [34]	Koupio (Finland), 1981-1982	>1month	546	42	4
Fine, 1996 [37]	Meta-analysis	>18	33,148	-----	5.1 13.6 (hospitalized) 36.5 (ICU admitted)
Santos de U, 1998 [21]	Mallorca, 1992-1994	>14	91	26	0
Almirall, 2000 [27]	Maresme, 1993-1995	>14	241	61.4	5 8 (hospitalized)
Gutiérrez, 2006 [28]	Vinalopó (Alicante), 1999-2001	>14	493	73.2	4.8 0 (outpatients) 6.6 (hospitalized)
Ochoa, 2008 [29]	Tarragona, 2002-2003	>65	946	75	13.6 2 (outpatients) 15 (hospitalized)

N=number of CAP studied.

The effectiveness of the 13-valent polysaccharide vaccine, the forerunner of the current PPV-23 was demonstrated in randomized controlled trials in young adults in the 1970s [41-43].

Since 1984, vaccination has been recommended for persons aged ≥65 years and people aged ≥2 years with risk factors [25, 44, 45].

The current consensus is that observational studies have shown that the PPV-23 is effective in preventing invasive pneumococcal disease [42, 46, 47]. However, vaccination coverages are not high in some countries due to doubts about the effectiveness of the PPV-23 in preventing non-bacteremic pneumococcal pneumonia [42, 46, 47].

Although bacteremic pneumococcal pneumonia is the most severe form, non-bacteremic forms are much more frequent [25], with only 10-20% of cases of pneumococcal pneumonia in adults being bacteremic. Since laboratory

methods for the diagnosis of bacteremic pneumococcal pneumonia have a low sensitivity and specificity, all-cause pneumonia has been proposed as a suitable outcome to evaluate the effectiveness of vaccination [42]. If a significant proportion of cases of CAP admitted to hospital are of pneumococcal origin, and vaccination is effective against bacteremic and non-bacteremic forms, this should result in a reduction in hospitalizations for all-cause CAP.

In 1999, various Spanish regions introduced PPV-23 vaccination for patients aged ≥65 years into the vaccination schedule [45], in accordance with international recommendations [44].

Vaccine coverage in some regions reached 35% in 2001, and 40% thereafter [48]. This coverage and the large number of hospitalizations for CAP in Spain made it possible to establish the objective of this study: To evaluate the effectiveness of PPV-23 in preventing cases of CAP requiring hospitalization in people aged ≥65 years.

1. Methods

Study design

A matched case-control study in patients with CAP admitted to five hospitals in three Spanish regions between 1 May 2005 to 31 January 2007 was carried out [49].

A case was defined as a person aged ≥65 years admitted to hospital through the emergency department who presented with an infiltrate on chest X-ray compatible with pneumonia and one or more of the following symptoms or signs of acute lower respiratory tract infection: cough, pleuritic chest pain, fever >38ºC, hypothermia <35ºC or dyspnea within the past 24 hours [25, 50]. Exclusion criteria were institutionalized patients, patients with nosocomial pneumonia (onset ≥2 days after hospital admission), patients whose initial diagnosis of pneumonia was not confirmed during the hospital stay and cases of CAP in whom the pneumococcal and influenza vaccination status could not be determined

Three hospital controls were selected for each case. Controls were admitted through the emergency department with a diagnosis other than pneumonia, selected from the admission lists of each participating hospital. On selection, the vaccination status of controls was not known and, if the status could not be determined later, they were excluded.

For each case and control information on age, sex, dates of hospitalization and discharge (alive or dead), history of pneumonia, visit to the doctor in the past year, smoking, risk-consumption of alcohol and the presence or absence of underlying conditions was obtained. The pneumococcal and influenza vaccination status was also collected.

Each case was classified according to the level of risk and the degree of immunosuppression associated with the underlying disease: Stratum I (high risk) included all patients with conditions associated with immunocompromise, stratum II (moderate risk) included immunocompetent patients with one or more high-risk medical conditions and stratum III included patients not included in strata I or II (Table 6).

Each case was matched with three control subjects by sex, age (+/- 5 years), date of hospitalization (+/-30 days) and underlying disease and/or stratum.

Patient information was obtained through two sources: a) Review of written hospital medical records (underlying diseases, alcohol consumption, and history of pneumonia and vaccination status) and b) Interview of the patient or close relatives (spouse or offspring) for visits to the doctor in the past year, alcohol consumption and vaccination status using a questionnaire

Table 6. Distribution of cases and controls by stratum.

Stratum I	Stratum II	Stratum III
Solid organ neoplasia	COPD*	Patient not included on stratum I/ II.
Hematologic neoplasia	Diabetes mellitus	
Solid organ o bone marrow transplant	Heart failure grade 3 or 4	
Immunosuppressive therapy	Chronic renal failure	
Radiotherapy	Chronic liver disease	
Corticosteroid therapy (20 mg/d/15 day)	Asymptomatic infection HIV	
Autoimmune disease		
Asplenia		
Chronic renal failure requiring hemodialysis		
AIDS**		
Disabling neurologic disease		

*COPD: Chronic obstructive pulmonary disease.
** AIDS: Acquired immunodeficiency syndrome.

completed by qualified staff. Vaccination status was also obtained from the vaccination card and health care centre vaccination registers.

The vaccination status was ascertained by staff blinded as to whether the patient was a case or control.

Patients were considered vaccinated when the vaccine had been given ≥15 days before the onset of pneumonia for cases or ≥15 days before the date of hospitalization for controls**.** The same criteria were used to determine prior influenza vaccination (IV) status.

We calculated the minimum required sample size according to Schlesselman criteria [51].

We assumed a prevalence of vaccination in the control group of 0.35 [52] and vaccine effectiveness (VE) against all-cause pneumonia of 35 %. With an alpha error of 0.05 (two-tailed), a beta error of 0.20 and three controls per case, we calculated that 269 cases and 807 controls would be needed. Because vaccination coverage was estimated to be lower in some of the participating regions, we increased the number of cases to 405 and controls to 1,215. The study was approved by the ethic committee of each hospital.

Statistical Analysis

The differences observed between cases and controls according to the study variables were analysed using paired tests. The McNemar chi square test or binomial distribution test, when appropriate, was used for categorical variables and the paired t-test for continuous variables. A two-tailed distribution for all p values and considered $p<0.05$ to be statistically significant was assumed.

Conditional logistic regression (CLR) to account for the effects of confounding variables was used. The variables introduced in the CLR analysis were influenza vaccine status, variables potentially related to the vaccination response and those with a p value <0.1 in the univariate analysis**.** In the final analysis**,** variables with a significance of $p<0.05$ were included in the model. Adjusted odds ratios (ORs) for immunosuppressed (stratum I) and immunocompetent patients (stratum II and III) separately and for all three strata combined were calculated.

VE was estimated using the formula VE = (1-OR) x 100.

The statistical analysis was performed using the SPSS v15.0 statistical program.

2. Results

489 cases and 1,467 controls were included in the analysis; of 489 sets: 200 (41%) in stratum I, 190 (39%) in stratum II and 99 (20%) in stratum III.

The distribution of study variables was similar in the two groups, although more cases than controls had had a previous episode of pneumonia [119 (25.3%) vs 192 (13.7); p<0.001]. The only significant differences in the distribution of underlying diseases between cases and the three controls were in the proportions with solid organ neoplasia [51 (10.4%) in cases vs 304 (20.7%) in controls; p<0.001], hematologic neoplasia [43 (8.8%) vs 53 (3.6%); p<0.001], chronic obstructive pulmonary disease (COPD) [180 (36.8%) vs 442 (30.1%); p=0.006]; diabetes mellitus [108 (22.1%) vs 393 (26.8%); p=0.04] and corticosteroid therapy [24 (4.9%) vs 37 (2.5%); p=0.009] showed significant differences between cases and controls.

Of 489 sets, 200 were immunosuppressed and 289 immunocompetent.

Vaccination effectiveness

The unadjusted and adjusted VE according to immune status are shown in Table 7. The overall adjusted VE for all three strata combined was 23.6% (95% CI: 0.9 to 41.0). For overall VE the significant variables included finally in the model were history of pneumonia, solid organ neoplasia, hematologic neoplasia, chronic obstructive pulmonary disease and diabetes mellitus.

Table 7. Effectiveness of 23-valent pneumococcal polysaccharide vaccination in preventing hospitalization for pneumonia [49].

Group	n	Vaccinated	Unadjusted Analysis		Adjusted Analysis[#]	
			OR (95% CI)	VE % (95% CI)	OR (95% CI)	VE % (95% CI)
Overall						
Cases	489	229 (46.8)	1.0	--	1.0	--
Controls	1,467	750 (51.1)	0.795 (0.628-1.007)	20.5 (-0.7-37.2)	0.764 (0.590-0.991)	23.6 (0.9-41.0)
Immunosuppressed						
Cases	200	99 (49.5)	1.0	--	1.0	--
Controls	681	327 (54.5)	0.793 (0.561-1.119)	20.7 (-11.8-43.9)	0.790 (0.525-1.187)	21.0 (-18.7-47.5)
Immunocompetent[*]						
Cases	289	130 (45.0)	1.0	--	1.0	--
Controls	867	423 (48.8)	0.797 (0.576-1.102)	20.3 (-10.2- 42.4)	0.764 (0.544-1.072)	23.6 (-7.2-45.6)

Data are presented as n, n(%) or % unless otherwise stated.
OR: Odds ratio. VE: vaccination effectiveness. CI: confidence interval
[*]Strata II and III combined.
[#]For overall effectiveness, we adjusted for history of pneumonia, solid organ neoplasia, hematologic neoplasia, chronic obstructive pulmonary disease and diabetes mellitus.
For immunosuppressed patients, we adjusted for history of pneumonia, solid organ neoplasia, hematologic neoplasia, and chronic obstructive pulmonary disease.
For immunocompetent patients, we adjusted for history of pneumonia, diabetes mellitus and tobacco use.

The adjusted VE for immunosuppressed cases was 21.0% (95% CI: -18.7 to 47.5). For immunosuppressed patients, the significant variables included in the model were history of pneumonia, solid organ neoplasia, hematologic neoplasia, and chronic obstructive pulmonary disease.

When strata II and III were combined into one group of immunocompetent patients, the adjusted VE was 23.6% (95% CI: -7.2 to 45.6). For immunocompetent patients, the significant variables included in the model were a history of pneumonia, diabetes mellitus and smoking.

3. Discussion of results

We studied the effectiveness of the PPV-23 in preventing CAP requiring hospitalization and found an effectiveness of 23.6% (95% CI: 0.9% to 41.0%).

Before a vaccine is licensed, its clinical efficacy is evaluated in randomized clinical trials [52]. Once licensed for general use, the vaccination effectiveness can be assessed in observational studies, which are necessary when clinical trials have not provided conclusive results or have not been made in the population group for which the vaccine is recommended.

The 13-valent pneumococcal polysaccharide vaccine, the forerunner of the PPV-23 was evaluated in randomized clinical trials including young adult South-African miners [41], and the results encouraged research on its effectiveness in the population groups for which it was recommended: people aged ≥65 years and those with underlying disease of risk. The results of clinical trials in these groups were mostly inconclusive [53-57], suggesting that people susceptible to infection may not have an adequate immune response to the pneumococcal vaccine evaluated.

However, Fedson *et al.* showed that these studies did not have a sample size large enough to obtain conclusive results [42], and pointed out that the outcomes assessed in these studies were not always the correct ones. They recommended that two outcomes should be assessed in evaluations of the effectiveness of the PPV-23: invasive pneumococcal disease (pneumococcal bacteremia) or all-cause pneumonia, while other outcomes that have been studied, such as pneumococcal pneumonia, lower respiratory tract infections, pneumonia-related deaths and all-cause mortality not being acceptable [42]. This approach is based on the lack of diagnostic methods with sufficient sensitivity and specificity to correctly identify non-bacteremic pneumococcal pneumonia.

The current consensus is that observational studies have demonstrated the effectiveness of the PPV-23 in preventing invasive pneumococcal disease [42, 46, 47]. However, vaccination coverages in some countries are not high, partly due to doubts about the efficacy of the PPV-23 and its effectiveness in preventing non-bacteremic pneumococcal pneumonia [42, 46, 47, 58].

Our results show that the effectiveness of the PPV-23 in preventing hospitalization for pneumonia was 23.6% (95% CI: 0.9% to 41.0%).

Although the evidence is limited, some observational studies have shown some protective effect of the PPV-23 against hospitalization for CAP (Table 8). Nichol *et al.* [59, 60] and Wagner *et al.* [61] found that vaccination reduced hospital admissions due to all-cause CAP, and reduced the all-cause pneumonia case-fatality rate. Nichol *et al*. carried out a retrospective cohort study [59] which included 1,898 patients aged ≥65 years with COPD, and found that patients vaccinated with PPV-23 had a lower associated risk of hospitalization for CAP [relative risk (RR)= 0.57 (95% CI: 0.38 to 0.84)] and a lower risk of death from all causes [RR=0.71 (95% CI: 0.56 to 0.91)]. Wagner *et al.* [61] performed a case-control study of 1,077 residents in a geriatric hospital and found a significant reduction in the risk of pneumonia in people who received the PPV-23 [OR = 0.28, p <0.001] and a significant reduction in the risk of death from all causes [OR = 0.27, p <0.001] and death due to pneumonia [OR = 0.33, p <0.001]. Vila-Córcoles *et al.* [62] in a prospective cohort study of 11,241 subjects, confirmed the protection obtained by vaccination with PPV-23 against hospitalization for all-cause pneumonia [HR: 0.74 (95% CI: 0.59 to 0.92)] and against death from pneumonia [HR: 0.41 (95% CI: 0.23 to 0.72)].

However, Jackson *et al.* [63] in a retrospective cohort study of 47,365 people aged ≥65 years between 1998 and 2001, found no reduction in hospitalizations due to all-cause CAP [VE= -14% (95% CI: -28% to -2%)] despite finding a significant reduction in pneumococcal bacteremia [VE= 54% (95% CI: 13% to 76%)] and all-cause mortality [VE= 12% (95% CI: 5% to 17%)] in the 38,207 immunocompetent patients.

Likewise, neither Ansaldi *et al.* [64] or Skull *et al.* [65] found a reduction in hospitalizations due to CAP in patients vaccinated with the PPV-23. Ansaldi *et al.* [64] retrospectively studied 9,170 subjects of all ages (85.4% aged ≥64 years) for a period of 547,139 person-months, of which 71.7% were before and 28.3% after vaccination. They found that, in unvaccinated patients, the risk of hospitalization for asthma or otitis media was significantly higher, but that although risk of hospitalization for pneumonia was somewhat higher in people not vaccinated with PPV-23 (8.8 % in unvaccinated *versus* 7.8% in vaccinated subjects), the differences were not statistically significant [RR=1.12 (95% CI:0.91 to 1.38)].

Skull *et al.* [65] evaluated the effectiveness of the PPV-23 in preventing hospitalization for CAP in a case-cohort study of patients with a mean age of 78.4 years, but did not find that vaccination provided any benefits [RR= 0.99 (95% CI: 0.82 to 1.19].

In our study, the effectiveness of PPV-23 in preventing hospitalization for pneumonia [23.6% (95% CI: 0.9% to 41.0%)] was close to that found by Vila-Córcoles *et al.* [26% (95% CI: 8% to 41%)] [62] and Nichol *et al.* [27% (CI: 95% -13% to 52%)], although in the latter study, the results were not statistically significant [60]. The VE in our study was lower than that found by Wagner *et al.* (72.1%) [61].

Table 8. Observational studies about PPV-23 effectiveness.

Author, year, place	Type of study	Patients	Number of subjects	Outcome studied	Vaccine effectiveness % (95% CI)
Nichol, 1999, USA [59]	Retrospective cohorts (2 years)	≥ 65 years with COPD*	1,898	Hospitalization for all-cause pneumonia	43% (16-62)
				Death due to all cause	29% (9-44)
Wagner, 2003, Vienna [61]	Cases-controls (2 years)	Residents in a geriatric hospital >70 years	359 cases 718 controls	All-cause pneumonia	72% p<0.0001
				Death due to all cause	73%
				Death due to pneumonia	67%
Vila-Córcoles, 2006, Tarragona [62]	Prospective cohorts (3 years)	Residents ≥ 65 years assigned to 8 CAP	11,241	Hospitalization for all-cause pneumonia	26% (8-41)

*Chronic obstructive pulmonary disease.

A recently published meta-analysis of randomized clinical studies in older people failed to demonstrate protection of PPV-23 vaccination against all- cause pneumonia [66]. The study evaluated the efficacy of the PPV-23 in the prevention of certain clinical outcomes, including all-cause pneumonia. It also assessed the methodological quality of the trials analyzed. This meta-analysis included 22 trials of which the current PPV-23 was only evaluated in 8. Prevention of all-cause pneumonia was investigated in 19 trials, reducing to 11 who were studied in elderly patients or with chronic lung disease. The meta-analysis results for these 11 trials showed a RR = 0.89 (95% CI: 0.69 to 1.14), but these trials did not specify in which assessed the PPV-23, (and only in 8 of the 22 trials analyzed was the PPV-23 used in the study). According to information provided, it appears that only 5 of the 11 evaluated the PPV-23.

A 2008 systematic Cochrane review [67] of English-language studies evaluating the efficacy and effectiveness of the PPV-23 included 15 randomized trials and 7 observational studies. The observational studies provided evidence of protection against invasive pneumococcal disease in populations where the PPV-23 is usually used [OR= 0.48 (95% CI: 0.37 to 0.61)]. The meta-analysis of the clinical trials also showed the PPV-23 provided protection against invasive pneumococcal disease [OR= 0.26 (95% CI: 0.15 to 0.46)]. The effectiveness of the PPV-23 against all-cause pneumonia was not proven because, although the OR was significant [OR = 0.71 (95% CI: 0.52 to 0.97)] with a VE of 29% [95% CI: 3% to 48%], the meta-analysis had substantial statistical heterogeneity. The PPV-23 was associated with a not significant reduction in all-cause mortality [OR = 0.87 (95% CI: 0.69 to 1.10)].

Our study found an effectiveness of the PPV-23 in preventing hospitalization for all-cause CAP of 23.6%. It is estimated that only 30-50% of cases of CAP are due to *S. pneumoniae* [25] and therefore the effectiveness of the PPV-23 against pneumococcal pneumonia (bacteremic and non-bacteremic) would be expected to be higher. In the study by Austrian *et al.* [41] carried out in South African miners with the 13-valent pneumococcal polysaccharide vaccine, the VE was 82% against bacteremic pneumococcal pneumonia and 78.5% against pneumococcal pneumonia (diagnosed by sputum and blood cultures). Observational studies have shown that the PPV-23 prevents 50-70% of cases of invasive pneumococcal disease (due to all serotypes) [25, 42]. If 30-50% of all cases of CAP in our population are caused by *S. pneumoniae,* our findings suggest that if the level of protection against all-cause CAP was 23.6%, the level of protection against pneumococcal pneumonia would be near the level of protection (50-70%) found in observational studies of invasive pneumococcal disease [46].

Our study, like other observational studies, has strengths and limitations. One strength was the size of the sample (489 cases and 1,467 controls), which yielded statistically significant results for the overall study population. The adjusted overall VE was 23.6% (95% CI: 0.9% to 41.0%). The lack of statistical significance in immunocompetent patients may be due to a sample size that was not sufficient to study the effectiveness of groups according to the immune status.

In case-control studies of vaccination there is always a possibility of bias that distorts the results and reduces the validity of the findings [68]. One source of bias is the assessment of vaccination status in patients. However, in our study, information on the vaccination status was obtained retrospectively by investigators who were unaware of whether the patient was a case or a control, using the records of the same primary care centres for cases and

controls, and therefore this point is moot. Moreover, the vaccination status was investigated in all primary care centres to which the patient was assigned since the PPV-23 was introduced in the vaccination programme (2000).

To control for confounding variables, controls were matched with cases by date of hospitalization, age, sex and underlying diseases that could influence the disease incidence. Even so, statistically significant differences between cases and controls were observed for some variables: history of pneumonia, solid organ malignancy, hematologic malignancy, corticosteroid treatment, diabetes mellitus and COPD. The explanation is that pairing was carried out taking into account the disease with greater immunosuppression or the longest evolution, but many patients had more than one disease. To avoid the possible confounding effect of these variables, we adjusted the results using conditional logistic regression. Influenza vaccination could have a confounding effect when assessing the protective effect of the vaccine. However, we believe that this possibility was minimized as the variable was always introduced into the conditional logistic regression analysis.

4. Conclusion

The incidence of CAP is always greater in the elderly and persons with underlying diseases, and between 30 and 50% of cases of CAP requiring hospitalization are caused by *S. pneumoniae*. Therefore the reduction of pneumonia-related morbidity partially will depend on the improved use of preventive strategies such as immunization against *S. pneumoniae*.

In our study, the overall effectiveness of the PPV-23 in preventing hospitalization for pneumonia is estimated at 23.6% (95% CI: 0.9% to 41.0%). Current recommendations on PPV-23 vaccination are based on studies of vaccine effectiveness against invasive pneumococcal disease. Our results suggest that the PPV-23 is effective and reduces hospital admissions due to pneumonia in the elderly, thereby reinforcing the application of vaccination programmes in this age group.

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6. Green chemistry: Ecotoxicity and biodegradability of ionic liquids

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Abstract. Green chemistry plays a very important role in the sustainable development, seeking to reduce and prevent pollution at its source, minimizing the hazard and maximizing the efficiency of the chemical processes. Ionic liquids (ILs) are a new generation of chemicals that have a great potential for contributing to the greenness of chemical processes and developing new applications, both being of interest for the pharmaceutical industry. This work deals with the development of ILs as greener alternatives for some of the processes within the frame of green chemistry. It focuses on the environmental impact of the ILs, their ecotoxicity and potential biodegradability, compiling results of different ecotoxicological studies. ILs have the reputation of being "green" chemicals, but not all of them can pass favourably the tests evaluating their environmental effects.

Introduction

Green chemistry can be defined as the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances, along with the minimization of waste production. Green

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chemistry can be applied across the life cycle of a chemical product, including its design, manufacture, and use. Chemical and pharmaceutical industries are among the main sources of pollution and hazardous waste generation, but they are also responsible for the prevention of any potential danger to the environment.

P.T. Anastas and J. C. Warner are considered to be the promoters of green chemistry. In their book "Green Chemistry Theory and Practice" published in 1998 [1], they formulated the 12 basic principles of green chemistry. The main ideas underlying the principles are to use less chemicals and energy, more secure raw materials, solvents and processes, to achieve energy efficient processes, to minimize waste production and reduce derivatization steps. Green chemistry promotes the use of safe, environment-benign substances, including solvents, whenever possible, the control of waste in real time and the increase of the amount of raw material present in the final product.

Most of the processes that involve the use of chemicals have the potential to cause a negative impact on the environment. For this reason it is essential to eliminate or at least reduce the involved risk to an acceptable level. The simplest form to express the risk is by multiplying hazard and exposure. The traditional way to minimize the risk of chemical processes has been to limit exposure by controlling the so-called circumstantial factors, such as the use, handling, treatment and disposal of chemicals. In contrast, green chemistry seeks to minimize risk by minimizing hazard. It means that the green chemistry shifts control from circumstantial to intrinsic factors, such as the design or selection of chemicals with reduced toxicity and of reaction pathways that eliminate by-products or ensure that they are benign. Green chemistry has become a major component of the science and of the sustainable development in general. Academic interest in green chemistry involves worldwide research aimed at cleaner processing and it has increased significantly in the past few decades.

1. Green chemistry metrics and pharmaceutical industry

A key question is how one can judge whether new processes do indeed have a reduced environmental impact. A series of green chemistry metrics of different categories (mass, energy, safety, ecotoxicity, etc.) can be used in order to evaluate the efficiency and potential environmental impact of the chemical processes [2]. The first one formulated was the Sheldon's Environmental factor (E-factor) [3]. It represents the weight of waste per unit weight of product and has been widely used by chemists. This metric is very simple to understand and use. It emphasizes the waste produced in the

process, instead of in a reaction, thus helping those who try to fulfill one of the twelve principles of green chemistry, namely, to avoid waste production. The E-factor incorporates yield, stoichiometry and solvent utilization and can be used to assess multi-step reactions (step by step or in one calculation).

Another green chemistry metric used is Atom Economy (AE) which gives an idea of how much of the reactants remain in the final product [4, 5]. It is expressed by the ratio (percentage) between the molecular weight of the final product and the molecular weights of the reactants. Its drawback is that, for example, catalysts and solvents are ignored, as they are not incorporated into the final product. AE is focused on the molecular weights and not on the mass or stoichiometric yield.

Glaxo Smithkline (GSK) developed two other metrics [6] one of them being carbon efficiency, which can be calculated by the percentage of total carbon amount in the product compared to that in the reactants. The other one is the reaction mass efficiency, which compares the mass of the final product with the mass of the reactants (in percentage). It takes into account atom economy, the amount of product obtained in a chemical reaction and stoichiometry. Like carbon efficiency, this measure shows the "greenness" of a reaction but not of a process. They do not take into account the global amount of waste produced. These two metrics could describe a process as "very green", but they would not be taking into account any of the solvents used, nor the energy issues.

Another metric is Process Mass Intensity (PMI) [7] that is defined as the ratio between total mass of materials used and mass of product. PMI is similar to the E-factor and it is easy to calculate. It includes all materials used in the synthesis of 1 kg of product (e.g. reagents, solvents, water, etc) not only the waste, as it happens in the E-factor. It is important to take into account that in the life cycle analysis of a pharmaceutical process the waste's contribution is much lower than all the materials used in the process. The pharmaceutical industry, through the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable, has selected PMI as the key mass-based green metric to know the greenness of processes and uses it to obtain greater efficiency and innovation in the pharmaceutical and fine chemicals industries.

The EcoScale is a recently developed metric tool for evaluation of the effectiveness of a synthesis reaction [8]. It is characterized by its simplicity and general applicability. The EcoScale evaluates the quality of the organic preparation based on yield, cost, safety, conditions and ease of workup/purification. It uses a scale from 0 to 100 with 100 representing the ideal reaction (100% yield and minimal risk for the operator and environmental impact). The EcoScale score is then calculated by lowering the

maximum value of 100 by any applicable penalty points, which take into account both the advantages and disadvantages of specific reagents, set-ups and technologies. By calculating the EcoScale score, a quick assessment of the "greenness" of reaction protocol is obtained, and the areas that need further attention are clearly indicated, which finally can lead to the improvement of reaction conditions. The considered penalty points and its quantification are described by Van Aken et al. [8].

Among all the mentioned metrics, E-factor or PMI seem to be the most adequate to evaluate the “greenness” in the pharmaceutical industry, because they include solvents, catalysts, reaction media and any other substance involved in the processes. E-factor is useful in highlighting the fine chemicals and pharmaceutical industries (Table 1) as the areas in which green chemistry is likely to have its most immediate impact.

It is important to emphasize that these green chemistry metrics are an oversimplification and do not allow to know some environmentally important properties, such as the toxicity, the biodegradability, the bioaccumulation and the fate of the chemicals used. However, these factors are often used by chemical and pharmaceutical industries because they are easy to use and provide information that allows the comparison between different processes.

The pharmaceutical industry's constant drive to lower the spending is helping to speed up the adoption of green chemistry because it can also offer significant cost advantages. The savings involve more efficient syntheses that avoid exotic reagents, minimizing energy use, replacing organic solvents, etc. Many pharmaceutical companies are finding innovative ways to reduce their impact on the environment during drug manufacturing. Pfizer [9] is applying the principles of green chemistry in the production of Viagra®, Lyrica®, Lipitor® and Vfend®. For example, in the process of synthesis of Viagra®,

Table 1. Environmental impact as measured by the E-factor [3].

Industry	Product tons per year	E-factor (waste/product ratio by weight)
Oil refining	10^6 - 10^8	~ 0.1
Bulk chemicals	10^4 - 10^6	< 1-5
Fine chemicals	10^2 - 10^3	5-50
Pharmaceuticals	10^0 - 10^3	25->100

the number and quantity of solvents involved has been reduced [10]. The Eli Lilly Company has redesigned the synthesis of one anticonvulsive drug, LY300164. Aventis has developed a new synthesis of hydrocortisone using microorganisms. BHC Company (BASF Corporation) developed an improved efficient method to make ibuprofen using only three steps instead of the former six. In this case, all starting materials are converted to product, reclaimed as by-product or completely recycled in the process. Thus, the generation of wastes has been practically eliminated [11].

All of these examples can be cited as industry models of environmental excellence in chemical processing technology and are implementing the basic principles of green chemistry.

2. Ionic liquids: Properties and applications

In the pharmaceutical industry, organic solvents are a major source of waste because they are used as reaction media or in separation operations and their efficient control can produce a substantial improvement in the environmental impact of a process [2]. The best way to avoid problems with solvents is not to use them, an approach that has been widely exploited in the paints and coatings industries. Most reactions do, however, require a solvent, and a green chemical process must necessarily involve solvents that are environmentally acceptable. One of the new classes of solvents that offer opportunities to move away from traditional chemical processes to new, clean, green technologies are the ionic liquids [12]. BASF designed a new process for scavenging acids in the chemical synthesis of phosphorus compounds using ionic liquids: Basil^{TM} (Biphasic Acid Scavenging Utilizing Ionic Liquids), which offers significant advantages over the conventional system. This is the first large-scale industrial process worldwide that uses ionic liquids, and their developers received the "Innovation Award" of the "European Chemical News" trade journal and the BASF Innovation Award in October 2004.

An ionic liquid (IL) is generally defined as a salt in which the ions are poorly coordinated, with a melting point below 100 °C [13]. Some ILs can be liquid even at room temperature [14] and then they are called room temperature ionic liquids (RTILs). Most of the ILs used up to date have a bulky organic cation (imidazolium[a], pyridinium[b], pyrrolidinium[c], piperidinium[d], ammonium[e], phosphonium[f], etc.), substituted with alkyl chains of different length (from C_1 to C_{22}) and inorganic anions such as halogen (Cl^-, Br^-, I^-), $[N(CN)_2]^-$, $[BF_4]^-$, $[PF_6]^-$, etc. (Fig. 1). New ionic liquids, with linear aliphatic anions are currently being developed [15].

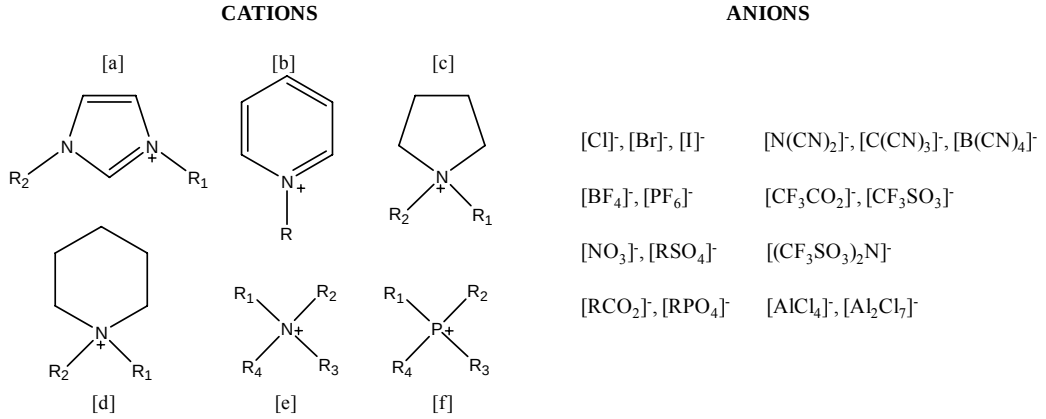

Figure 1. Principal ions present in most ILs.

Ionic liquids (ILs) are not newly discovered compounds, they have been known for almost a century. The first documented observation of ionic liquids by chemists was the so-called 'red oil' formed during Friedel–Crafts reactions in the mid-nineteenth century. The structure of the red oil was later identified by NMR as a stable intermediate composed of a carbocation and a tetrachloroaluminate anion [16, 17]. The earliest example of a room temperature ionic liquid was ethylammonium nitrate ($EtNH_3^+[NO_3]^-$) with a melting point of 12 ºC. It was described by Paul Walden in 1914 and obtained by the neutralization of ethylamine with concentrated nitric acid. One of the first 1,3-dialkylimidazolium RTILs was reported in the early 1980s by Wilkes and co-workers. It was obtained through the mixing of 1-ethyl-3-methylimidazolium chloride with aluminum trichloride [16]. Organo-aluminate ILs have a limited range of applications due to the high reactivity of the chloroaluminate anion towards water [18]. In the early 1990s, Wilkes reported two new ILs such as 1-butyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3-methylimidazolium hexafluorophosphate [16]. The cation 1-ethyl-3-methylimidazolium has been the most widely studied until 2001, and nowadays, 1,3-dialkylimidazolium salts are one of the most popularly used and investigated class of ILs [19]. Research into synthesis and possible applications of new ILs is growing in exponential form. The 20 papers published in 1994 have become more than 2,500 in 2008 [20].

The physicochemical properties of the ILs, like all other materials, depend upon the intermolecular and intramolecular forces and, subsequently, upon the structure of the cation and the anion. A significant number of investigations have been conducted for ILs on the relationship between their physicochemical properties and the structure of their cation and anion. [21-24]. In general, ILs have a negligible vapor pressure so they are not volatile and no atmospheric pollution can be expected due to their use. ILs are also

thermally stable so they can be used in chemical processes that require heat input. These and other characteristics (Table 2) such as nonflammability make them useful for many applications, and make that they meet up to the criteria of green chemistry.

There are literally millions of different structures that may be formed by combining different cations and anions and the number of possible combinations is estimated to be as high as 10^{18} [28, 29]. This enormous quantity of possible ILs would permit, based on physicochemical characteristics, to select the most appropriate for a particular purpose. In particular, RTILs are often called "designer solvents" because it is possible to create an IL with a required property. RTILs have been used for several other applications, and their development continues at a considerable rate owing to their peculiar physical and chemical properties such as high thermal and chemical stability, lack of inflammability, low volatility, and tunable solubility in several organic compounds [30]. Due to their low volatility, many of the RTILs have been used as greener alternatives to conventional toxic and volatile organic solvents by taking advantage of their unique properties [31]. This set of properties allows the design of very attractive reaction systems that can solve some of the main drawbacks of currently used methods of synthesis or to obtain new procedures for making various products [32, 33]. Polarity, hydrophilicity/hydrophobicity and other properties of ILs can be adjusted by an appropriate combination of cations and anions [34].

As the unique properties of the ILs were being discovered, there was a rising interest in applying them as a reaction medium in a wide variety of chemical transformations that until recently could only be carried out in organic solvents. The literature describes numerous uses of ILs, some of them being: reaction media for many organic transformations [35], in separations

Table 2. Some physico-chemical characteristics of ionic liquids [25-27].

Decomposition point	150 – 500 ºC
Dielectric constant	Implied ≤ 30
Electrical conductivity at 25 ºC	< 0.6 S m^{-1} (maximum value 11.9)
Flammability	Non-flammables
Melting point	< 100ºC (maximum value 239 ºC)
Temperature range liquid phase	From - 96 to 300 ºC
Thermal conductivity	0.117 – 0.199 W/m/K
Vapor pressure	Negligible
Viscosity	Usually 0.013 – 0.22 Pa.s (maximum value 1.02)

and extractions [36], as electrolytes for electrochemistry [37], in nanotechnology [38], in biotechnology [39], and in engineering processes [40], absorption of gases (CO_2) [13], as catalysts in organic synthesis [41], aldol condensation [42] and organometallic and radical polymerization [43]. Other authors have described ILs specific applications for extraction of active ingredients from medicinal plants [44-46]. Some of the ILs applications are presented in Fig. 2.

The great number of potential ionic liquids and the possible applications makes their classification a very difficult task because different criteria can be used (physical, chemical or structural characteristics, industrial applications, etc.). So, according to the chemical properties, ILs can be divided into protic (PILs) and aprotic (AILs). The distinguishing feature between both is that all PILs have a proton available for hydrogen bonding [47], whereas AILs have not.

However, for some authors, the most useful way of grouping them is based on the properties that have conditioned the evolution of their use. According to Hough et al. [48] the first generation includes ILs for which the accessible physical properties such as decreased vapor pressure and high thermal stability are often unique. Second generation ILs have potential use as functional materials (energetic materials, lubricants, metal ion complexing agents, etc.) which utilize novel tunable physical and chemical property sets. The third and most recent generation of ILs involves biological properties combined with chosen physical and chemical properties. This third generation of ILs with biological properties may be a breakthrough for the pharmaceutical industry because it opens up many possibilities to generate

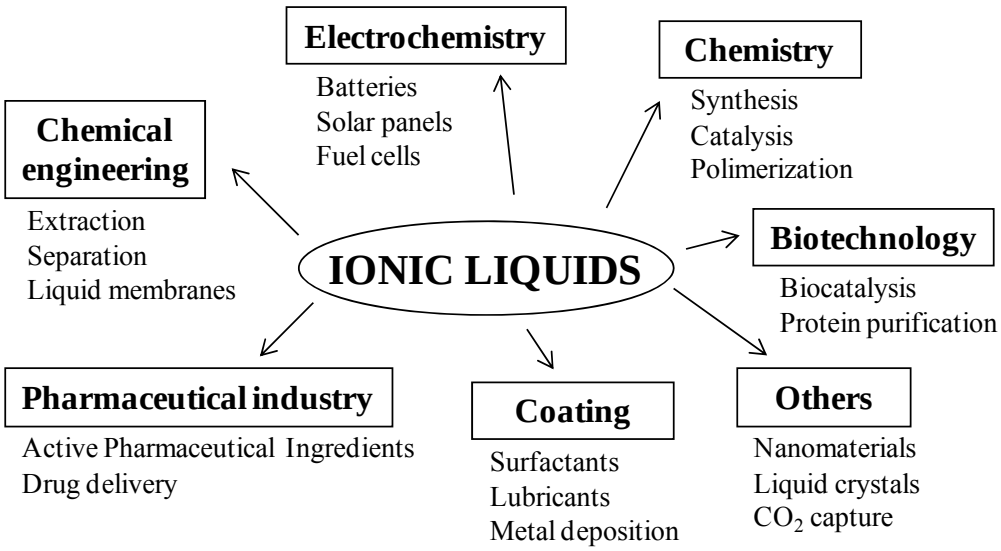

Figure 2. Possible applications of ionic liquids [51].

active pharmaceutical ingredients (APIs) in the form of ionic liquids (IL-APIs). Most of APIs are crystalline salts that present some problems related to dissolution, transport, bioavailability and polymorphism control, which can reduce the pharmacological activity [49, 50]. A possible way to overcome the drawbacks of a drug with an ionic active group is to change the complementary ion for another one able to bring IL physico-chemical properties to the new substance and thus modify its initial pharmacokinetic properties.

The design of IL-APIs with dual biological activity is a step beyond drug development. This can be achieved by the combination of a cation and an anion (both being APIs) to generate an IL that maintain the pharmacological activities of each drug while improving their pharmacokinetic properties [50, 52]. Hough et al. [48] have synthesized dual IL-APIs, like ranitidine docusate and didecyldimethylammonium ibuprofenate from ranitidine hydrochloride (histamine H_2-receptor antagonist) and sodium docusate (emollient), and didecyldimethylammonium bromide (antibacterial) and sodium ibuprofenate (anti-inflammatory), respectively. The same authors have described synergistic effects in the case of lidocainium docusate, an IL-API prepared by the combination of an analgesic (lidocaine hydrochloride) and an emolient (sodium docusate). Bica et al. [53] have paired salycilate and acetyl salycilate with a set of cations with variable biological activity covering analgesic, local anesthetic, antiarrhythmic, antimicrobial or antibacterial activity to obtain dual functional liquids salts. However although such ILs with aspirin could be prepared, they suffer from limited stability and slowly decompose into the corresponding salycilate ILs when exposed to moisture.

Recently, MEDRx Co. Ltd. (a Japanese pharmaceuticals company) and IL Pharma Inc. (a subsidiary of MEDRx Co., Ltd.) are developing Etodolac Patch (MRX-7EAT) for treatment of pain and inflammation using ILTS® (Ionic Liquid Transdermal System). MRX-7EAT is a pharmaceutical topical patch containing etodolac, a non-steroidal anti-inflammatory drug [54]. The oral etodolac products have been widely known and used to relieve pain and inflammation. However, MRX-7EAT will be the first topical etodolac patch in the world. The results of non-clinical studies in some animals have shown the safety and efficacy of MRX-7EAT, and the results of clinical trials in human have also shown the safety and tolerability.

On the other hand, some considerations should be taken into account when trying to bring new substances to market. The current legislation on chemicals in the European Union [55] called Registration, Evaluation, Authorization, and Restriction of Chemical Substances (REACH) regulates the safety of chemical products, their manufacturing, toxicity, biodegradability, transport and use in the industrial sectors. Even though ILs

have potentially "green" profile, they are basically chemical products, and as such, have to fulfill the REACH criteria, taking into account their possible commercial use. In this sense, a very important aspect that has to be studied in sufficient depth is the potential negative impact of ILs, both on the environment and humans. The findings for the toxicity of ILs must comply with the requests of the REACH.

3. Ecotoxicity of ionic liquids

Even though the ILs are considered as non-volatile and thus cannot contribute to the air pollution, the water solubility of many ionic liquids is not negligible. The potential release of ionic liquids into aquatic and terrestrial environment may lead to water and soil pollution, and related risks. So, it is important to evaluate ecotoxicity, biodegradability, bioaccumulation and environmental fate of these chemicals. Taking into account the possible use and commercialization of ILs, they should pass the REACH evaluation, which recommends that the tests used for the assessment of the potential toxicity of chemicals have to be done in accordance with OECD guidelines. These tests can be divided into three groups: effects on biotic systems, health effects, and degradation and accumulation.

There is a wide range of OECD tests that can be implemented in order to assess the potential ecotoxicity of ILs on biotic systems. Some of the most frequently used up to date are: Freshwater Alga and Cyanobacteria, Growth Inhibition Test [56], *Daphnia* sp. Acute Immobilisation Test [57], *Daphnia magna* Reproduction Test [58], *Lemna* sp. Growth Inhibition Test [59], Earthworm Acute Toxicity Tests [60], Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test [61], Soil Microorganisms: Nitrogen Transformation Test [62], Soil Microorganisms: Carbon Transformation Test [63], Collembolan Reproduction Test in Soil [64]. There are other non OECD methods that are also commonly used to evaluate the ecotoxicity on aquatic organisms: Bioluminiscence inhibition assay in *Vibrio fischeri* [65], snails [66], and zebra mussel [67].

Several properties of ionic liquids and their effects on aquatic organisms have been investigated in different works. Toxicological research studies concerning ionic liquids have been undertaken in the past decade and some of these findings will be summarized below.

Green algae are ecologically relevant organisms that are at the base of the food chain. The algal test has an advantage over tests done with e. g. fish or invertebrates because it measures a population-level response. Algae have short life cycle which makes them ideal for toxicological studies, as they can respond quickly to environmental change [68]. Cho and co-workers [69] have

found that the toxicity of 1-butyl-3-methylimidazolium, 1-butyl-3-methylpyridinium, 1-butyl-1-methylpyrrolidinium, tetrabutylammonium and tetrabutylphosphonium bromides was between two and four orders of magnitude greater than those of the organic solvents examined (methanol, dimethylformamide and 2-propanol). This group was investigating other series of imidazolium based ILs and the conclusion was that the toxicity of ILs increased with the increase in side chain length [70]. Other authors [71] indicate that ILs derived from imidazolium present an acute toxicity to *Daphnia magna* higher than benzene and some organochlorine solvents (tri- and tetra-chloromethane). Latała et al. [72] have examined the effect of imidazolium based ILs on other species of Baltic algae (*Oocystis submarina* and *Cyclotella meneghiniana*). They found that the response of the two species differed dramatically being *Oocystis submarina* more resistant to the toxicity of ILs than the other one, and that the toxicity was reduced in more saline waters. Matzke et al. [73] investigated the effect of imidazolium based ILs with C_4 and C_8 side chains on *Scenedesmus vacuolatus* species. Their findings confirmed other authors conclusions, i.e. that the toxicity strongly depends on the side chain effect.

Ecotoxicological literature on ILs toxicity to invertebrates mainly focuses on the use of *Daphnia magna* as a test organism because it is an important link between microbial and higher trophic levels. The results of all studies noted the link between toxicity and alkyl chain length of the tested ILs containing imidazolium, pyridinium or quaternary ammonium as cations

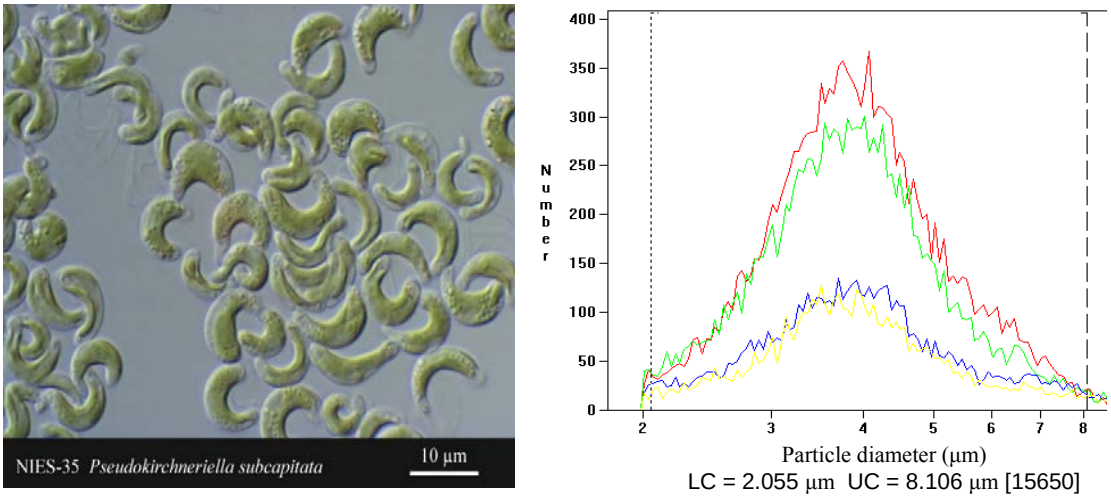

Figure 3. Morphology of *Pseudokirchneriella subcapitata* (photo from National Institute for Environmental Studies of Japan) [74] and growth algae inhibition curves showing the ecotoxicity of 100 mgL^{-1} of 1-butyl-3-methyl imidazolium (blue and yellow lines) in comparison with a control (red and green lines). Unpublished results from the authors.

[71,75-78]. Similar results were obtained in other studies on the snail *Physa acuta* with imidazolium and pyridinium based ILs [79] and also on *Dreissena polymorpha* (zebra mussel) [80]. In these tests a positive relationship between alkyl chain length and toxicity of ILs was also demonstrated, as well as the lower sensitiveness of *Physa acuta* than *Daphnia magna* to ILs.

Zebrafish (*Danio rerio*) plays an important role in ecotoxicology as an important model vertebrate. Concerning toxicity of ILs to the zebrafish, Pretti et al. [81] evaluated the toxicity of commonly used (imidazolium and pyridinium ILs) and new AMMOENG® ILs (quaternary ammonium derivates). The results of this study revealed that ILs may cause a completely different effect on fish than expected according to their chemical structures. Imidazolium, pyridinium and pyrrolidinium could be regarded as non-highly lethal towards zebrafish. On the other hand, some ammonium salts showed LC_{50} remarkably lower than that of organic solvents and tertiary amines. They also demonstrated that fish species are less sensitive to ILs toxicity compared to other species belonging to lower trophic levels.

The duckweed, *Lemna minor*, is a common aquatic plant that has been frequently a focus of the investigation of phytotoxicity of ILs [73, 82-84]. In general, 1-alkyl-3-methylimidazolium compounds with longer alkyl chains were more toxic to *Lemna minor* than those with short alkyl chain lengths. Imidazolium and pyridinium cations with butyl groups had similar EC_{50} (the concentrations that produced a 50% reduction in root growth) while the equivalent ammonium cation had a much higher EC_{50} and thus proved to be less toxic.

Microtox® is a standardised toxicity test system which is based on the reduction of the bioluminescence of the marine bacteria *Vibrio fischeri* when exposed to a range of concentrations of the tested chemical. This is a rapid, sensitive, reproducible, ecologically relevant and cost effective test. It is recognised and used throughout the world as a standard test for aquatic toxicity testing. Docherty and Kulpa [85] investigated the toxicity of imidazolium and pyridinium ILs and found that the hydrophobicity, which corresponds to the increasing alkyl chain length of the IL cation induces rising toxicity. These findings are consistent with those from other authors [73, 86]. Couling and co-workers [75] have expanded the range of investigated ILs and noted that the quaternary ammonium compounds were less toxic than the imidazolium and pyridinium analogues. In comparison to some commonly used industrial solvents such as phenol, toluene and benzene, long chain (> C_4) ILs are more toxic. The values of EC_{50} for the most frequently used organic solvents for the Microtox® test are presented in the Table 3.

Table 3. Acute toxicity of organic solvents to *Vibrio fischeri* [87].

Solvent	**log EC_{50} (μM)**
Methanol	3.50
Acetonitrile	2.77
Acetone	2.52
Benzene	2.03
Phenol	1.49

Generally speaking, all of the aquatic toxicity tests showed that the head group (cationic part of the molecule) was responsible for the toxicity of the ILs. In most cases there was no influence of the anionic part of the ILs molecule to the toxicity. Only the anion bis[(trifluoromethyl)sulfonyl]imide $[(CF_3SO_2)_2N]^-$ showed higher toxicity than others. The side chains on the head groups were proven to have a very strong influence on the toxicity. The longer and more branched the side chain is, the more toxic is the ionic liquid. Most toxic ionic liquids have an alkyl chain with more than eight carbons. These results point to the fact that some ILs may be more toxic than the commonly used organic solvents, and not as green as expected.

In Table 4 the toxicity of ILs to different levels of biological complexity is presented.

Table 4. Toxicity of different ILs expressed as logEC_{50} (μM): 1-butyl-3-methylimidazolium (C_4mim), 1-hexyl-3-methylimidazolium (C_6mim), 1-octyl-3-methylimidazolium (C_8mim), 1-butylpyridinium (C_4py), 1-butyl-3-methylpyridinium (C_4mpy), 1-butyl-3-methylpyrrolidinium (C_4mpyr), 1-butyl -3-methylpiperidinium (C_4mpip). N.A. non available.

Ionic liquid	*Vibrio fischeri*	*P. subcapitata*	*Lemna minor*	*Daphnia magna*
[C_4mim]Cl	2.95 [85]	2.34 [73]	2.82 [73]	1.93 [75]
[C_4mim]Br	3.07 [86]	3.46 [88]	N.A.	1.57 [75]
[C_4mim][BF_4]	3.55 [86]	N.A.	2.49 [82]	1.68 [75]
[C_4mim][PF_6]	3.07 [89]	2.20 [78]	N.A.	1.85 [75]
[C_6mim]Br	1.42 [75]	2.57 [70]	N.A.	0.78 [75]
[C_6mim][BF_4]	3.18 [86]	N.A.	N.A.	N.A.
[C_8mim]Cl	1.19 [89]	1.46 [78]	N.A	N.A
[C_8mim][BF_4]	1.41 [86]	N.A.	N.A	N.A
[C_4py]Cl	3.18 [84]	N.A.	2.32 [84]	N.A.
[C_4mpy]Br	2.12 [85]	3.46 [88]	N.A.	1.76 [75]
[C_4mpyr]Cl	>4.30 [84]	3.67 [88]	N.A.	N.A.
[C_4mpip]Br	4.27 [84]	3.27 [84]	0.47 [84]	N.A.

Peric et al. (unpublished results) have studied the toxic effect on aquatic organisms of a new family of water soluble PILs which are composed of ammonium substituted organic salts. The cationic moiety is mono-, di- or tri-hydroxiethylammonium while the anionic moiety is an alkylcarboxylate ($\leq C_5$). The results show no toxicity to aquatic organisms, with EC_{50} values being between 460 and 2600 mg L^{-1} for the tests of aquatic toxicity (Microtox® test and green algae growth inhibition test). According to the EU regulation [90] they have no toxic effects on aquatic organisms (all of the EC_{50} are above the limit value of 100 mg L^{-1}). Within this group of PILs the toxicity increases with the increase of complexity of the molecule. Comparing the EC_{50} values obtained for the aprotic ILs it can be seen that the new PILs are less toxic than the AILs studied up to date.

Studies on the effects of the ILs on soil and sediment organisms are very limited or still missing so far. Terrestrial organisms such as the spring tail *Folsomia candida*, a soil invertebrate [73], the earthworm *Eisenia foetida* [91] or *Caenorhabditis elegans* (a soil roundworm) [92] have been tested. In this last case, the authors suggest the use of *Caenorhabditis elegans* as a model organism for inexpensively and quickly exploring toxicological effects of 1-alkyl-3-methylimidazolium chloride ILs.

Concerning higher plants, Matzke et al. [73] investigated the toxicity of ILs derived from imidazolium with different anions to wheat (*Triticum aestivum*) and cress (*Lepidium sativum*). The side chain effect was once again confirmed, with slightly diverse patterns of toxicity depending on the anion used. Matzke et al. [93] investigated the influence of different clay minerals and clay concentrations on the toxicity of the anionic moieties of imidazolium based ILs towards wheat plants. The obtained data showed that 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide appeared to be the most toxic, independently of the type of clay added to a reference soil. The toxicity of different ILs with the same cationic moiety (1-butyl-3-methylimidazolium chloride, tetrafluoroborate and hydrogen sulfate) was mainly dependent on the cation and the observed effects varied according to the added clay type and clay concentration to the reference soil. An increase of smectite clay content resulted in less inhibitory effects of these ILs. Studzinska and Buszewski [94] have proved that hazardous effects of imidazolium ILs are closely connected with organic matter content in soil. Soil with more organic carbon was observed to sorb IL cations more extensively than soil with little or no organic matter; hence, the more fertile the soil, the lower probability of hazardous effect of ILs to plants.

Wang and co-workers [95] conducted a study on the effect of 1-butyl-3-methylimidazolium tetrafluoroborate on wheat seedlings. The increase of this IL concentration in soil showed a significant negative effect both on

germination and roots and shoot length of the wheat plants. At low concentrations, 1-butyl-3-methylimidazolium tetrafluoroborate did not inhibit, and even promoted wheat seedling growth. At high concentrations, this IL inhibited wheat seedling growth significantly and decreased chlorophyll content, thereby reducing photosynthesis and plant growth. In another research, the phytotoxicity tests of chiral ILs containing (-)-nopyl derivatives were carried out in a plant house using spring barley (*Hordeum vulgare*) which is a monocotyledonous plant, and a common radish (*Raphanus sativus* L. subvar. *radicula* Pers.) which is a dicotyledonous plant [96]. According to the obtained results, increasing the concentration of ILs resulted in a systematic decrease in the crop fresh weight of total sprouts and the crop fresh weight per plant, both for spring barley and for common radish. It could also be noted that barley was more resistant than the radish and tolerated higher concentrations of ILs in soil.

A study conducted by Peric et al. [97] evaluated the toxicity of three protic ionic liquids: hydroxyethylammonium formate, dihydroxyethylammonium propionate and trihydroxyethylammonium pentanoate to terrestrial organisms by performing different bioassays with plants (onion, grass, and radish) and soil microorganisms involved in the most important biogeochemical cycles (carbon and nitrogen mineralization). The PILs analyzed in the study showed no toxicity, with EC_{50}s above 1000 mg kg^{-1} in all assays except for *Raphanus sativus* plant test with trihydroxyethylammonium pentanoate (EC_{50} = 826 mg kg^{-1}) (Fig. 4). Within the group of terrestrial organisms, the higher plants (the three plant species tested) have shown to be more sensitive to the presence of PILs than the soil microbiota, with *Raphanus sativus* being the most sensitive to the presence of PILs. From the results it can be deduced that, in general, the compounds with more complex molecular structure have a greater tendency to cause inhibition in the organisms tested than the compounds with the smaller molecule and simpler structure. The three analyzed PILs seem to be non toxic in the terms

Figure 4. Effect of trihydroxyethylammonium pentanoate on germination and growth of *Raphanus sativus* plants [97].

of chronic toxicity for plants and C and N cycles. Comparing the results from this test with those obtained by other authors for the group of aprotic imidazolium based ionic liquids [73, 95, 98], it was observed that the analyzed PILs are less toxic than the AILs. Even though the plant species used in the test were not the same as those used by other authors, the values of EC_{50} for the AILs were generally one order of magnitude lower than EC_{50} for the tested PILs.

Although the investigation of the phytotoxicity of ILs and their effect on soil has not been intensive, the available data can give a boost for the environmental scientists to start dealing more with the potential impact of ILs towards plants and soil.

4. Biodegradability of ionic liquids

Some methods for the determination of biodegradability in water and soil are described in the Section 3 (Degradation and accumulation) of the OECD guidelines for the testing of chemicals. The most widely used, that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium, are included in the Test No. 301: Ready Biodegradability [99]. The methods are: the dissolved organic carbon (DOC) Die-Away test, the CO_2 Evolution test (Modified Sturm Test), the MITI (Ministry of International Trade and Industry, Japan) test, the Closed Bottle test, the Modified OECD Screening Test and the Manometric Respirometry Test. One of the most frequently used parameters for the assessment of biodegradation in aqueous medium is biodegradation percentage, which represents the ratio beetwen the biological oxygen demand (BOD) and the Theoretical Oxygen Demand (ThOD) calculated from the chemical formula of the compound. The pass level for ready biodegradability is 60% of ThOD for respirometric methods, reached within 28 days. Compounds reaching or exceeding the pass level after more than 28 days (the usual duration of the tests) are not to be considered as readily biodegradable. The biodegradability in soil can be assessed by means of the test No. 307 Aerobic and anaerobic transformation in soil [100] and it is also expressed as the biodegradation percentage. Other methods as test No. 217, Soil microorganisms: carbon transformation test [63] and ASTM D 5988-96 have been used by different authors to evaluate indirectly the biodegradation process [101].

The biodegradation potential of ILs in aqueous media has been addressed in some works. Wells and Coombe [78] investigated the biodegradability of ammonium, imidazolium, phosphonium and pyridinium compounds by measuring BOD. They observed that the cations with short side chains (C_4) were not biodegradable. A strong inhibitory potential to the inoculum used in

the test was observed for series with longer side chains (C_{12}, C_{16} and C_{18}), indicating the toxicity of these ILs towards the microorganisms used. Docherty and co-workers [102] examined the biodegradability of *N*-methylimidazolium and 3-methylpyridinium compounds substituted with butyl, hexyl and octyl side chains and bromide as the anion. A dependency between biodegradability and the side chain length was found in the DOC Die-Away tests and in tests monitoring the changes in the total dissolved nitrogen concentration. Another IL, 1-octylpyridinium bromide, met the OECD criterion for being classified as readily biodegradable, whereas 1-hexylpyridinium bromide exhibited a decreased degradation rate. Compared to the pyridinium ILs the mineralisation of the imidazolium ILs was lower. The 1-methyl-3-octylimidazolium cation showed significant degradation rates, but those were not high enough for a classification as readily biodegradable. For the pyridinium and imidazolium head groups carrying a butyl side chain no significant biodegradation was observable. In another study, Gathergood et al. [103] found that the influence of anion was important only in a case of the octyl sulfate anion, which proved to be considerably more biodegradable than the other commonly used anions. The introduction of an ester group in the side chain of the 1,3-dialkylimidazolium cation led to biodegradation values very close to the pass level of the Closed Bottle test.

Stolte et al. [104] investigated the biodegradation of different *N*-substituted imidazoles, imidazolium, pyridinium and 4-dimethylaminopyridinium compounds bearing various alkyl side chains. They found a significant biodegradation for ecotoxicologically unfavourable compounds carrying long alkyl side chains (C_6 and C_8). In contrast for ecotoxicologically more recommendable imidazolium ILs with short alkyl ($\leq C_6$) and short functionalised side chains, no biological degradation could be

Figure 5. Manometric respirometers used to determine carbon mineralization and indirectly the biodegradation of chemicals in water and soil (Photo from the authors).

found. The introduction of different functional groups into the side chain moiety, thus offering a higher chemical reactivity, did not lead to the expected improvement of the biological degradation potential. After an incubation period of 24 days for the 1-methyl-3-octylimidazolium cation different biological transformation products carrying hydroxyl, carbonyl and carboxyl groups were identified. Furthermore, shortened side chain moieties were identified indicating the degradation of the octyl side chain via β-oxidation.

Gathergood and co-workers [105] intended to design, synthesize and evaluate biodegradable ILs containing ester or amide groups in the alkyl side chain. The introduction of a group susceptible to enzymatic hydrolysis greatly improves the biodegradation, compared with the commonly used dialkylimidazolium ILs, 1-butyl-3-methylimidazolium tetrafluoroborate and hexafluorophosphate. For the 3-methyl-1-alkyloxycarbonylmethylimidazolium bromide series, the greatest biodegradation was observed when alkyl side chain had 4 or more carbons. The corresponding amide analogs proved to be poorly biodegradable. In the next phase of their investigation [106] they tried to establish the influence of the anionic moiety on the biodegradability of ILs. Different C_4mim cations combined with Br^-, $[BF_4]^-$, $[PF_6]^-$, $[N(CN)_2]^-$, $[(CF_3SO_2)_2N]^-$ and octylsulfate as the counter ion were analyzed using the Sturm and Closed-Bottle test protocols. No compound showed significant degree of biodegradation with the exception of the IL containing octylsulfate anion which had higher levels of biodegradability. The same group did a further research toward the discovery of biodegradable ILs [103]. The aims of the study were to incorporate additional structural modifications in order to improve biodegradability. They incorporated 2-methyl group into the molecule of imidazolium ILs to provide an additional site for metabolism, parting from a fact that 2-methylimidazole is significantly more biodegradable than imidazole. The incorporation of a methyl group in the 2-position of imidazolium cation had no significant effect on biodegradability. The commonly used 1-butyl-3-methylimidazolium core showed negligible levels of degradation. Part of this team continued their research by trying to design, synthesize and evaluate biodegradable pyridinium ILs. Harjani et al. [107] prepared ILs bearing an ester side chain moiety, using either pyridine or nicotinic acid. These ILs showed high levels of biodegradation under aerobic conditions and can be classified as ‘readily biodegradable’. In contrast, pyridinium ILs with alkyl side chains showed significantly lower levels of biodegradability in the same test.

The fact that only the long side chains in ionic liquids improved biodegradability creates a conflict of aims between minimizing the toxicity and maximizing the biodegradability. The issue of biodegradability seems to be a problem in the development of environmentally safer ionic liquids.

Peric et al. (unpublished results) have studied the biodegradability in water of three ILs from the new family of PILs, derived from substituted ammonium salts (hydroxyethylammonium formate, diydroxyethylammonium propionate, and trihydroxyethylammonium pentanoate). Almost all of the analyzed PILs showed to be readily biodegradable with biodegradation rates of 57 to 86% (expressed in terms of theoretical oxygen demand) in a 28 days period. These results can be due to the fact that these PILs do not show toxicity towards microorganisms and also to their non cyclic simple structure. Even though they have cations and anions composed of short alkyl chains ($\leq C_5$), these chains have hydroxyl and carboxyl functional groups which can increase the biodegradability rate, in accordance with the findings of other authors related to ester and amide functional groups [105].

Like in the case of the terrestrial ecotoxicity, the data on the biodegradability in soil is scarce. Kumar et al. [108] investigated the fate of 1-butyl-3-methylimidazolium tetrafluoroborate when in contact with soil-microorganisms, wastewater microorganisms, *Pseudomonas putida* and *E. coli*. Although 1-butyl-3-methylimidazolium tetrafluoroborate was indicated to be recalcitrant in Sturm and Closed-Bottle test assays as mentioned above, it was observed in this study that *P. putida* was able to break down 1-butyl-3-methylimidazolium tetrafluoroborate after 15 days of incubation.

The aerobic biodegradation processes of ionic liquids in soil were monitored for the first time by Modelli et al. [101] , working with four ionic liquids obtained from the 1-butyl-3-methylimidazolium and 1-methoxyethyl-3-methylimidazolium cations combined with the tetrafluoroborate and dicyanamide counter anions, by measuring the total production of CO_2 over six months, according to ASTM D 5988-96. The results indicate that the biodegradability rate ranges between 17 and 52% for 1-butyl-3-methylimidazolium and between 0.1 and 3.6% for 1-methoxyethyl-3-methylimidazolium, with dicyanamide and tetrafluoroborate anions respectively. In both cases the biodegradation rate did not exceed 10% in 28 days of the test duration.

Peric et al. (unpublished results) have performed other type of studies of biodegradability of ILs in soil, according to test No. 217, Soil microorganisms: Carbon transformation [63], using three ILs of the new family of PILs, (hydroxyethylammonium formate, dihydroxyethylammonium propionate, and trihydroxyethylammonium pentanoate). The results indicate that the biodegradation rate ranges between 60 and 90%, with the most complex ILs showing the lowest biodegradation rate. The ILs from the new family of PILs are clearly less toxic than the ILs derived from imidazolium, pyridinium, pyrrolidium, etc.; and also less toxic than the conventional solvents, with a notable rate of biodegradation in water and soil. This can be a path to follow

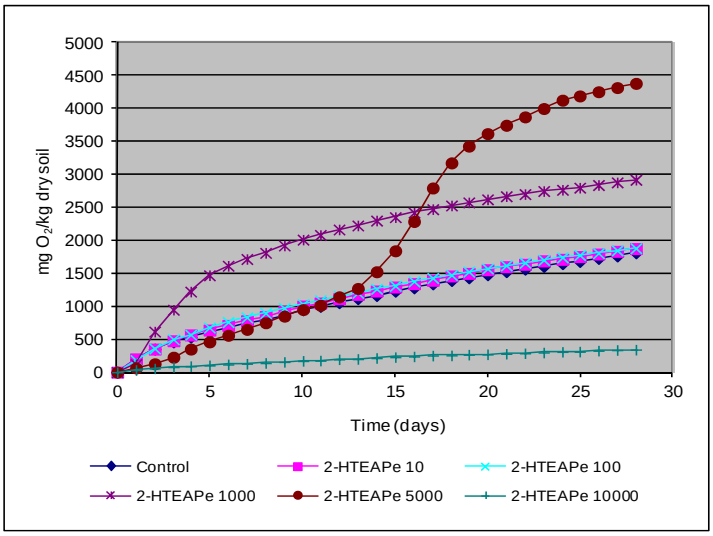

Figure 6. Cumulative consumed oxygen of different concentrations of trihydroxyethylammonium pentanoate (2-HTEAPe) compared to the control soil, according to OCDE test 217 [63], to observe inhibitory effect (10,000 mg kg^{-1}) and the biodegradation (1,000/5,000 mg kg^{-1}) of IL. Data from the authors.

regarding a synthesis of really green ILs, formed by a pair of organic ions, with simple (short and lineal) structure and functional groups which facilitate biodegradation.

5. Conclusion

The large number of possible ILs structures and their unique physical, chemical and biological properties make it feasible to consider them as an opportunity to contribute to the greenness in the various fields in which they can be applied. Recent studies show that not all ILs synthesized to date are as green as expected. Some of the cyclic AILs, especially the ones derived from imidazolium and pyridinium with long side chain substituents, have proven to be even more toxic for aquatic organisms than the classical organic solvents that they are aiming to replace. However, the constant synthesis of new ILs presents an opportunity for the design of compounds which can comply with the most demanding technical requisites while presenting low levels of ecotoxicity and high biodegradability. A good example of this is the new familiy of PILs based on ammonium substituted organic salts.

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7. Strategies for the synthesis of enantiopure compounds focused on organocatalysis

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Abstract. The preparation of enantiomerically pure compounds (EPC) is a continuous social demand due to the clinical advantages that enantiopure drugs offer over the racemic forms. Here, the best well-established synthetic strategies to access to single-enantiomer compounds are briefly described and compared. In particular, the enantioselective catalysis is introduced paying special attention to the organocatalysis, an emerging and fruitful area in the EPC-synthesis. Of particular interest is the use of small organic molecules as catalysts in cascade reactions. Organocascade reactions involve the formation of several chemical bonds and often generate stereogenic centers with excellent stereoselectivity. Such one-pot reactions avoid time-consuming and costly step-by-step processes and are environmentally friendly as they occur in the absence of metals. Additionally, the chemical waste of the organocatytic cascade reactions is drastically reduced since the intermediates are not isolated and purified.

Introduction

In the pharmaceutical field, stereochemistry is placed in an extremely relevant position. The tridimensional structure of a compound is very important when interacting with a chiral medium as the human body, the

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biological effect directly depending on the stereochemistry of the exogenous compound and the receptor. Thus, a single-enantiomer drug can be pharmacologically interesting whereas its mirror image can be inactive or display a different desirable or non-desirable activity. Therefore, the administration of enantiopure drugs brings benefits in terms of improved efficacy, more predictable pharmacokinetics and reduced toxicity [1]. These advantages forced pharmaceutical companies [2] and health authorities [3] to place stereochemically pure substances in a privileged position. Consequently, it is not strange that 7 out of the top 10 most selling-drugs worldwide in 2010 are commercialized as enantiopure forms. Among them, the top three positions are for Nexium® (esomeprazole), Lipitor® (atorvastatin), and Plavix® (clopidogrel), with a whole invoicing of 15 billion dollars [4].

The large demand of enantiopure products has broken out the progress of the *asymmetric synthesis*, considered in the last years as one of the most important areas of research in both industry and academia. Nowadays, the number of synthetic methods available for the preparation of chiral molecules has permitted to efficiently gain access to a myriad of enantiomerically pure compounds.

1. Strategies for the elaboration of enantiopure compounds

In 1980 Prof. Seebach [5] introduced the term "*EPC-synthesis*" (synthesis of enantiomerically pure compounds) to include all the processes for the preparation of chiral enantiopure compounds. The three main synthetic approaches in EPC-synthesis are listed below (Fig. 1) [6]:

- *Resolution of racemates.*
- Synthetic transformations from an enantiomerically pure starting compound. In the particular case of an easily available natural compound it is called synthesis from the *chiral pool*.
- *Stereoselective reactions* that involve an enantiopure reagent as a source of chirality, in stoichiometric (auxiliary) or catalytic amounts, which is not included in the final product.

Next, the three main strategies to obtain enantiopure compounds are outlined.

The first optical resolution of a racemic mixture was performed by Prof. Pasteur in 1848, who was able to manually separate the two kinds of hemihedral crystals of racemic tartaric acid salts [7]. This fact represented the discovery of the molecular chirality and of the spontaneous resolution [8]. In

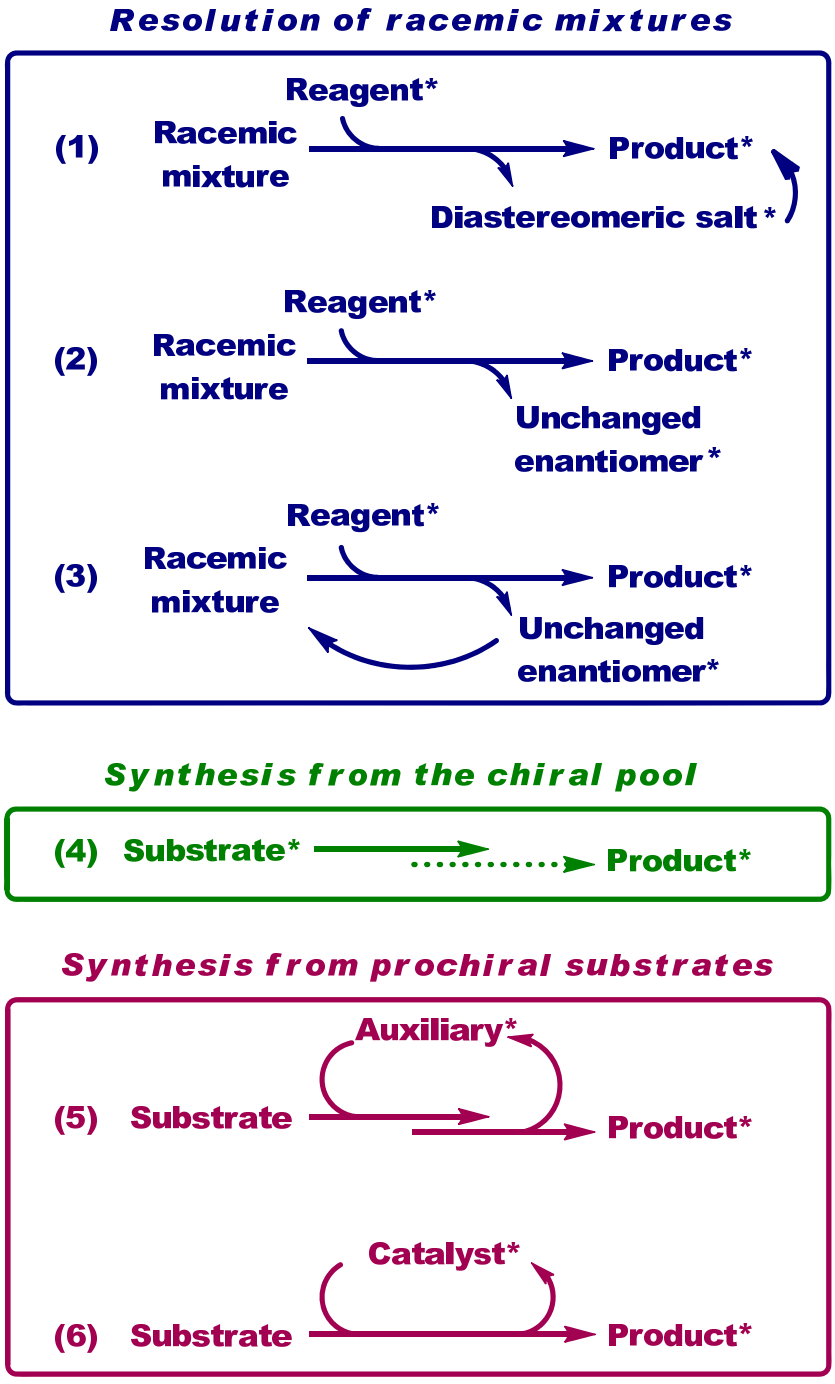

Figure 1. Strategies for the synthesis of enantiopure compounds.

spite of the simplicity of this separation technique it is limited to conglomerates, since both enantiomers of the substance deposit in equal quantities as enantiomorphous crystals [9]. It is worthy of note that only between 5 to 10% of the total chiral organic solids reveals as conglomerates. Notably, the vast majority of resolutions involve the conversion of a racemate, by treatment with an enantiomer of a chiral substance, into

diastereomeric salts (Eq.1, Fig. 1). The different solubility properties of the diastereomeric salts allow the separation of both products and the subsequent treatment with a base or an acid give access to both enantiomers.

Apart from the aforementioned resolution process based on physical properties like solubility, there are other resolution processes as the *kinetic resolution*. The kinetic resolution relies on the unequal reaction rates of the enantiomers with a chiral nonracemic reagent (Eq. 2, Fig. 1). In that case, the reaction rates should be different enough to recover the less reactive or non-reactive enantiomer. The maximum theoretical yield for a kinetic resolution is 50% for each enantiomer and one of them is chemically modified. Of particular interest is the *dynamic kinetic resolution* that permits the total conversion of a racemic mixture into a single enantiopure product (Eq. 3, Fig. 1). This strategy involves a standard kinetic resolution and an *in situ* racemization process of the less reactive enantiomer (unchanged enantiomer), which must be a labile chiral substrate for the easy conversion into the racemic mixture again [10].

In the *chiral pool* synthesis the chiral source is a natural enantiopure compound that will remain included in the structure of the final product (Eq. 4, Fig. 1). This methodology is therefore more useful when the desired final product and the chiral compound used are structurally similar. The chiral pool arsenal is integrated by carbohydrates, amino acids, hydroxy acids and terpenes.

The *stereoselective synthesis* from prochiral substrates is a potent tool that allows the preparation of a broad variety of enantiopure compounds. This methodology involves chemical reactions that introduce one or more elements of chirality in a substrate molecule producing stereoisomeric products (enantio- or diastereoisomers) in unequal amounts [11]. A chiral auxiliary or a catalyst is responsible for the asymmetric induction.

The use of auxiliaries is based on the temporarily incorporation of a chiral moiety in a prochiral substrate (Eq. 5, Fig. 1) generating new elements of chirality selectively. The process involves: 1) the introduction of the auxiliary; 2) high diastereoselectivity in the process of generation of the new elements of chirality, 3) separation of the formed diastereoisomers and finally 4) recovery of the chiral auxiliary. As chiral auxiliaries are required in stoichiometric quantities, their preparation has to be easy and inexpensive.

Nowadays, different well-known chiral auxiliaries allow to selectively perform a great number of reactions [12]. Among them the Evans' oxazolidinones [13] or the Oppolzer's sultames [14] constitute robust examples. Remarkably, the use of chiral amino alcohols pioneered by A. I. Meyers [15], and extended for other research groups [16], played an important role as chiral auxiliary strategy and nitrogen source for the efficient synthesis of alkaloids and nitrogen-containing bioactive compounds.

Chiral auxiliaries become very popular 30 years ago due to their efficiency in the generation of stereoselectivity, affording 100% pure enantiomers after the separation of the diastereoisomers. However, the need for using stoichiometric quantities of the chiral auxiliary and the disadvantage of requiring two additional synthetic steps, introduction and removal of the auxiliary, prompted synthetic chemists to divert their attention to the asymmetric or enantioselective catalysis.

In the *enantioselective catalysis* the chiral information is transferred by an enantiopure catalyst, which means that substoichiometric quantities of a chiral molecule activate the substrate in a reversible manner to accelerate the reaction (Eq. 6, Fig. 1). As the interaction between the catalyst and the substrate is reversible the catalyst is not consumed during the process and can be introduced in a new catalytic cycle. The atom economy of the process is optimal, minimizing the waste generated [17]. An additional advantage of this methodology is the multiplication of the chirality [18], since stoichiometric quantities of enantioenriched product are obtained from substoichiometric quantities of catalyst.

In contrast to the use of chiral auxiliaries that affords enantiopure compounds through diasteromeric intermediates, asymmetric catalysis furnishes directly enantiomers from prochiral compounds allowing the preparation of a broad variety of chiral compounds with high enantiomeric excess.

A relevant datum of the importance of asymmetric catalysis in Chemistry is that Profs. W. S. Knowles, R. Noyori and K. B. Sharpless were awarded with the Nobel Prize in 2001 due to their research on the field (Fig. 2).

Enantioselective catalysis can be divided in three main areas, biocatalysis, organometallic catalysis and organocatalysis, depending on the nature of the chiral catalysts employed.

In the *biocatalysis* an enzyme is responsible for the acceleration of the process [19]. Due to their complex tridimensional structure of *L*-amino acids, the biocatalysts are involved in highly chemo-, regio-, diastereo- and enantioselective processes. Moreover, these reactions are carried out under mild conditions to avoid side products and the processes are environmentally harmless.

The *organometallic catalysis* is responsible for the most part of the enantioselective catalysis published in the last years [20]. The success of this area is mostly due to the particular affinity of metals to complex with functional groups and to the structurally well defined metal-organic ligand complexes which is translated into an efficient asymmetric induction.

An important contribution in the field dates from the seventies when Prof. W. S. Knowles and co-workers [21] demonstrated that complexes of rhodium and phosphine ligands with C_2 symmetry catalysed the addition of

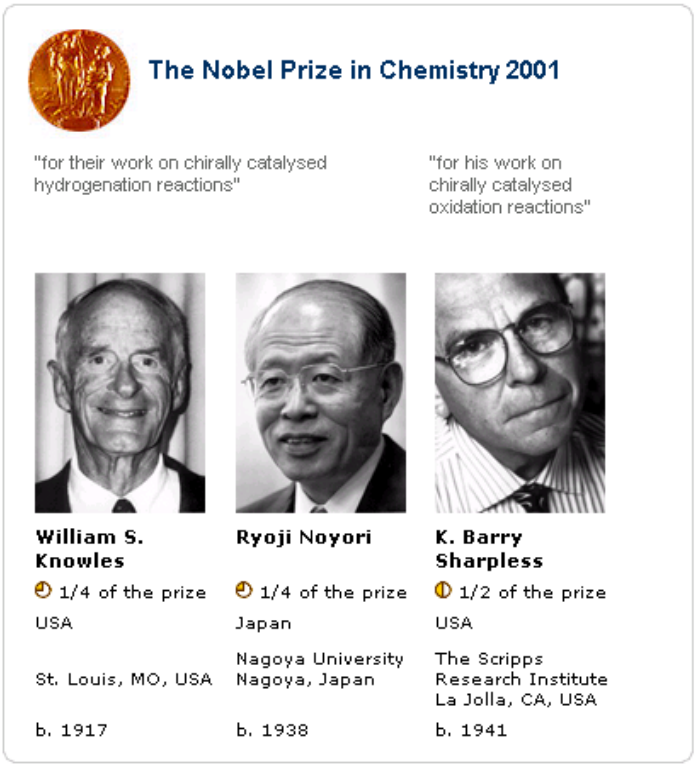

Figure 2. Nobel Prize in Chemistry (2001) shared by W. S. Knowles, R. Noyori and K. B. Sharpless. (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2001/)

hydrogen to one of the faces of a prochiral olefin to generate a stereogenic C-H center with high enantioselectivity (Fig. 3). Apart from giving a boost to the development of ligands with a C_2 symmetry axis, the main application of this reaction was the industrial preparation of *L*-dopa [22].

Nowadays, the organometallic catalysis enjoys an elevate grade of development including participation in a great variety of oxidations, reductions, insertions to σ bonds, activations of π bonds and reactions catalysed by Lewis acids. In spite of this advantage, some organometallic complexes are expensive, demanding on strict reaction conditions, and metals are toxic which is negative from both the environment and the contamination of the final product point of view.

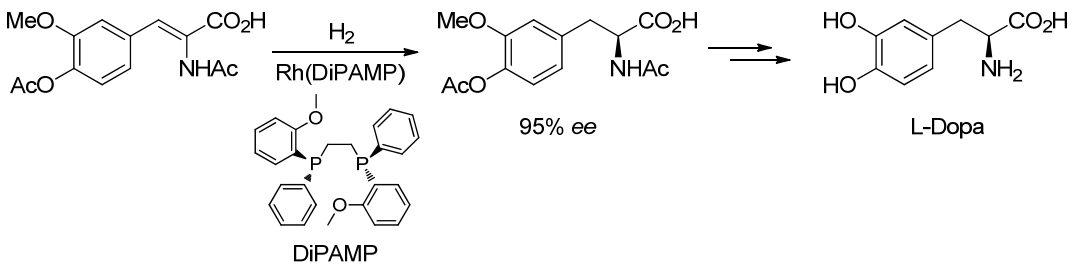

Figure 3. Enantioselective hydrogenation catalysed by rhodium-DiPAMP complex.

Environmental and health regulations gave strong support to cleaner and non-toxic chemical processes avoiding the use of toxic reagents. Chemical companies consider that get rid of transition metals in catalytic reactions is highly desirable. In fact Nature, our source of inspiration, does not use metals for the most part of biocatalytic processes. In this sense, the efforts made for the scientific community to get synthetic sequences free of metals have driven to the configuration of a new area within the enantioselective synthesis: the *organocatalysis*.

The organocatalysis is defined as the acceleration of chemical reactions by small organic molecules in the absence of metals [23]. Although the organocatalysis is a field that has been incredibly developed in the last decade, the use of organic molecules to catalise chemical reactions has been known for more than a century [24]. In 1896, Emil Knoevenagel used a secondary amine (piperidine) to accelerate the condensations that received his name (Fig. 4) [25].

The first example of an asymmetric organocatalysis was described by Mackwald in 1904 using the brucine alkaloid in a decarboxylation process [26]. In 1912, Bredig and Fiske described the asymmetric addition of HCN to benzaldehyde catalysed by cinchona alkaloids [27]. However, those examples occurred with a low enantiomeric excess, below 10%. Later on, in 1960 Pracejus published the organocatalytic methanolysis of a ketene with higher enantioselectivity levels (76% *ee*) [28]. In the seventies a remarkable hit in the organocatalysis was achieved when the first asymmetric aldolization (Hajos-Parrish-Eder-Sauer-Wiechert reaction) catalysed by *L*-proline was described (Fig. 5) [29].

In the following decades (1980-2000), two new fields in the catalysis with organic molecules started with the publication of the two first examples in the phase transfer catalysis [30] and the activation through hydrogen-bonding interactions [31].

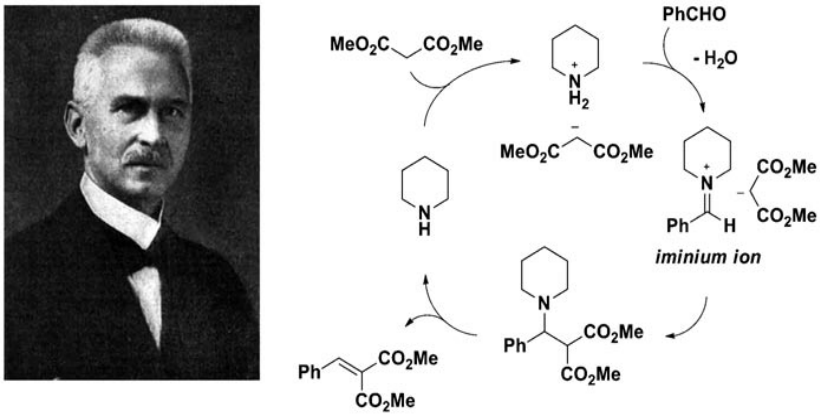

Figure 4. Prof. Emil Knoevenagel (1865-1921). Piperidine catalysed the reaction of diethyl malonate with benzaldehyde.

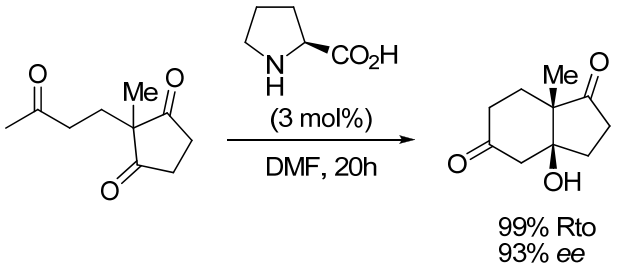

Figure 5. Hajos-Parrish-Eder-Sauer-Wiechert reaction.

In 2000, a study carried out by List, Lerner and Barbas III demonstrated the ability of small molecules to catalyse reactions that until then were promoted by bigger organic molecules or enzymes through similar mechanisms [32]. The same year, MacMillan and co-workers developed the first highly enantioselective organocatalytic Diels-Alder reaction catalysed by a secondary chiral amine, an imidazolidinone [33].

This new application and the conceptualization of the word "organocatalysis" by MacMillan, represented the revival of the secondary amines and other organic molecules as catalysts. The strong support of the scientific community to this field was due to the numerous advantages that the small molecules represent for catalysis. Some of them are listed below:

- The organocatalysts usually are non-sensitive to the humidity and the atmospheric oxygen. This stability makes catalysts easy to handle because it is not necessary the use of dry boxes, inert atmosphere or anhydrous solvents, improving the reproducibility of the results.
- Nature provides enantiopure compounds that can be used directly as organocatalysts. Thus, these molecules are easily available in considerable quantities and in both enantiomeric series.
- The organocatalysts are low or non-toxic substances, respectful with the environment. Moreover, they can be easily isolated from the reaction mixtures avoiding the contamination of the final product. It is noteworthy that the most part of the organocatalytic reactions are performed in high concentration or in the absence of solvent minimizing the expenses of solvent and the formation of additional residues. These properties increase the level of safety and reduce the cost of research either in the academic or industrial areas.

Nowadays, the organocatalysis is a potent synthetic tool situated in a privileged position between the two main catalytic strategies (the bio and metal catalysis) and complements them.

The interest in catalysis with organic molecules is indicated in the increasing number of publications related to this subject in the last decade. The number of publications containing the concept of "organocatalysis" rose steadily from 2000 to 2007 (Fig. 6) rising to 821 publications in the year 2010. Prior to 2000 the number of articles containing the indicated keyword was almost negligible.

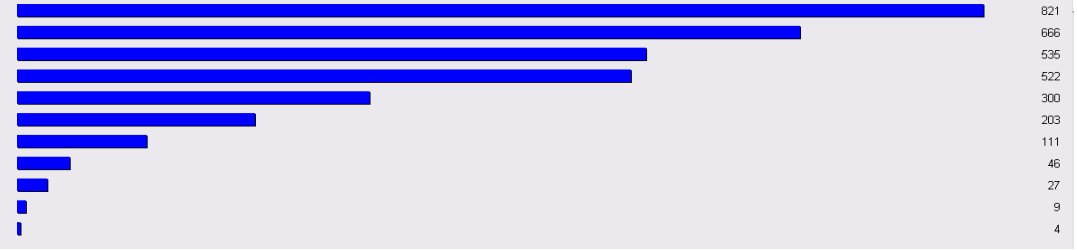

Figure 6. Number of publications including the term "organocatalysis" in the title or in the abstract from the year 2000 (4) to 2010 (821) in the SciFinder Scholar (2007) database.

This tremendous advance in the organocatalysis has given access to a great number of enantiomerically pure compounds with an added value from the biological and structural point of view including drugs and natural complex products [34].

The development of novel activation methods is responsible for the rapid and continuous progress on the organocatalytic area gaining applicability to wide organic reactions. In a first level, the different activation methods can be classified taking into account if the reversible interaction between the catalyst and the substrate is non covalent (1) or covalent (2). The former is also divided depending on the implication of ionic pairs or hydrogen bonds. The latter is classified into *N*-heterocycles carbene catalysis or amine catalysis, also known as *aminocatalysis*. The reactive species that is formed when the catalyst interacts with the substrate determines the kind of aminocatalysis involved. Next, this classification is underlined and the different headings are briefly described.

1. Non covalent interactions:
    a. Formation of ionic pairs:
        i. Phase transfer catalysis
        ii. Brønsted base catalysis
    b. Formation of hydrogen bonds
2. Covalent interactions:
    a. Catalysis with *N*-heterocyclic carbenes
    b. Catalysis with amines (via iminium ion, enamine, dienamine, radical cation or ammonium ion)

The enantioselective phase transfer catalysis (PTC) (1.a.i) is performed in a heterogeneous medium and uses quaternary ammonium salts to facilitate the migration of the reagent from one phase to the other. The asymmetric induction is based in the formation of a chiral ionic pair soluble in the organic phase that is where the stereoselective reaction occurs. The most used ammonium salts are the cinchona alkaloids derivatives and binaphthylamine derivatives [35]. The Brønsted base catalysis (1.a.ii) [36] is based on the use of a chiral base able to deprotonate a (pro)nucleophile (chiral ionic pair) increasing the reactivity in front of eletrophiles. The most employed Brønsted bases as chiral catalysts are the cinchona alkaloids and derivatives, due to their availability and their catalytic and inductive efficiency. This family of compounds is found in Nature as two pseudoenantiomeric forms (Fig. 7) and each of them furnishes one of the two possible enantiomers of the reaction product.

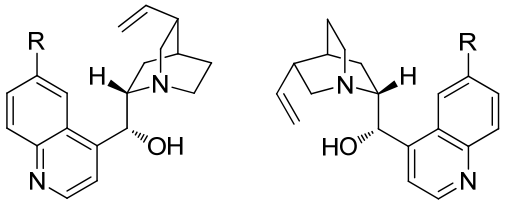

Figure 7. Pseudoenantiomeric cinchona alkaloids.

Cinchona alkaloids have been considered as one of the most privileged chiral inductors in organic synthesis [37]. Different structural features of cinchona alkaloids are responsible for the efficient activation and transmission of the chiral information to the substrate [38]: 1) the presence of a basic nitrogen atom included in a bulky quinuclidine nucleus and a secondary alcohol that can activate the substrate establishing hydrogen-bonding interactions; and 2) the presence of a quinoline ring that can adopt different conformations in the reaction media acting as a "molecular wall" for the preferential approach of the reagent. When the basic nitrogen of the quinuclidine activates the (pro)nucleophile and the hydroxyl group interacts with the electrophile through hydrogen-bonding interactions, cinchona alkaloids are considered to be bifunctional catalysts as a double simultaneous activation occurs in the process [39].

The formation of hydrogen bonds is very important in biological systems and in metabolic terms such interaction between enzymes and substrates means the acceleration of a wide variety of reactions. This biological strategy of activation is mimicked by different catalysts (1.b) [40]. Among them,

phosphoric acids and thioureas stand out due to their efficiency in the formation of highly organized transition states, a very important factor for the discrimination of the enantiotopic faces of a substrate.

In the catalysis through covalent interactions between a catalyst and a substrate is highlighted the use of *N*-heterocyclic carbenes (2.a) [41] and the catalysis using amines (2.b) [42]. The aminocatalysis is based on the use of amines as catalysts and represents an important part of the organocatalysis. There are different activation modes in aminocatalysis that allow to establish a classification depending on the reactive species that is generated in the interaction between the substrate and the catalyst.

In the iminium ion catalysis [43] the reactive species is an iminium salt formed by the reversible reaction between a primary or secondary amine (catalyst) and a carbonyl compound (substrate). Thus, a lowering in the energetic potential of the lowest unoccupied molecular orbital (LUMO) is observed facilitating the reaction with nucleophiles. The Knoevenagel condensation and the Michael addition are two examples of reaction in which this activation strategy has been successfully applied. When the substrate is an α,β-unsaturated ketone or aldehyde, the iminium ion catalysis favors the β-functionalization of the carbonyl compound (Fig. 8).

The catalysis via enamine [44] involves the generation of an enamine that results from the tautomerization of an iminium ion intermediate. In that case, a primary or secondary amine activates the substrate increasing the energetic potential of the highest occupied molecular orbital (HOMO). The HOMO-raising activation facilitates the reactivity of the α-carbonyl position with electrophiles. This strategy has been frequently used in aldol and Mannich reactions (Fig. 9).

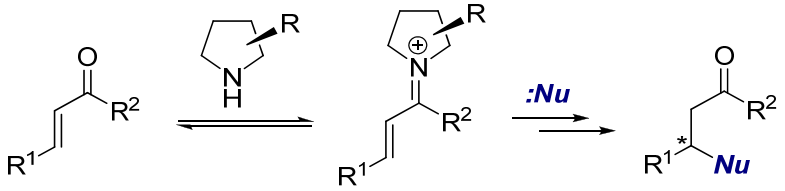

Figure 8. Activation via iminium ion (*Nu* = nucleophile).

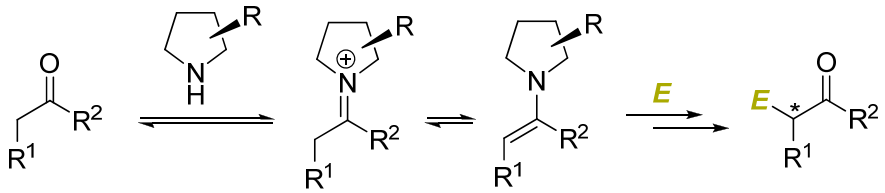

Figure 9. Activation via enamine (*E* = electrophile).

The catalysis via dienamine, introduced in 2006 by Jørgensen and co-workers [45], can be considered as a kind of catalysis via enamine and it has been used for the functionalization of the γ position of α,β-unsaturated aldehydes with electrophiles.

In the last years, a new class of catalysis via radical cation has been developed [46]. In this case, SOMO (single electron occupied molecular orbital) activation is produced allowing the introduction of different substituents in the α carbonyl position. This new methodology has expanded the field of organocatalysis merging aminocatalysis with radical chemistry.

Finally, the catalysis via ammonium ion implies tertiary amines [47] as catalysts. The formation of ammonium enolates [48] typically with cinchona alkaloids, and the formation of acyl-ammonium with DMAP analogues [49] are representative examples.

The transmission of the chiral information from the catalyst to the final product must be very efficient in order to achieve stereoselective chemical processes. In the particular case of the aminocatalysis, the stereoelectronic properties of the catalyst (amine) are responsible for the asymmetric induction. Thus, the stereocontrol of a reaction can be determined by steric factors, electronic factors or a combination of both. Chiral catalysts bearing bulky groups can prevent an arbitrary approach of the reagent to the substrate forcing an oriented bond formation that avoids the steric hindrance. In an opposite way but complementary, catalysts with hydrogen-bond donating groups can establish interactions of electronic affinity with hydrogen-bond accepting groups present in the reagent, thus facilitating the reaction between the two reacting partners in a well-defined manner.

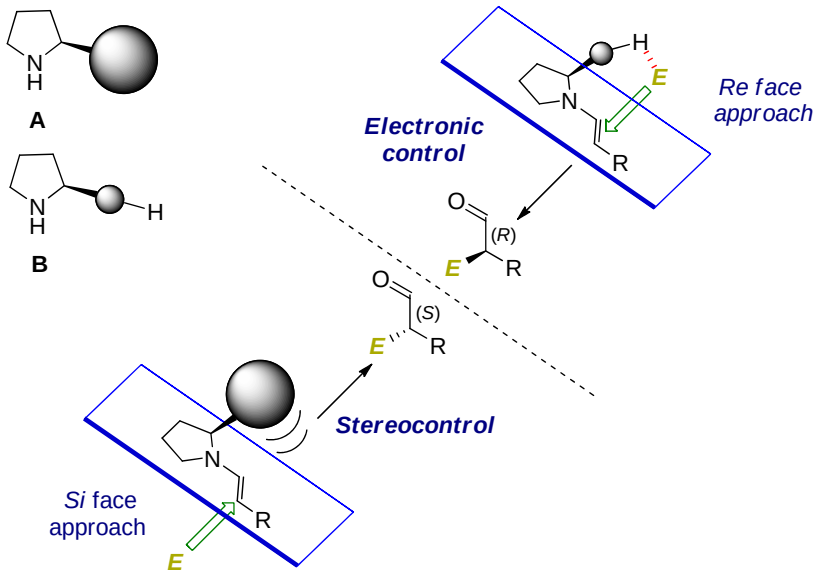

Figure 10. Stereoelectronic control of the reaction by the catalyst.

Figure 10 depicts how different substituted chiral pirrolidines (**A** and **B**) can orientate and control the addition of aldehydes to electrophiles through an enamine like intermediate. The presence of bulky substituents in the organocatalyst (**A**) can shield the enamine *Re* face forcing the electrophile to a *Si* face approach. However, the use of pirrolidines with hydrogen-bond donor groups (**B**) would facilitate the approach of the electrophile preferentially for *Re* face of the enamine. Thus, both strategies would drive to opposite enantiomers.

2. Cascade reactions

Evolution provided living organisms with biosynthetic processes that convert efficiently simple molecules into complex molecular systems. One of the key features of the biosynthetic routes in Nature is the achievement of cascade reactions. As an example, the pharmacologically and structurally very interesting diterpene taxol is proposed to be biosynthetically prepared in just five steps involving few enzymes [50] (Fig. 11). However, the most efficient synthesis of taxol achieved in the laboratory by Wender´s group involved 37 steps from verbenone with 0.44% overall yield [51].

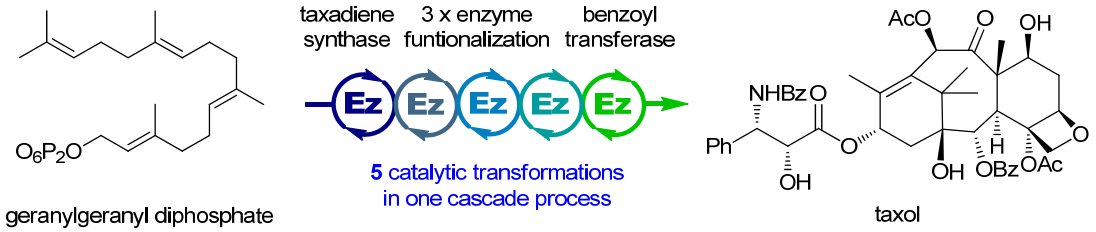

Figure 11. Proposed biosynthesis of taxol.

In the laboratory, the preparation of complex chiral molecules requires several synthetic steps and involves the isolation and purification of intermediates. This operational strategy prevents, in most cases, a low-cost fast access to enough quantities of interesting natural products and bioactive compounds for the benefit of the community.

The attempts to mimic Nature for avoiding these limitations led to the development of new *one-pot* strategies. The one-pot reactions are carried out in a simple vessel allowing the formation of various chemical bonds and stereogenic centers without the isolation or purification of intermediates. Thus, the efficiency of the synthesis increases since the number of synthetic steps is reduced. As a consequence, there is a minimum consumption of chemicals and a minimization of waste, reducing environmental contamination. Therefore, chemical companies concerned about economic

and ecological profitability are really interested in one-pot reactions. Merck Research Laboratories described an impressive synthetic sequence for the preparation of the anti-migraine drug telcagepant (Fig. 12) [52]. A symbiosis between one-pot reactions and organocatalysis using Jørgensen's catalyst is the key feature of a brilliant synthesis with the isolation of only three intermediates and without chromatographic purification. This example illustrates the preparation on an industrial scale drug by an environmentally friendly process with the high quality standards that the pharmaceutical production demands.

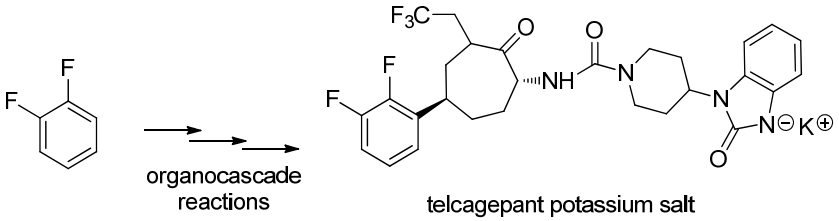

Figure 12. Synthesis of telcagepant potassium salt from 1,2-difluorobenzene by Merck Research Laboratories.

Recently, different terms to describe one-pot processes have been considered. Tietze [53] described a *domino* reaction as the process involving two or more sequential bond-forming transformations which take place under the same reaction conditions. In a *tandem* process the transformations occur simultaneously sometimes using two or more different catalytic processes [54]. However, the term *cascade* reaction is used to include the above mentioned one-pot reactions [55].

The advantages and continuous development of this bio-inspired strategy has converted cascade reactions in a useful tool for the rapid access to molecular complexity [56].

The combination of enantioselective catalysis, one of the most efficient methods in EPC-synthesis, with cascade reactions is one of the most powerful approaches for the preparation of chiral complex molecules, and it is known as *organocascades* or *organocatalytic cascades* (Fig. 13) [57].

The organocatalytic approach in cascade reactions frequently involves only one catalyst that usually interacts with carbonyl substrates. The organocascades are based on the easy tautomeric iminium ion-enamine conversion and the different reactivity of the resulting tautomers to furnish consecutive reactions. The catalysis via iminium ion allows the addition of nucleophiles in the β-position of α,β-unsaturated carbonyl compounds. This addition generates an enamine-like intermediate that can react with the electrophiles present in the reaction media (Fig. 14).

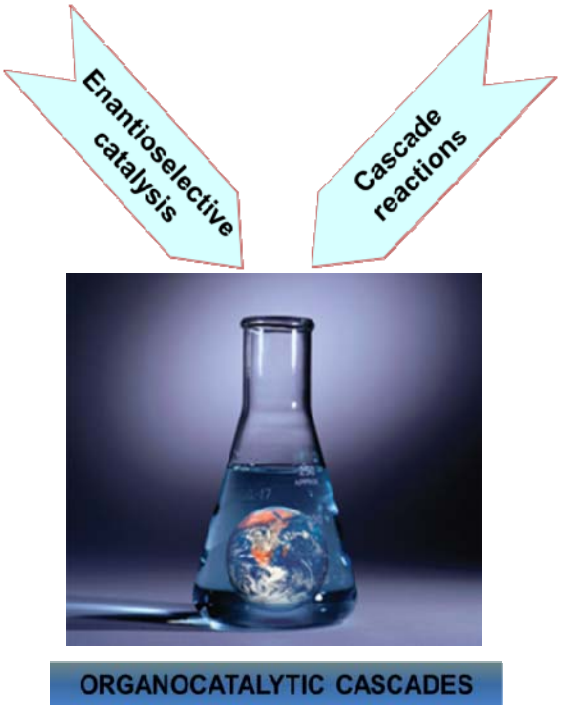

Figure 13. Organocatalytic cascades.

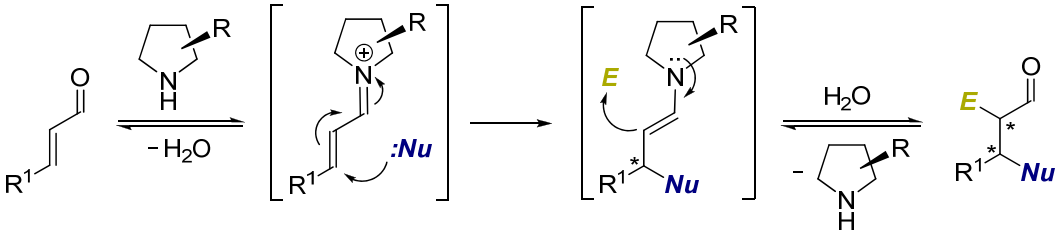

Figure 14. Domino reaction via activation iminium ion-enamine.

The combination of sequential activations via iminium ion-enamine has provided numerous organocatalytic cascade syntheses of enantiomerically pure molecules. It is noteworthy the triple cascade reaction via activation enamine-iminium-enamine reported by Enders and co-workers for the synthesis of pentasubstituted cyclohexanones (Fig. 15) [58].

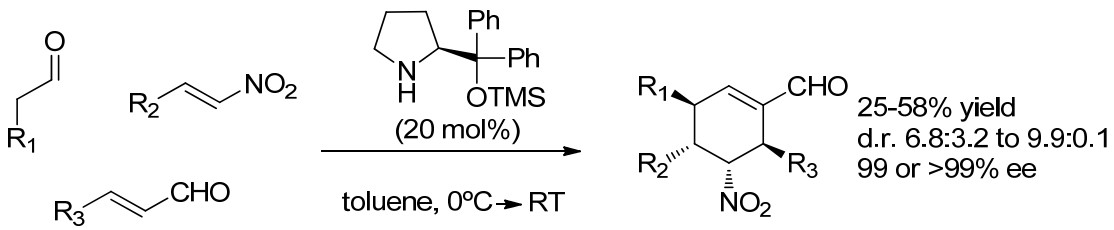

Figure 15. Synthesis of pentasubstituted cyclohexanones by Enders *et al*.

3. Cooperative catalysis

The structural diversity present in the biosynthetic molecules is due to the combination of catalytic cascades that involve different enzymes, which activate different substrates. For the success of this catalytic combination, it is essential the capability of different enzymes to coexist in the same media without undesirable interactions. Moreover, some reactions need the participation of coenzymes and metallic cofactors as further substrate activators. For this reason, the efficient combination of organocatalysts with other catalysts, which are able to activate different functional groups, is highly desirable to increase the molecular diversity of the synthetic products. *Cooperative catalysis* between organic and metal catalysts has evolved rapidly in recent years and its improvement offers to the synthesis of enantiomerically pure compounds levels of reactivity, selectivity and diversity that are very difficult to get using other methodologies [59].

Remarkably, this innovative strategy not only promotes single reactions but also enables multiple transformations in a one-pot process for the generation of previously unattainable compounds.

4. Conclusion

Therefore, the design and development of new catalytic strategies is a continuing challenge at the forefront of synthetic chemistry. Stereoselective organocatalytic reactions have proved to be a powerful tool for the synthesis of enantiomerically pure molecules. In addition, catalytic cascade sequences allow the minimization of time, cost, effort and waste of synthetic processes, thus becoming ideal biomimetic approaches to gain molecular complexity efficiently and ecologically.

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8. Bicelles: New nano systems for skin applications

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Abstract. Bicellar systems are lipid nanostructures formed by long- and short-chained phospholipids dispersed in aqueous solution. Because of their attractive combination of lipid composition, small size and morphological versatility, bicelles became new targets for skin research. Bicelles modify the skin biophysical parameters and modulate the skin barrier function acting as enhancers for drug penetration. Moreover, these aggregates have the ability to penetrate through the narrow intercellular spaces of the skin stratum corneum and to reinforce its lipid lamellae. Their structures allows for the incorporation of different molecules that can be carried through the skin layers. The remarkable versatility of bicelles is their most important characteristic, which makes it possible their use in different fields. These aggregates represent new nanosystems for skin applications. In this work we provide an overview of the main properties of bicelles and their effects on the skin.

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Introduction

Bicelles consist in nanostructures formed by long and short chain phospholipid molecules dispersed in aqueous solution [1, 2]. These structures represent a fascinating category of versatile and robust lipid assemblies whose uses are expanding in several research fields. Bicelles have been described as discoidal nanostructures in which a long chain phospholipid, usually dimyristoyl-phosphatidylcholine (DMPC), forms a flat bilayer and a short chain phospholipid, normally dihexanoyl-phosphatidylcholine (DHPC), stabilizes the rim of the structure (Fig. 1). Although this description has been observed in several bicellar systems, currently it is known that bicelles may display different morphologies depending on the sample preparation and experimental conditions.

The bicellar structure was developed by Sanders and Schwonek in 1992 to solve experimental problems of lipid vesicles and micelles as membrane models for NMR studies of protein characterization [3]. However, the interesting combination of lipid composition, small size and morphological versatility made bicelles new targets for skin related studies. The lipid bilayered structure of this system, with diameters in the range of 15–50 nm and thickness about 4–6 nm, presents optimal conditions as potential platforms for applications related to skin research. This is because of the structural resemblance of bicelles and lipid layers of the skin stratum corneum (SC), the absence of surfactants in the composition of bicelles and the possibility of encapsulating different molecules in the their structures.

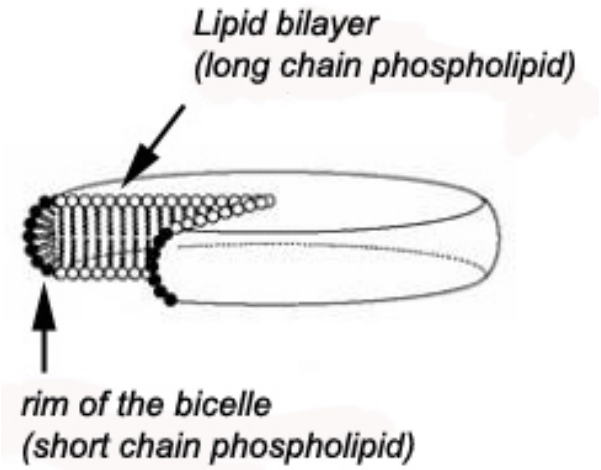

Figure 1. Schematic representation of a bicelle showing the flat bilayer region composed of long chain phospholipid, surrounded by a rim of short chain phospholipid.

The SC is a bilayered lipid-rich matrix structure with embedded keratinocytes that builds the upper strata of skin [4]. One of the SC key functions is to control permeability being the main target and the main barrier for transdermal drug delivery [5-7]. Perturbations in the structure and lipid composition of SC are associated to different diseases [8-10]. In order to replace the SC lipids or to provide drugs and other substances needed to restore skin functionality, several systems have been designed as skin carriers, delivery systems, penetration enhancers, etc. Micelles and liposomes are perhaps some of the most used systems for these purposes. However, despite their demonstrated beneficial effects, their use has some limitations [11, 12]. In fact, the large size of liposomes (usually in the range of 200-500 nm) makes their penetration in the skin improbable since the approximated thickness of the SC intercellular spaces are around 6 - 10 nm [13, 14]. In case of micelles, their surfactant components normally produce skin irritation [15, 16]. The use of bicelles presents advantages over these two classical systems because bicelles exhibit dimensions small enough to pass through the narrow SC lipid lamellae, they contain a bilayer for molecules encapsulation and they are formed exclusively by lipids [17, 18].

These interesting features of bicelles have been the basis for the new research line that is being developed with bicelles. In the last years bicelles have been investigated as regard to their structure, function and interaction with the skin tissue. The potential of this nanostructures for skin research is outstanding since their applications would range from model membranes to study the SC lipid behavior to applications such as SC lipids regenerators, skin carriers, penetration enhancers or retarders and drug delivery systems [19]. These studies gave rise to a consistent basis for considering bicelles as new nanosystems for skin applications.

In this work, we review the main properties of bicelles and the effects of their interaction with the skin *in vitro* and *in vivo*. To this aim we focus on two systems: the classic one formed by dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) and another more compatible with the skin characteristics formed by dipalmitoylphosphatidylcholine (DPPC) and dihexanoylphosphatidylcholine (DHPC).

1. Preparation and morphological characteristics of bicelles

A typical preparation of bicelles involves hydrating the desired mixture of long- and short-chain phospholipids, followed by a series of cycles of freezing, thawing, and gentle vortexing until a clear solution is obtained. The archetypal model of bicelles is formed by dimyristoyl-phosphatidylcholine (DMPC) as large chain phospholipid, and dihexanoyl-phosphatidylcholine (DHPC) as short-chain

one. However, in order to imitate better the environment of biological membranes or to adapt the systems for an specific effect, bicelles with different lipid compositions have been prepared. In this way, DMPC can be doped with phospholipids that have identical chain lengths but different headgroups (e.g., dimyristoyl-phosphatidylglycerol (DMPG), dimyristoyl-phosphatidylserine (DMPS), dimyristoylphosphati-dylethanolamine (DMPE) [20, 21], palmitoyl-stearoyl-phosphatidylcholine (PSPC) [22] or with cellular lipids such as cholesterol and cardiolipin) [23]. Bicelles can also be prepared with dipalmitoyl-phosphatidylcholine (DPPC) or dilauryl-phosphatidylcholine (DLPC) to vary the total bilayer thickness, or with an unsaturated lipid, palmitoyl-oleoyl-phosphatidylcholine (POPC) to obtain bicelles capable of forming micrometre-scale lipid domains [24]. Apart from DHPC, the rim of bicelles can be formed by a bile-salt derivative such as 3-(cholamidopropyl) dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPSO) [25]. The so-called ideal bicelle model supposes a strict segregation between the two main phospholipids [26].

Depending on the composition, lipid concentration (c_L) and the long/short chain phospholipid molar ratio (q), bicelles display different morphologies. In general, the bigger is the q value, the bigger is the size. Inversely, the bigger is c_L, the smaller is the size. Temperature also exerts important effects on bicelles. In temperatures higher than the melting or transition temperature (T_m) of the long chain phospholipid, bicelles undergo some morphological transitions and may align in magnetic fields.

The spontaneous alignment of bicelles is their most important characteristic regarding protein characterization by NMR techniques. It is generally accepted that bicelles with q values between 2.8 and 6.5 and lipid concentration in the range of 3 – 40% w/w spontaneously align in magnetic fields in such a way that the bilayer plane is parallel to the magnetic field, giving rise to a ^{31}P NMR spectrum showing two well-resolved resonances. The high field resonance corresponds to the long chain phospholipid localized in the planar surface of the aggregate, whereas the low field resonance is attributed to the short chain phospholipid distributed in the torus [3, 27, 28]. The ^{31}P NMR spectra of bicelles are therefore used to diagnose the formation of bicelles and to verify the quality of the sample orientation. Also these spectra give information on the morphology of the aggregates.

Although the classic description of DMPC/DHPC bicelles as disk-shaped objects formed by a DMPC bilayer and closed on the edges by DHPC molecules is well accepted for bicelles with $q < 2.8$ at temperatures below the T_m of the DMPC, in the last decade, this characterization has been extensively debated for bicelles with $q > 2.8$ and temperatures above DMPC T_m [27-29].

The disk-shaped model is not consistent with the mechanism of bicellar alignment as a function of temperature in the magnetic field. According to

Ottiger and Bax, disk fusion would be necessary to reach an appropriate size for cooperative alignment [30]. The changes in viscosity and sample transparency that occur under temperature variations corroborate this hypothesis. Rowe and Neal added that the classical model does not explain the increase of viscosity at temperatures where alignment begins, which suggests the formation of large aggregates [31].

Based on their observations, Nieh et al. proposed a model for bicelles with q = 3.2, both with and without lanthanide cations (Ln^{3+}) [29, 32, 33]. At temperatures below the T_m of DMPC, the bicelles were disk shaped. As the temperature rose and the systems changed from the gel to the liquid crystalline phase in the presence of Ln^{3+}, the bicelles fused together in an end-to-end manner to form lamellar sheets with perforated holes that were lined with DHPC. Further increases in temperature caused phase separation, with the formation of DHPC-rich mixed micelles and DMPC-rich oriented lamellae that incorporated the DHPC-rich mixed micelles, even at higher temperatures [34]. In the absence of Ln^{3+}, bicelles were disk-shaped in the gel phase and chiral nematic or "wormlike micelles" in the liquid crystalline phase. These bicelles became multilamellar vesicles at higher temperatures.

While some authors have proposed that the aligned samples correspond to elongated aggregates, which are present when samples become viscous [35], others claim that the aligned bicelles are perforated lamellar sheets, which are present when the sample viscosity drops and its appearance becomes milky [28, 30, 36]. This latter model is known as the "Swiss cheese model" (Fig. 2). In a comprehensive study, Triba et al. [27] found that both elongated and perforated lamellar structures are compatible with the NMR aligned spectra, although they did not disprove the possibility of discoidal structure in the aligned phase.

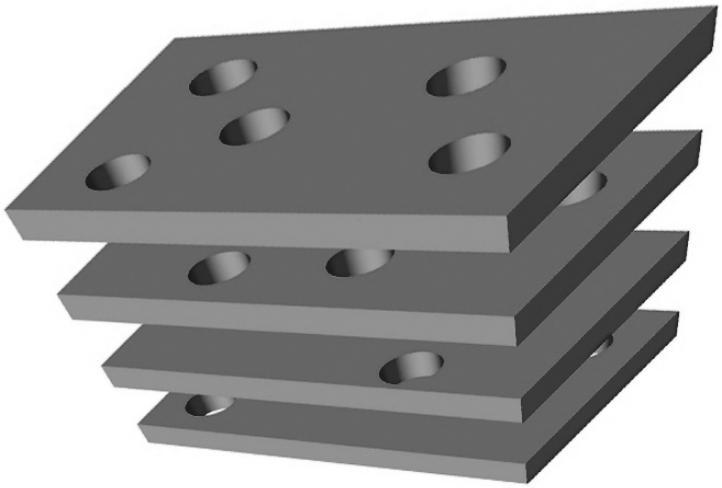

Figure 2. Representation of the Swiss cheese model consisting of flat bilayer sheets of long chain phospholipid perforated with surrounding short chain phospholipid.

Recently, a comparative study related the morphologies of alignable (q = 3.5) and non-alignable (q = 2) bicellar systems, c_L =20%, with temperature changes [37]. ^{31}P-NMR spectra indicated that the q = 2 bicelles exhibited isotropic behavior at all temperatures (20-60 ºC), while the q = 3.5 bicelles varied considerably with temperature. Even at 20 ºC, a low field resonance peak and a broad higher field signal suggested partial orientation of the sample with the magnetic field. Just above the DMPC T_m, at 25 ºC, the spectrum showed two broad resonances near the positions of those observed at 20 ºC. At higher temperatures, the lines became more intense, indicating increased magnetic alignment that reached a maximum at 40 ºC. These peaks disappeared at higher temperatures and were replaced by a broad signal at higher field resonances, which are characteristic of a phase transition from bicellar aggregates to larger, slow-moving structures, such as vesicles. There was also a gradual up field shift of both peaks with increasing temperature, which represents an increase in DMPC bilayer order and the gradual incorporation of DHPC molecules into this bilayer. This study suggested that increasing temperatures promote the migration of DHPC molecules from the edges of the structures to the bilayer area of the alignable bicelles. Depletion of DHPC molecules at the edges leads to bilayer fusion, which increases the bicellar diameters and improves the alignment up to a certain point at which bigger and/or non-flat structures are formed and the alignment is lost.

Electron microscopy techniques have proven to be very useful as complementary techniques to determine samples' morphology. The study of these samples by Cryo-SEM and TEM found round aggregates of approximately 20 nm for bicelles with q = 2 below T_m and elongated aggregates of approximately 2000 nm above T_m. These larger aggregates do not align in magnetic fields, although their size and morphology explain the increase in viscosity with temperature. At q = 3.5, discoidal bicelles of approximately 40 nm were observed below T_m, and extended areas of stacked lamellar sheets were observed above T_m. The authors considered these aggregates to be the most ordered phase because the best alignments in the ^{31}P-NMR experiments were obtained at the same temperatures.

Van Dam et al. applied Cryo-TEM technique to characterize DMPC/DHPC q = 3.2 and c_L=5% w/w doped with DMPG and CTAB [28]. Through this technique they observed disk shaped structures viewed face-on and edge on, threadlike cylindrical micelles and branched toroidal structures at 20 ºC, 25 ºC and 30 ºC, respectively. In another study, these authors used this technique to investigate the morphology of DMPC/DHPC samples over a wide range of c_L and q, covering isotropically tumbling bicelles and larger aggregates [38]. They concluded that temperature and the ratio q are the dominating variables for changing sample morphology, while c_L to a lesser

extent affects the aggregate structure. They observed at q = 0.5, small, possibly disc-shaped aggregates with a diameter of approximately 6 nm. At higher q-values, they observed distorted discoidal micelles that tend to sort cylindrical micelles similar to those reported by Barbosa-Barros et al. [37].

Extensive and comprehensive studies have been reported on bicelle morphology and phase behavior [34, 39-42]. Throughout this work, researchers have reported that (a) bicelles are disk-shaped nanoaggregates at temperatures below the T_m of the long-chain phospholipid; (b) increases in temperature cause an initial increase and subsequent drop in viscosity; and (c) when the temperature is raised above the long-chain phospholipid T_m, bicelles with $q > 2.8$ undergo morphological transitions from disks to elongated micelles, perforated lamellar sheets and mixed multilamellar vesicles.

However, the studies disagree on the exact morphology of the structures when $T \geq T_m$ and the sample exhibit magnetic alignment. Works describing the morphology of bicelles with $q < 2.8$ at temperatures above the long-chain phospholipid T_m are scarce. For these reasons, most recent reports use the term “bicelle” to refer only to the sample composition and not the disk-shaped morphology.

2. Effects of bicelles on the skin

The motivation for using bicelles in the skin arises from the resemblance of these structures to micelles and liposomes. Bicelles combine some of the most attractive characteristics of these systems and therefore present several advantages for skin applications compared to either micelles or liposomes. Bicelle structures contain a bilayer that allows for the incorporation of different molecules but are much smaller (approximately 15-40 nm) than a regular liposome (≅200 nm). This is due the presence of DHPC molecules on the edges of the structures, which control the diameter of the assembly. These molecules are responsible for discoidal-shaped bicelles and the formation of other structures, such as small vesicles. In fact, systems formed by long alkyl chain phospholipids and DHPC have been reported to produce reasonably monodisperse unilamellar vesicles that are thermodynamically stable, with radii ranging between 10 and 40 nm [43-46]. Several kinetic studies have shown that discoidal bicelle morphology is a precursor to small vesicle morphology [46, 47]. DHPC molecules solubilize the DMPC bilayer similar to a surfactant; however, DHPC is a phospholipid with the same polar head group as DMPC. These two lipids differ only in the lengths of their hydrophobic chains. The use of systems composed only of lipids avoids damage to the skin barrier function caused by surfactants, which is

characterized by breaking of the corneocyte envelopes and disorganization of the intercellular lipid structures.

As already mentioned, above the long chain phospholipid T_m (approximately 23 °C for DMPC), DMPC/DHPC bicelles undergo phase transitions, changing from small aggregates of approximately 15-20 nm to structures bigger than 500 nm. Because skin intercellular spaces lie in the range of 6-10 nm and physiological skin temperatures are near 37 ºC, this phase transition would present a handicap for skin penetration. DMPC/DHPC bicelles are likely not able to penetrate into SC intercellular spaces, and their effects are limited to the skin's surface.

A different scenario is obtained if the long chain phospholipid DMPC is substituted by another phospholipid with higher T_m, such as DPPC. This phospholipid has two additional carbons in its hydrophobic chain and a T_m of 41 °C. At physiological temperatures, bicelles composed of DPPC and DHPC are still small aggregates. Some studies have found that at 37 °C, DMPC/DHPC bicelles these structures have dimensions of approximately 15 nm in diameter and 5.4 nm in thickness. These structures have been shown to penetrate through the intercellular spaces of the skin SC and achieve deeper internal layers [48].

In vitro studies

Experiments using human and pig SC samples treated with bicelles to study the effects of these systems on the SC microstructure have been reported. In one of these studies, fresh human SC samples were incubated with DMPC/DHPC bicelles with c_L = 20% and q = 2 for 18 h at 25 °C. Treated and untreated SC samples were cryofixed, cryosubstituted and visualized by TEM. No differences, including microstructural alterations and/or apparent damage, were observed in images of the treated SC compared with untreated samples (Fig. 3A and 3B).

Given the small sizes of bicelles (20 nm in diameter and 4.5 nm in thickness) and their bilayered structures, the lipid dispersion through the SC lamellae and the reinforcement of the SC bilayer area would be expected. However, this was not observed, suggesting that these bicelles were not able to penetrate or disperse through the SC lipid area [19]. This result was clarified by Rodriguez et al. [49] using ATR-FTIR. Th authors reported that the application of DMPC/DHPC q = 2 bicelles caused phase transitions in the SC lipid conformation from the gel state to the liquid crystalline state. This transition would be promoted by the incorporation of phospholipids from bicelles in the SC lipid lamellar structure. This process involves an increase in the fluidity and/or disorder of the lipids. An analysis of phosphate vibrations only detected effects from DMPC/DHPC bicelles on lipids at the

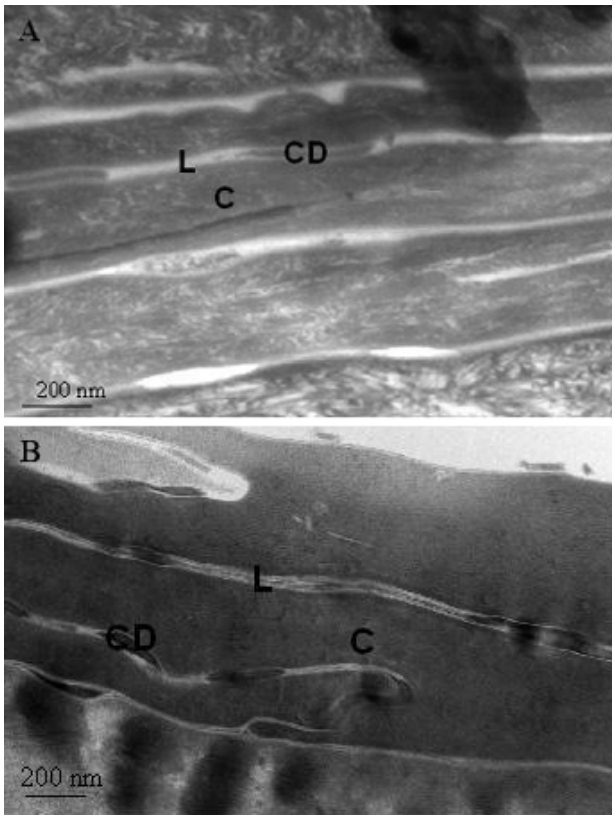

Figure 3. FSTEM micrograph of (A) native SC and (B) SC treated with DMPC/DHPC bicelles. Both images show regular areas of corneocytes, (C), lipid intercellular spaces (L) and corneodesmosomes (CD) [19].

outer layer of the SC, suggesting that the majority of DMPC/DHPC bicelles remained in the outermost part of the tissue. This is likely why Barbosa-Barros et al. [19] did not visualize microstructural differences between untreated and treated SC; transitions occurring mainly on the SC surface do not imply structural modifications that can be visualized in EM experiments.

In another study, DPPC/DHPC bicelles with c_L = 10% and q = 3.5 were incubated with fresh pig skin SC samples for 18 h at 37 ºC. This DPPC/DHPC system was specially developed to obtain improved effects on the SC microstructure and better skin penetration at physiological conditions. Because the DPPC T_m is 41 ºC, the structures do not undergo phase transitions at physiological temperatures (approximately 37 ºC), and small bicelle structures are favored during the incubation process. After incubation, both treated and untreated SC samples were high pressure frozen (HPF) from an initial temperature of 37 ºC, freeze fractured and observed using Cryo-SEM [48]. These bicelles produced quite different results in the SC than the DMPC/DHPC q = 2 system. The DPPC/DHPC system could penetrate and interact with the SC, forming lipid vesicles and new lamellar-like structures observed by Cryo-SEM (Fig. 4). To study this phenomenon, Rodriguez et al.

[50] studied the effects of DPPC/DHPC on SC lipids using ATR-FTIR spectroscopy coupled to a tape-stripping methodology. Analysis of the lipid organization in terms of chain conformational order and lateral packing showed that bicelles hampered the temperature-dependent fluidization of SC lipids in the most superficial layers of the SC and led to a lateral packing corresponding to a stable hexagonal phase. CH_2 stretching and phosphate vibrations in the ATR-FTIR spectra of subsequent stripping indicated that DPPC/DHPC bicelles penetrated into and were widely distributed in deep layers of the SC. These results corroborate the presence of different DPPC/DHPC structures inside the SC layers, and bicellar reinforcement of the SC structures was observed.

Various authors have reported studies of bicellar state-transitions from disks to vesicles that were induced by dilution or temperature changes. There is a general consensus that these transitions occur in progressive steps, implying the coexistence of different aggregates in the medium [28, 39, 51]. To study the bicelle-to-vesicle transition, DLS studies were performed by Barbosa-Barros et al. 2008 [48]. A DPPC/DHPC sample with q = 3.5 and c_L = 10% was sequentially diluted with water in seven steps, and each diluted sample was measured using DLS at 37 °C to imitate physiological conditions. The DLS curves indicated that the hydrodynamic diameter (HD) of the structures increased upon dilution from 11.3 nm (assigned to bicelle disks) to aggregates larger than 1 μm. The morphologies of the aggregates were analyzed using EM, which confirmed the DLS results, indicating a dilution-induced transition from disks to vesicles.

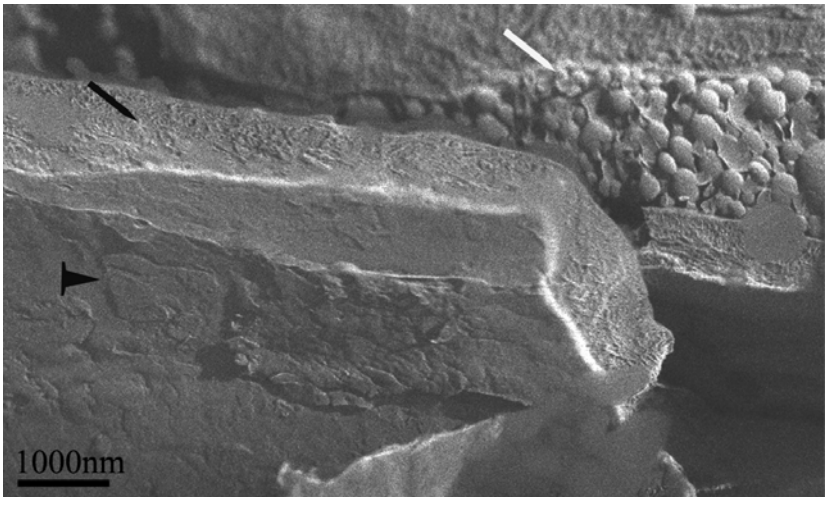

Figure 4. Cryo-SEM micrograph of SC treated with DPPC/DHPC bicelles. The white arrow shows vesicle structures with sizes around 200 nm and the arrowhead points to the lamellar-like structures in the intercellular lipid areas. The black arrow shows a corneocytes area [48].

In bicellar systems, the DHPC molecules are found mainly on the edges of the disk structures and in the water (as monomers). With increasing dilutions, the DHPC concentration in water decreases, and DHPC is transferred from the bicelle edges into solution, maintaining monomeric equilibrium. Hence, disk diameters increase, and high dilutions lead to the fusion and closure of large bilayered disks, forming vesicles. This bicelle-to-vesicle transition explains the presence of vesicles in the SC intercellular spaces treated with DPPC/DHPC [48]. This process would have been promoted by the dilution of aggregates because the SC pieces were washed with water after incubation. Bicelles that have been transformed into vesicles presumably follow a process similar to that observed in the DLS observations of diluted bicellar solutions outside of the skin.

Regarding the ability of bicelles to encapsulate drugs, Rubio et al. [52] performed percutaneous penetration studies with DMPC/DHPC and DPPC/DHPC systems with c_L = 10% and q = 2 to evaluate their effects on the skin penetration of dichlofenac diethylamide (DDEA). The authors reported that the incorporation of DDEA in the bicelles led to markedly decreased bicelle sizes, indicating that DDEA tends to be located at the bicelle edges, similar to DHPC. Fig. 5 shows Cryo-TEM images of DPPC/DHPC bicelles with and without DDEA. Both systems decreased the percutaneous absorption of the DDEA compared to an aqueous solution of DDEA, suggesting a retarded effect after treatment with bicelles. This effect was more marked for the DMPC/DHPC bicelles. This could be related to the different T_m values for the two systems. At 37 ºC, DMPC/DHPC ($T_m \approx$ 23 ºC) bicelles were in the liquid crystalline phase, in contrast to the gel phase SC lipids ($T_m \approx$ 60 ºC). However, DPPC/DHPC bicelles ($T_m \approx$ 41 ºC), were in gel phase, similar to the SC lipids. Different mixing behaviour of lipids from bicelles with SC lipids could induce different effect on the retention of DDEA in the upper layers of the skin. This retarder effect was ascribed in part to rigidity in the head groups of bicelle phospholipids caused by the carboxyl groups of DDEA. This rigidity would hinder the penetration of DDEA through the skin. However, DDEA was likely unable to diffuse out of the bicellar systems because of its high affinity for its vehicle.

A second percutaneous penetration assay was also performed by these authors to evaluate the potential of bicelles as penetration enhancers. In this experiment, the skin was pretreated with the bicellar systems (DMPC/DHPC and DPPC/DHPC systems with c_L = 10% and q = 2) before the application of an aqueous DDEA solution. The global results obtained showed that pretreatment of the skin with bicelles promotes an increase in the percutaneous absorption of DDEA, with no significant differences between DMPC/DHPC or DPPC/DHPC systems. These studies suggest that treatment

with these bicelle systems prior to drug treatment can enhance drug absorption. This enhancement is ascribed to an initial interaction of bicelles with the SC that causes some disorganization of the intercellular lipids, which are responsible for the SC barrier functionality. These results suggest a route to aid the absorption of DDEA through the skin.

In vivo studies

Non-invasive biophysical studies performed with healthy volunteers have evaluated the effects of bicelles on skin *in vivo*. These studies report mainly on measurements of skin hydration, elasticity, erythema and transepidermal water loss (TEWL). Skin hydration and elasticity are useful measures of skin water content, distensibility, extensibility and tonicity [53, 54]. Erythema indicates skin tolerance [55], while TEWL indicates barrier function integrity [56, 57].

The application of DMPC/DHPC bicelles with c_L = 20% and q = 2 to the skin of healthy volunteers has been reported [19]. In this experiment, intra-individual comparisons of three test areas on the volar forearms of 6 healthy Caucasian female (ages 25–38) volunteers with no visible skin abnormalities were performed. In the test areas, bicelles, deionized water and control (non-treated) areas were randomized regarding the test sites on each subject. The solutions were applied daily over a period of 10 days, and the skin properties were measured each day before application.

Successive bicelle applications led to an increase in TEWL from days 0 to 11. This increase was moderate and did not reach pathological levels, which are from 25 to 40 g/m^2/h [58, 59]. Decreasing of the skin hydration was also observed. Elasticity, in turn, showed improvement with the application of bicelles. The changes in the erythema of the skin, considering inter- and intra-individual variability, were not indicative of an irritation process [60]. The bicelles were found to promote increases in TEWL and skin elasticity and harmless decreases in skin hydration.

Bicelles act to enhance penetration, causing phase transformations in lipid domains that may be relevant to skin permeation [61]. As reported by Rodriguez et al. [49], the phase transition of the SC lipid conformation from the gel state to the liquid crystalline state, which causes the fluidity of these lipids, explains the increase of TEWL *in vivo*. Compared to other enhancers, bicelles would have the additional advantage of not causing skin irritation [62].

Another study reported on the effects of the application of DPPC/DHPC bicelles with c_L = 20% and q = 2 on the skin of healthy volunteers. The results were similar to those obtained with the DMPC/DHPC system, although they were more discrete. In a similar way, this system led to an

increase in TEWL and a decrease in skin hydration. However, the effects were approximately 75% and 50% less intense, respectively, than those obtained with the DMPC/DHPC system [63]. This result is not unexpected because the DPPC/DHPC system contains less DHPC, so the aggregates formed are slightly bigger and have longer bilayer areas, which would exert a protective effect on the SC. In addition, as observed in the *in vitro* studies, this system penetrates into the skin SC and reconstitutes its lipids in lipid vesicles inside of the skin lamellae, which reinforce the lipid structure of the tissue.

3. Conclusion

The use of bicelles for skin applications is a scientific novelty. This new lipid system represents a unique versatile structure that has different effects on the skin depending on the self-assembly adopted. Bicelles are effective skin carriers due to their size, structure and composition. Although bicelles have no aqueous internal compartment for encapsulating drugs, their bilayered structure allows for the encapsulation of lipophilic and amphiphilic compounds. Additionally, because of their ability to increase the permeability of the SC, these structures enhance the penetration of hydrophilic components dissolved in aqueous medium. Further, the conversion of bicelles into vesicles inside the SC hinders their migration outside of the tissue and allows a lipid reinforcement effect on the skin. By modulating their physical and chemical characteristics, bicelles may be useful for a wide range of applications. Bicelles are therefore promising nanostructures with the potential to become new multifunctional, skin-compatible delivery system.

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9. Advances in the research of new biosurfactants and their potential use in the biomedical and pharmaceutical industry

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Abstract. Biosurfactants (BS) are a structurally diverse group of surface-active substances produced by microorganisms. Interest in BS production has markedly increased during the past decade, although large-scale production has not been possible because of low production yields and high total costs. At present, BS have gained importance in environmental applications, while new applications in the pharmaceutical, biomedical, cosmetic and food industry, with a high added value, are still developing. This article describes classical and new BS producer bacteria together with their new BS applications. With these specialized and cost-effective applications, BS can be considered as an interesting option for the near future.

1. Introduction

Surfactants are amphipathic molecules that accumulate at interfaces, decrease interfacial tensions and form aggregate structures such as micelles [1]. Surface active agents or surfactants are an integral part of our everyday life and are products with a worldwide production. The enormous market

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demand for surfactants is currently met by numerous synthetic, mainly petroleum-based, chemical surfactants. About 54% of the total surfactant output is utilized in household/laundry detergents, with only 32% destined for industrial use [2]. Chemical surfactants are available in many forms, and are generally classified based on charge as anionic, non-ionic, cationic and amphoteric. Cationic surfactants are the most toxic and have historically been used as antimicrobials, while anionics are less toxic and are more active against Gram positive than Gram negative bacteria, and non-ionics are often considered non-toxic. Important surfactants include linear alkylbenzene sulfonates (LAS), fatty alcohol ethoxylates (FAEO) and lauryl ether sulfate (LES) [3].

The growing awareness towards the use of renewable-based products and "green products" has stimulated the development of alternatives to these chemical surfactants. Biosurfactants (BS) are an example of such environmental friendly options [4]. BS can be obtained either by chemical synthesis from renewable resources, by microbial fermentation processes or by enzymatic syntheses [5].

The amphiphilic nature of BS allows the partition at water/air, oil/air or the oil/water interfaces where it lowers surface and/or interfacial tension. Such characteristics enable emulsifying, foaming, detergency and dispersing properties [6]. One important feature of BS is that they have very low critical micelle concentrations (CMC). This means that BS are effective at low concentrations, lower than many chemically made surfactants. The fact that only small amounts of BS are needed to reduce surface tension coupled with their known biodegradability makes them excellent candidates for "green" detergents and surfactants.

Comparing with chemical surfactants, BS have several advantages such as lower toxicity, higher biodegradability and diversity, effectiveness at extreme temperatures or pH values and widespread applicability. Additionally, their unique structures provide new properties that classical surfactants may lack. BS occur naturally in soil, which makes them acceptable from a social and ecological point of view [2].

The most remarkable difficulty for BS commercial application is the BS production at low concentrations, which makes product recovery difficult and expensive. In order to reduce their production costs and increase their competitiveness, various researches have been working in the development of new and cheaper processes and/or in the use of low-cost raw materials [4].

2. Natural Roles of Biosurfactant

Recent research indicates that there are a number of reasons why microorganisms make BS; mainly these reasons relate to the need to change

surface or interfacial properties of the cell or local environment. Prokaryotic cells communicate with the environment through their envelopes. Smaller cells have a higher surface/volume ratio, and thus can have a more efficient exchange of nutrients with their surrounding than larger cells. To have an effective relationship with the environment, microorganisms need surfactants [7]. When considering the natural roles of BS, it is important to emphasize that they are produced by a wide variety of diverse microorganisms and have very different chemical structures and surface properties. This diversity makes difficult to generalize about the natural role of BS [8]. Nevertheless, BS appear to play a role whenever a microbe encounters an interface.

BS regulate the attachment-detachment of microorganisms to and from surfaces. If a BS is excreted, it can modify the interface and enable or inhibit bacterial attachment. BS that low interfacial tension are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation [1,8]. BS may be used as carbon and energy storage molecules, as a protective mechanism against high ionic strength, and may simply be byproducts released in response to environmental change such as extracellular coverings [1]. Surface or interfacial tension changes are needed in the complex social responses that control cell development.

BS are important for motility (gliding and swarming motility as well as de-adhesion from surfaces) and cell-cell interactions (biofilm formation, maintenance and maturation, quorum sensing, amensalism and pathogenicity). In response to certain environmental signals, bacteria will differentiate from an independent free-living or to an interdependent surface attached mode of live. Surfactants such as rhamnolipids may be able to maintain open channels in biofilm formations by affecting cell-cell interactions and the attachment of bacterial cells to surfaces. Rhamnolipid synthesis is an active mechanism whereby the bacteria exploit intracellular interaction and communication to actively maintain these channels [9]. Rhamnolipid production not only guarantees the structure, and therefore the nutritional balance of the biofilm, but these surfactants also prevent outside invading bacteria from colonizing the open spaces in the resident biofilm [10].

Much research has been directed towards determining how microorganisms can enhance acces to poorly soluble substrates prior to either passive or active uptake into the cell. BS may play a role mediating interactions between the hydrophobicity of the cell surface and the substrate surface. It is known that exogenously added BS can increase the apparent water solubility of organic compounds and alter its bioavailability. BS are used to complex with heavy metals. Rhamnolipid eliminated cadmium toxicity when added to the medium by a combination of metal complexation and rhamnolipd interaction with the cell surface to alter cadmium uptake [8,11,12]. It has been described

that di-rhamnolipid removed from artificial contaminated soil, not only the leachable or available fraction of Cd and Pb but also the bound metals. Additionally, the microbial population of the contaminated soil was increased after removal of metals, indicating the decrease of toxicity of heavy metals to soil microflora. Heavy metals removal from sediments in a continuous flow configuration also was possible whith rhamnolipids [13].

3. Biosurfactant Classification

BS are classified based on their chemical structure. A typical BS is composed of a hydrophilic component (mainly amino acids, peptides or proteins, mono/disaccharides, polysaccharides) and a hydrophobic component (mainly saturated or unsaturated fatty acids, hydroxyl fatty acids or fatty alcohols) [6]. BS molecular masses generally range from 500 to 1500 Da and their CMC values range from 1 to 200 mg/L [1].

BS are structurally diverse and can have various chemical structures mainly consisting of glycolipids, lipopeptides, phospholipids, neutral lipids, polymeric surfactants and particulate BS, depending on the producing microorganism, raw matter and process conditions [5,6]. Low molecular weight BS are able to reduce the surface tension of water to 25-30 mN/m. The main property of high molecular weight BS (polymeric and particulate) is their ability to stabilize oil/water emulsions and they are therefore called bioemulsifiers [8,14]. A brief description about each class of BS is given below.

Glycolipids: Most known BS are glycolipids. They are commonly mono or disaccharides acylated with long chain fatty acids or hydroxyl fatty acids. Among them, rhamnolipids, trehalose lipids and sophorolipids are the best studied. Cellobioselipids and mannosyl erythritol lipids are also included in this group.

- Rhamnolipids are exoproducts of the opportunistic pathogen *Pseudomonas aeruginosa* and were described as a mixture of four congeners. Naturally produced rhamnolipids are always found as mixtures of different congeners, as observed with various strains of *P. aeruginosa* [15,16,17]. The development of more sensitive analytical techniques has lead to the further discovery of a wide diversity of rhamnolopid congeners and homologues that are produced at different concentrations by various *Pseudomonas* species. Recently, bacteria belonging to other families, classes or even phyla have been isolated and described as rhamnolipid producers [18].
- Different trehalose containing glycolipids are known to be produced by several microorganisms belonging to mycolates group, such as *Mycobacteria, Corynebacteria*, *Arthrobacter, Nocardia*, *Gordonia*

and specially *Rhodococcus*. Most of the trehalose lipids synthesized by this group are bound to the cell envelope and are produced mainly when microorganisms are grown on hydrocarbons probably as strategy to overcome the low solubility of hydrocarbons and enhance their transport. Microbial commercial success of trehalose lipids is scarce because its high cost of production related to the fact that are bound to the cellular envelope and requires non-renewable *n*-alkane carbon sources [14].

- Sophorolipids are produced by various yeast species, the most intensively studied being *Candida bombicola*. There are generally considered as the most promising glycolipids, because the producing microorganism is non-pathogenic and because of the high substrate conversion, high productivity and product recovery [3].

Lipopeptides: Several types of cyclic lipopeptides with surface active properties are produced mainly by members of the *Bacillus* sp. Up to now, lipopeptides are the most effective surfactants with high efficency, that is, low BS concentration produces significant reduction of surface tension. Antimicrobial activity is a frequent property present in this group.

- *Bacillus subtilis* produces a cyclic lipopeptid called surfactin, which has been reported to be one of the most active BS that has been discovered to date [8].
- *Bacillus licheniformis* produced lichenysin, a lipopeptide (1006 to 1034 Da) with a lipid moiety mixture of 14 linear and branched beta-hydroxy fatty acids (C_{12}-C_{17}) and aminoacids such as glutamic acid, aparagine, valine, leucine and isoleucine. This BS is a powerful surface active agent (surface tension: 28 mN/m; CMC: 12 mg/L) and presents good antibacterial activity [19].
- The polymyxins are a group of lipopeptides produced by *Bacillus polymixa* and related bacilli. Polimixin B is a decapeptide in which amino acids 3-10 form a cyclic octapeptide with a branched-chain fatty acid connected to the terminal 2,4-diaminobutyric acid (DAB). The hydrophobic side-chain of the fatty acid together with the cationic γ-amino groups of the DAB residues give these antibiotics the surface-active properties of a catinoc detergent [8].

Phospholipids: Kretschmer *et al*. [20] described in 1982 the lipophilic compounds with surfactant properties of the culture suspension containing *Rhodococcus erythropolis*. Thirteen major lipids from organic crude extract were isolated. Non polar and polar lipids from which nonionic triglycerides

and α,α-trehalose corynomycolates were the most abundant lipids. From components described, phosphatidylethanolamines (PE) acted as the most powerful surfactants reducing the interfacial tension below 1mN/m.

Neutral lipids: The report of the production of surface active bile acids by *Myroides* SM1 bacterial strains was performed by Maneerat *et al* (2005) [21]. These compounds are composed of cholic acid, deoxycholic acid and their glycine conjugates.

The biosphere offers a vast natural resource of BS. Sampling and isolation of bacteria are the basis for screening of BS producing microbe. Hydrocarbon-contaminated sites are promising for the isolation of BS producing microbes. Mercadé *et al.* (1996) [22] isolated 5 BS producing strains from petroleum-contaminated soil samples by using waste lubricating oil as the sole carbon source being *Pseudomonas* sp. the most frequent isolate, followed by *Rhodococcus* sp. Espuny *et al.* (1995) [23] and Abalos *et al.* (2001) [15] also described the isolation BS producing strains from vegetal oil-contaminated soil sample. Nevertheless, undisturbed soils also can have BS producing bacteria. Strain 6.2S, isolated from volcanic soil and identified as *Sphingobacterium* sp., was recently reported as a new BS producer [7].

The exploration of the marine habitat which has been underexplored has brought about new microorganisms. This represents a great opportunity to discover new bioactive compounds (new functional products). Up to now, more than 10,000 metabolites with biological activities have been isolated from marine microorganisms, meeting the needs of the industry in the new era of the white biotechnology, the "green chemistry". Beside the well known soil microorganisms, BS-yielding marine microbes emerge as a wide source of BS producers.

A number of new BS has been recently described and accelerated advances in molecular and cellular biology are expected to expand our insight into the diversity of structures and applications of BS. Inside of glycolipid class, of special interest are the first *Ochrobactrum* sp. (*α-Proteobacteria*) and *Brevibacterium* sp. (*Actinobacteria*) isolates which produce a glycolipid with surface activity [24]. Some marine γ-proteobacteria as *Pseudomonas fluorescens,* produced a complex BS composed of trehaloselipids link to diacyl-monoglyceride-protein [25]. This complex renders the cell capable of degrading hydrocarbons. New *Halomonas* sp. glycoprotein has been described with a high emulsifying activity. Other strain of *Halomonas* sp. accumulated glycolipids (oligosaccharide-lipids) and a noteworthy sulfated heteropolysaccharie was produced by *Halomonas eurithalina* [26]. *Alcanivorax borkumensis*, besides to be a good polyhydroxy-alkanoate producer, produces an anionic glycolipid containing glycine for the uptake of hydrophobic substrates [27]. Other bacteria belonging to the β-proteobacteria

such as *Alcaligenes* sp. [28] produces glycolipids, or *Arthrobacter* sp. which produces a trehalose-lipid, very effective BS [29]. Recently, a marine *Azotobacter chroochocum* has been described as a BS producer [30].

From the phylum of the *Bacteroidetes,* the genus *Myriodes* (formerly *Flavobacterium*)*,* commonly found in marine habitat especially after oil-spills, produced ornithine-lipids often cell associated, which allow the cell to degrade hydrocarbons, however, surface active properties diminished in extreme conditions [31]. In the genus *Myroides*, recently *Myroides odoratus* has been reported to produce bile acids [21]. *Corynebacterium kutscher*, a glicopeptide-lipid producer, is a new BS producer from sea belonging to phylum *Actinobacteria* [32].

The family of the lipopeptides, a highly effective BS group, has been expanded with the description of *Bacillus circulans* as a new species producing a lipopeptide with low toxicity [33] and by a new type of lichenysin, produced by *B. licheniformis,* whose most novel characteristic is that the alkyl-chain size ranges between C_{12} to C_{17} [33]. Another lipopeptide type surfactant is produced by *Azotobacter chroococum* growing in motor lubricant oil. A sponge-associate *Actinomyces* has been recenly reported. It is notewortly to mention that a glycopeptide surfactant has been detected in a strain of *Bacillus megaterium* [34].

The diversity of BS is limited only by biological evolution and suggests that only a tiny fraction of BS has been characterized to date. New BS discoveries have relied on the employment of advanced analytical methods and, often, significant screening efforts [1]. Although the improvements obtained from these strategies to successfully compete with synthetic surfactants, novel microorganisms must be designed. Data on the genes involved on the production of BS are critical to designing organisms with improved features and new properties [36].

4. *Sphingobacterium* sp., a New Biosurfactant Producer Bacterium

Recently, the isolation of a new BS-producing bacterium was carried out on a soil sample of the Azores islands. From 10 volcanic earth samples 54 different isolate colonies were obtained and strain 6.2S was selected on the basis of its capacity to decrease surface and interfacial tension. Strain 6.2S was identified as a *Sphingobacterium* sp. and is the first strain in this genus to be reported as a BS producer.

After 48 h culture of *Sphingobacterium* sp. 6.2S in G-Mineral Salts Medium (G-MSM), of 3.8 mg/mL of protein and 190 mg/L crude extract were obtained. Compositional analysis revealed that the crude extract consisted primarily of

lipids (71.6%) and a minor fraction of carbohydrate (5.6%) and protein (4.4%). When *Sphingobacterium* sp. grew on G-MSM with n-alkenes the surface tension was reduced from 55 mN/m to 32mN/m. The crude extract (BS mixture) strongly reduced surface tension (22 mN/m at 10 g/L), producing one of the lowest values recorded for a microorganism-produced surfactant [7].

To purify crude extract, a modification of the method described by van Echten-Deckert (2000) [37] was used, and two fractions, fraction A and fraction B, were obtained (Figure 1), both with superficial activity [7].

Fraction A (28.7%, w/w of the total extract) decreased surface tension of distilled water to 33.0 mN/m, and presented a CMC of 180 mg/L. Three majority spots were visualized in thin layer chromatography with ninhydrin (amine group determination) and molybdenum blue (phospholipid determination) (Figure 1). These results indicated that the analyzed compounds belong to the family of phospholipids and some of them had in their structures nitrogen groups. An analysis of each spot with two dimensional-TLC using different standard phospholipids showed that fraction A was a mixture of phospholipids, being phosphatidyletanolamine the most abundant. Finally, these results were assessed

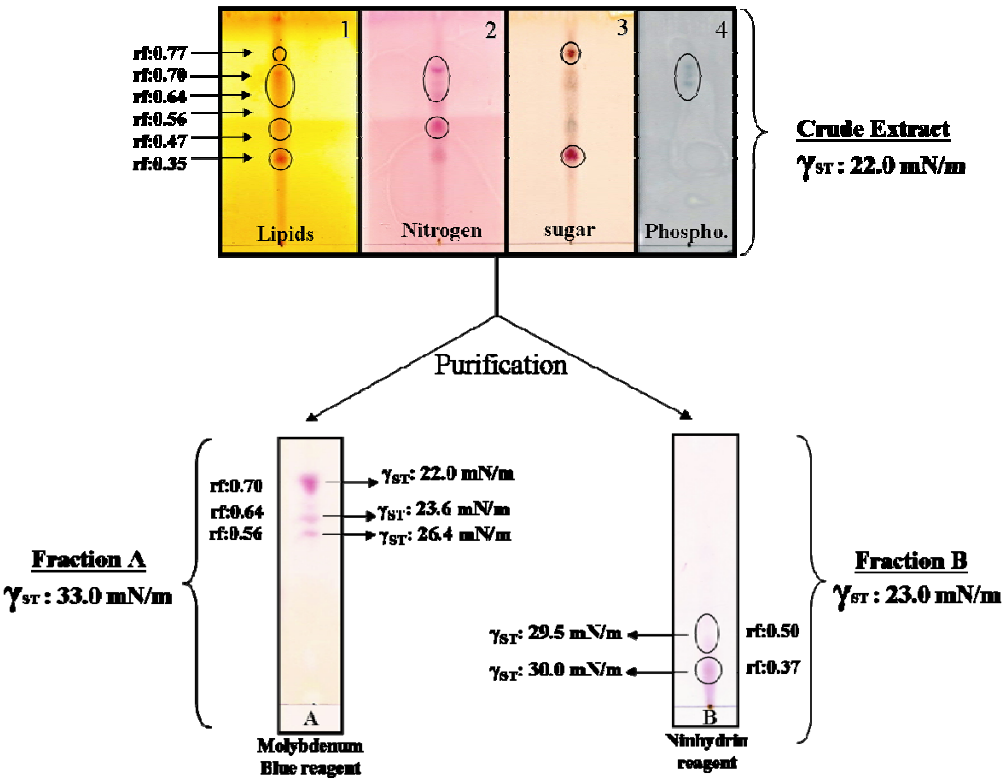

Figure 1. Analysis of the extract produced by *Sphingobacterium* sp. 6.2S. Crude extract developed by iodine vapours (lipids), ninhydrin (nitrogen), molish (sugar) and molybdenum blue (phospholipids). Fraction A: TLC obtained after purification and developed with Ninhydrin. Fraction B: TLC obtained after purification and developed with ninhydrin [7].

with a FTIR analysis, confirmating the presence of the functional groups characteristic of phospholipids.

Further analysis of fraction B (61.3%, w/w of the total extract) revealed that it was a mixture of lipopetides and at least one glycolipid. The surface tension-concentration curve showed two plateaux, the first of which can be attributed to a critical aggregation concentration of the BS with a protein (2.7 g/L) and the second to the true CMC in water (6.3 g/L) [7].

Emulsion capacity of supernatant produced by *Sphingobacterium* sp. was studied. At 25º C and pH 5, 70-65% of emulsion was measured and it was maintained during 5 days, and only a small decrease was observed (60-65% emulsion) after one month. A criterion used to identify bioemulsifiers is their ability to maintain at least 50% of the original emulsion volume after 24 h formation; therefore, 6.2S BS can be included in this family of compounds.

A thermal stability analysis was carried out between 0ºC and 100ºC and at pH 5, revealing that surfactant properties were maintained with the temperature increase and only a small decrease in interfacial and surface tension was observed after a thermal treatment of 121ºC. Nevertheless, it was found that after thermal treatment at 121ºC, the emulsification capacity of 6.2S BS decreased markedly from 65% to 25% (Figure 2). The surface activity was affected by pH. At acidic pH (1–5), surface and interfacial tension were lower (37 and 10 mN/m, respectively). At pH 7 an increase in surface tension and interface tension was observed. This change can be related to the pKa, indicating that the ionic form of the surfactant had lower surface activity. If developed to higher yields, BS from *Sphingobacterium* sp. 6.2S will lead to interesting applications.

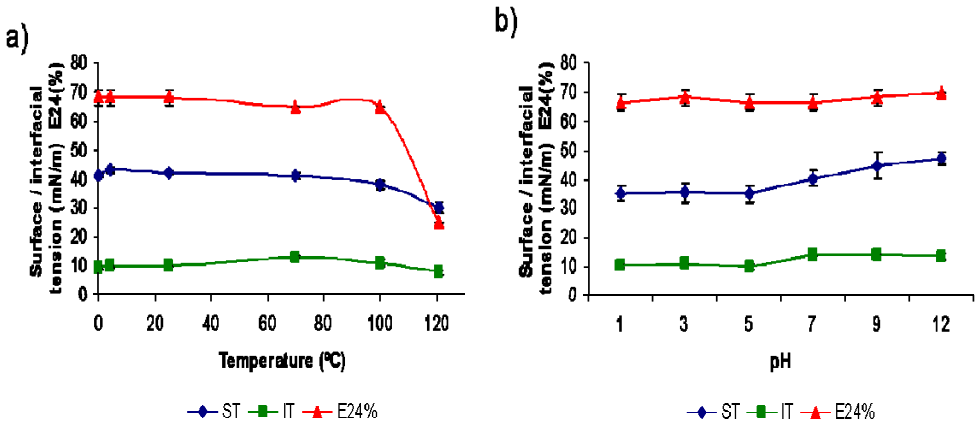

Figure 2. (a) Effect of temperature treatment on the stability of BS produced by *Sphingobacterium* sp., 6.2S grown on G-MSM. (b) Effect of pH on the stability of BS. Measurements were conducted on crude supernatant with unknown concentrations of BS at 25ºC [7].

5. Applications of Biosurfactants

Surfactants are an important class of chemical products with a high volume of use in a great variety of household and industrial applications. Most of these surfactants are petroleum based and are chemically synthesized [6]. BS are the natural choice because they are obtained by environmental friendly technologies and they possess many advantages over synthetic surfactants, such as lower toxicity, effectiveness at a wide range of physical conditions, but they are special as consequence of their biodegradability [6]. BS are more effective and efficient than many existing synthetic surfactants and they may provide new properties that the classical chemical surfactants lack [38,39].

BS find applications in an extremely wide variety of industrial processes that involve emulsification, foaming, detergency, wetting, dispersion and solubilization of hydrophobic compounds [19,40] and, in fact, the demand of BS is increasing worldwide in recent years [14,19,39,40,41,42]. At present, the main applications are the hydrocarbon bioremediation and oil and petroleum industry, in particular for microbial enhanced oil recovery (MEOR) due to their biodegradability avoiding environmental accumulation and toxicity [14]. In spite of their numerous advantages over the synthetic chemical surfactants the problem related with the large scale and cheap production still exits and is a major hurdle in economic competitiveness [6].

According to Syldatk and Hausmann (2010) [5] and Makkar *et al.* (2011) [6], the reasons for limited use of microbial surfactants in industry are the use of expensive substrates, limited product concentrations, low yields and formation of product mixtures rather than pure compounds. Nevertheless, their higher price is largely compensed by their environmental profile and performance benefits. In this regard, discovery of potent BS producing microorganisms would enhance their use and hence reduce total dependence towards the chemical synthetic surfactant industry [19].

The positive economical outlook can be enhanced by increasing their high throughput values or by harnessing other important properties such as pharmacological, antifungal and antiviral capabilities. Several BS have recently been used or anticipated to use in cost-effective applications in medicine, food and cosmetic industries [6]. BS have also potential applications as additives for agricultural use, food industry, mining and manufacturing processes, pulp and paper industries and as detergents or cosmetics [2,14,19,42,43].

6. Medical and Healthcare Applications

The biomedical and cosmetics area, in which low amount of high value BS is required in a high added value product, opens a large field where the

research seems to be at its infancy for new applications. Next, possible applications of BS in these areas are described. The use of BS in medical applications has been proposed, due to several biological properties such as antimicrobial ones [14,42]. BS also have potential for use in vaccines and gene therapy [42,44]. A judicial and effective combination of these strategies might, in the future, lead the way towards large-scale profitable production of BS [42].

Due to their biological origin, BS are generally considered safer than synthetic pharmaceuticals, although, to date there have been very few studies carried out to confirm their lack of toxicity [14,36]. In a study of the skin potential irritation of trehalose lipids produced by *Rhodococcus erythropolis* 51T7 in mouse fibroblast and human keratinocyte lines, results indicated that the BS was less irritating than SDS, and could be therefore used in cosmetic preparations [39].

Trehalose-6,60-dimycolate (TDM), or cord factor, has exhibited a number of different biological activities, including antitumor and immunomodulating functions [14], although Mycobacterial TDM use is limited by the relatively high toxicity of the molecule and the potential pathogenicity of producer strains. TDM produced by *Rhodococcus* sp. 4306 was demonstrated to exhibit lower toxicity, both *in vivo* and *in vitro*, than mycobacterial TDM [14].

Sophorolipids are other promising modulators of the immune response, being able to decrease sepsis related mortality *in vivo*, adhesion molecules levels, cytokine production and IgE levels [42]. Sophorolipids in lactone form have also been proposed as anti-ageing agents due to their capacity to stimulate skin dermal fibroblast cell metabolism and, more particularly, collagen neosynthesis [42].

Several BS exhibit antibacterial, antifungal and antiviral activities, which make them relevant molecules for applications in combating many diseases and infections. BS with known antimicrobial activity include surfactin and iturin produced by *Bacillus subtilis* strains, mannosylerythritol lipids from *Candida antarctica*, and BS isolated from *Streptococcus thermophilus* A and *Lactococcus lactis* [45]. Cyclic lipopeptides are often found to be antimicrobial. For example, the cyclic lipopeptide antibiotic surfactin has antibiotic, anticlotting, haemolytic and antiviral properties. In vesicle studies, surfactin was found to incorporate into membranes at low concentrations and induce slow leakage due to changes in membrane ultrastructure [1,46,47]. Two cyclic lipodepsipeptides isolated from *Pseudomonas fluorescens* have also shown antifungal and anti-weed activities [1]. A rhamnolipid mixture obtained from *P. aeruginosa* AT10 has exhibited inhibitory activity against the bacteria *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis*,

Serratia marcescens, *Mycobacterium phlei* and *Staphylococcus epidermidis* and excellent antifungal properties against *Aspergillus niger*, *Chaetonium globosum*, *Penicillium chrysogenum*, *Aureobasidium pullulans* and the phytopathogenic *Botrytis cinerea* and *Rhizoctonia solani* [15]. *Pseudomonas aeruginosa* 47T2, growing with waste frying oil as a carbon source produces a mixture of ramnolipids with wide antimicrobial activity [48]. Rhamnolipids are also able to inhibit the growth of harmful bloom algae species, *Heterosigma akashivo* and *Protocentrum dentatum.* The mannosylerythritol lipid (MEL), a glycolipid surfactant from *Candida antarctica*, has demonstrated antimicrobial activity particularly against Gram-positive bacteria [42].

Infections resulting from microbial adhesion to biomaterial surfaces have been observed on nearly al medical devices with severe economic and medical consequences. Biofilm infections pose a number of challenges. BS have been studied as antiadhesive agents in surgicals, representing an interesting approach because it may be possible to modify the surface properties to make it simultaneously anti-adhesive and give it antimicrobial activity [36, 42]. *Lactobacillus paracasei* sp. *paracasei* BS showed anti-adhesive activity against *Streptococcus sanguis* (72.9%), *Staphylococcus aureus* (76.8%), *Staphylococcus epidermidis* (72.9%) and *Streptococcus agalactiae* (66.6%). This finding is promising to control infections in the urinary, vaginal and gastrointestinal tracts, and in the skin [45].

Concerning the antiviral properties of BS, inhibition of growth of HIV in leukocytes by BS has been cited in the literature [49]. The sophorolipid produced by *Candida bombicola* and its structural analogues have been studied for their spermicidal, anti-HIV and cytotoxic activities [42].

The succinoyl-trehalose lipid of *Rhodococcus erythropolis* has been reported to inhibit herpes simplex virus and influenza virus and a 1% emulsion of rhamnolipids has been shown to be effective for the treatment of leaves of *Nicotiana glutinosa* infected with tobacco mosaic virus and for the control of potato virus-x disease [14,40,49].

Moreover, a trehalose lipid BS produced by *Rhodococcus* spp. has been shown to cause hemolysis of human erythrocytes through a colloidosmotic mechanism. This pore-type behavior can be explained by the formation of enhanced permeability domains in the erythrocyte membrane, as observed in model membranes [50,51].

Concerning the potential antitumoural activity of BS, first evidences of growth arrest, apoptosis and differentiation induced by glycolipids in different cancer cell line have been reported [14,42].

Another interesting application of BS could be as pulmonary surfactant in cases of deficiency. The isolation of genes for protein molecules of this surfactant and cloning in bacteria has made possible its fermentative production for medical applications [40,42,49].

BS are also very attractive in the health care and cosmetic industries. A large number of compounds for cosmetic applications are prepared by enzymatic conversion of hydrophobic molecules by various lipases and whole cells. The cosmetic industry demands surfactants with a minimum shelf life of three years. Therefore, saturated acyl groups are preferred over the unsaturated compounds. Monoglycerides, one of the widely used surfactant in the cosmetic industry, has been reported to be produced from glycerol-tallow by using *Pseudomonas fluorescens* lipase treatment [40,49].

Finally, BS have several promising applications in the food industry as food additives. Bioemulsifiers may stabilize oil-in-water (O/W) and water-in-oil (W/O) emulsions. The type of emulsion formed by the water and oil depends primarily on the nature of the emulsifying agent and, to a certain extent, on the process used in preparing the emulsion and the relative proportions of oil and water present. Lecithin and its derivatives, fatty acid esters containing glycerol, sorbitano, or ethylene glycol, and ethyoxylated derivatives of monoglycerides are currently in use as emulsifiers in the food industries worldwide [40, 49]. A novel bioemulsifier from *Candida utilis* has shown potential use in salad dressing [49]. Haba et al. [17] tested the ability of rhamnolipids to emulsify different oils used in a number of industries forming a stable emulsion with liseed oil. Trealose tetraester (THL) produced by *Rhodococcus erythropolis* 51T7, revealed effective emulsification with water and paraffin or isopropyl myristate. The composition of 11.3-7.5-81.8 (isopropyl myristate-THL-W) was stable for at least 3 months. To examine the solubilization behaviour of isopropyl myristate and paraffin oil in the multicomponent BS solutions, two-phase diagrams of the ternary system water–surfactant–oil were developed. Figure 3 shows the zone of 100% emulsion phase (shaded area) obtained after mixing different quantities of components. When paraffin was used as the oil phase, the diagram revealed a wide zone of total emulsion. Out of the shaded area, partial emulsion zones with different texture and aspect were observed.

Due to its more hydrophilic character, when isopropyl myristate was used as the hydrophobic component, the total emulsion zone moved to the left vertex, which is the area of higher water proportion. THL proved to be an effective emulsifier when there was a low amount of myristate oil in the mixture [52]. Apart from their obvious role as emulsifying agents, BS can have several other functions in food. For example, to control the agglomeration of fat globules, stabilize aerated systems, improve texture and

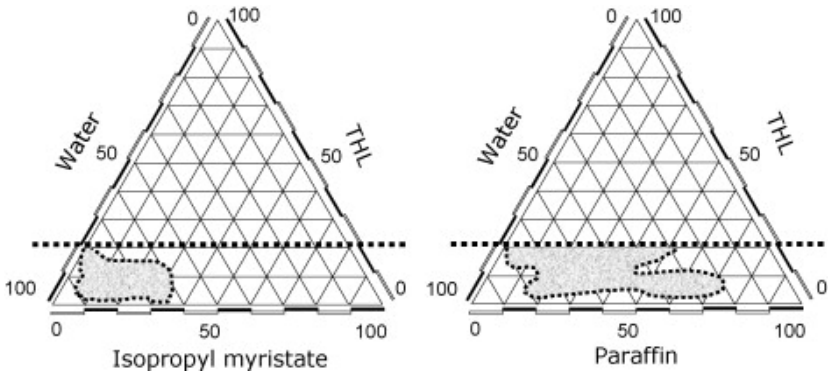

Figure 3. Phase diagram illustrating the emulsion area with isopropyl myristate (left) and paraffin (right) [39].

shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products. They are also utilized as fat stabilizers and antispattering agents during cooking of oil and fats. Improvement in dough stability, texture, volume and conservation of bakery products is obtained by the addition of rhamnolipid surfactants [42].

The involvement of BS in microbial adhesion and detachment from surfaces has also been investigated for food industry application. A surfactant released by *Streptococcus thermophilus* has been used for fouling control of heat-exchanger plates in pasteurizers, as it retards the colonization of other thermophilic strains of *Streptococcus* responsible for fouling. The preconditioning of stainless steel surfaces with a BS obtained from *Pseudomonas fluorescens* is able to inhibit the adhesion of *Listeria monocytogenes* L028 strain [42]. It is evident that the anti-adherent property of BS is very interesting in medicine. The use of plastic material (catether, sondes, etc) is a challenge consequence of bacterial adhesion. A BS pre-treatement on this material can confer anti-adherent and antimicrobial properties.

7. Conclusions

Surfactants are an important class of chemical products in view of the volumes sold and their great variety of applications. During the last decades, a wide variety of microorganisms have been reported to produce numerous types of BS. Their biodegradability and lower toxicity give them an advantage over their chemical counterparts. The most important factor limiting BS use is the production cost, however, the small amounts of product required and the special properties described makes BS high valuable molecules. Application of BS as food additives, specialty chemicals,

biocontrol agents and as a new generation molecules for health and beauty care permits to think in these compunds as the molecules of the future.

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10. Heavy metal accumulation by intestinal helminths of vertebrates

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Abstract. The relevancy of parasites as potential indicators of environmental quality has been increasing over the last years, mostly due to the variety of ways in which they respond to anthropogenic pollution. The use of fish parasites as bioindicators of heavy metal pollution in aquatic ecosystems has been widely studied. However, little information concerning terrestrial habitats is presently available. In fact, in the last two decades several studies have been performed worldwide in different habitats and/or conditions (theoretically both in polluted and unpolluted terrestrial ecosystems, but mainly in aquatic ecosystems) in order to investigate heavy metal pollution using parasitological models. Different groups of vertebrates (mainly fish, mammals and birds) and several parasitological models have been tested involving acanthocephalans mostly, but also cestodes and nematodes. It is not the aim of this chapter to do a complete revision of the available data concerning this subject. Instead, we emphasize some general aspects and compile a mini-review of the work performed in this

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field by our research group. The results obtained until now allow confirming several parasitic models as promising bioindicator systems to evaluate environmental cadmium and mainly lead pollution in terrestrial non-urban habitats, as it was already demonstrated for aquatic ecosystems. The present knowledge also allows confirming that parasites can reveal environmental impact. Environmental parasitology is an interdisciplinary field, which needs simultaneous expertise from toxicology, environmental chemistry and parasitology. Furthermore, environmental parasitology should be taken into account in order to increase the efficiency of environmental monitoring programs.

1. Introduction

Over the last decades parasites have been attracting attention as potential indicators of environmental quality due to the variety of ways in which they respond to anthropogenic pollution, particularly in aquatic ecosystems. In fact, several revision papers have been available for a long time now [1-6]. A pioneer study in this field addressed the issue of parasites as indicators of water quality as a whole, including the use of helminth transmission in marine pollution studies. Among other aspects, the study pointed out that: i) levels of infection of certain parasites could be used as early warning indicators of deteriorating water quality, ii) laboratory experiments should be undertaken to investigate the response of parasite transmission stages to selected pollutants, and iii) guidelines for the selection of the most appropriate host-parasite systems were needed [1]. Just a few years later, the first review on the use of fish parasites as bioindicators of heavy metals in aquatic ecosystems [2] presented some conclusions and perspectives on parasites as accumulation indicators. Some problems or limitations of this kind of studies were also evidenced, such as: i) helminth infestations may affect host sensitivity to heavy metals, ii) the vitality of fish aqueously exposed to cadmium was markedly reduced when they were infested with larval cestodes, and iii) adult parasites may have their own detoxification mechanisms or take up metals, which is in contrast to the extreme metal sensitivity of certain free-living parasite stages like cercariae. It was also pointed out that in order to evaluate the relationship between environmental exposure and acanthocephalan metal bioconcentration and to validate the role of parasites in environmental biomonitoring more laboratory studies on experimentally infected fish are needed. These studies should allow determining the bioconcentration factors, defined as the ratios of the metal concentration in the parasites to those in the host tissues ($C_{[parasite]} / C_{[host\ tissue]}$) [7].

Until recently little information was available on the simultaneous effects of parasites and pollutants on the physiological homeostasis of organisms.

However, today it is possible to assert that: i) parasites influence the metabolism of pollutants in infected hosts, ii) parasites interact with contaminants in synergistic or antagonistic ways, and iii) parasites induce physiological reactions in hosts that are thought to be pollutant-related. In addition, there is a close interaction between the effects of pollutants and parasites, which seems to be mediated at least partly by the endocrine system, which itself is closely related to the immune system of some hosts [8]. In fact, since it is well accepted that parasitism and pollution affect the physiological homeostasis of aquatic hosts [8], it seems reasonable to speculate that the same should occur in terrestrial hosts. The effects of pollution on parasitism are variable, i.e. pollution may increase parasitism if the pollutant mainly affects the host rather than the parasite (immunotoxic chemicals) or it may exercise a negative effect over certain parasites, which are more susceptible to the particular pollutant than their hosts. The interaction between parasites and pollution can be even more complex. In fact, some parasites may even have a "positive" influence on their hosts when exposed to environmental pollution or, on the contrary, they may have synergistic effects becoming more harmful to the host or modeling gonad development of the host [8]. It has been postulated that where hosts face a polluted environment parasitism might be advantageous. This hypothesis deserves further investigation, as it is unclear if the "negative" effects of parasites may be outweighed by the positive impact of reduced pollutant levels [5]. Therefore, it would be important to study this effect under realistic environmental conditions.

Concerning the main goal of this mini-review, it must be said that there are several works on the uptake and accumulation of metals by acanthocephalans of fish most of which have been summarized elsewhere [3, 4]. According to that data the most conspicuous metal accumulation was found in *Pomporhynchus laevis* parasitizing the chub, *Leuciscus cephalus*, which reduce the metal levels in fish host tissues [9]. This fact motivated some experimental works that evaluated the kinetics and processes of metal uptake by fish tissues and their parasites [10-12]. In fact, a model explaining metal uptake by *P. laevis* and the associated reduction of lead in host tissues was developed [10]. In this work the central element of the hypothesis is that acanthocephalans take up bile-bound lead in the small intestine. The liver expels lead ions by binding them to steroids in the bile and these complexes pass down the bile duct into the intestine. In uninfected fish, the intestinal wall reabsorbs bile-bound lead, which then runs through the hepatic intestinal cycle. This cycle can be interrupted by parasites by taking up bile-bound lead thus reducing the available amount of lead that could be reabsorbed by the fish host. As a consequence, infection reduces the amount of lead available

for accumulation in fish tissues. This fact has been partially analyzed in the terrestrial model *Rodentolepis microstoma* / *Mus domesticus* in El Hierro Island (Canary Archipelago, Spain) considering that this cestode inhabits within the duodenum near the bile ducts of this small mammal [13]. This uptake has also been described for other elements and even for important physiological cations [7,14,15]. For example, it was seen that the levels of several essential elements in the liver of *Perca fluviatilis* were negatively correlated with the size of the infrapopulation of *Acanthocephalus lucii* [14]. It appears that acanthocephalans take up these essential elements as a by-product when taking up bile salts with extreme efficiency, as most intestinal parasites are unable to synthesize their own steroids and fatty acids [16,17].

Acanthocephalans seem to meet most of the criteria commonly accepted for ideal bioindicators, even though their individual age cannot be determined. However, it seems unnecessary to specify their exact age, which is known to range from 50-140 days; in addition as metal uptake occurs more rapidly in the acanthocephalan than in host muscle tissue this ratio could provide information on the duration of environmental exposure and a higher ratio must be expected after a short-term exposure than in longer exposure periods [3, 18-20]. The accumulation capacity of some acanthocephalans such as *Acanthocephalus lucii* from natural infected perch, *Perca fluviatilis* was compared to other established free-living organisms such as the zebra mussel *Dreissena polymorpha* from the same site. The following conclusions were obtained: i) the zebra mussel allows to discriminate between gradients of pollution most likely due to its immobility as a consequence of being attached to the substratum by byssal threads and ii) the bioconcentration values of Cd and Pb in *A. lucii* (despite the high variability of metal burden values) were several times greater than those in the zebra mussel. These observations suggest that acanthocephalans are even more useful as environmental indicators for assessing metal pollution in aquatic habitats than other established indicator organisms [21, 22]. In this context we should answer and contextualize the question why do we need new accumulation indicators especially if we are dealing with endoparasites of vertebrates? Among other aspects, it can be said that the use of parasites supposes a contribution to the exploration of remote areas, by investigating whether or not a specific metal is present in a given habitat at bioavailable concentrations [15]. Another interesting possibility is to study the biological availability of noble metals such as the platinum group elements (PGE: palladium, platinum and rhodium) emitted with exhaust fumes from automotive catalytic converters [23]. Around a decade ago it was evident that most of the studies performed in this field were made in aquatic habitats

and only a few involved terrestrial hosts emphasizing that it would be worthy intensifying the research in terrestrial ecosystems [3]. Information using terrestrial vertebrates such as rodents is still quite limited even though they are thought to be useful in predicting environmental risk and in assessing environmental quality [24-26]. There is even less information about models involving small mammals and their helminth parasites, which can be summarized in only some experimental works [27-29] and in a reduced number of field studies [13, 30-32] involving cestodes and acanthocephalans as bioindicator models of heavy metal pollution (mainly Pb, Cd and Hg). Under natural conditions, there is a very limited amount of research dealing with the way parasitism and pollution can interact with each other in terrestrial organisms. Nowadays this subject continues to be more studied in the aquatic environment despite the increasing awareness that parasitism should be investigated in the light of the respective environmental conditions. In fact, since pollution can favour or decrease parasitism depending on an uncountable number of interacting variables, environmental parasitology is an interdisciplinary field which needs simultaneous expertise from toxicology, environmental chemistry and parasitology [1, 5, 33].

2. Materials and methods

Even though such studies have used a wide range of sample types and methodologies, there is a common denominator. Samples may include host tissues (liver, kidney, muscle, gonads, bone, feathers, etc.) as well as parasites (mainly larval and adult stages of several groups of helminths, either whole individuals or portions) that must be taken using stainless-steel or other non-contaminating instruments.

The analytical methodology varies according to different studies. Our particular work is performed at the "Centres Científics i Tecnològics de la Universitat de Barcelona" (CCiTUB), which holds the ISO 9001:2008 certifications. At the proper facilities, samples are weighed (if possible between 100-200 mg wet weight) and then digested in Teflon vessels with suprapure HNO_3 (2 mL) and H_2O_2 (1 mL) in an oven at 90ºC and left overnight. All material used in the digestion process is thoroughly acid-rinsed. After digestion, samples are diluted with Milli-Q water and then analyzed for trace elements by inductively coupled plasma-mass spectrometry (ICP-MS; Perkin-Elmer Elan 6000).

The whole process must be standardized and validated by use of certified standards and blanks. In our surveys two standards are mainly used (DORM-2 and DOLT-3; National Research Council, Canada). The accuracy of all results is higher than 90% and very close to 100% for As, Hg, Pb and Cd.

Detection limits are very low for each element and are calculated as the mean blank value plus three standard deviations of this mean blank.

All concentrations are usually presented as ppb (ng g^{-1}) or ppm (μg g^{-1}) wet weight. Bioaccumulation factors (BFs) in helminths are calculated as the ratios of the metal concentration in the parasite to those in the host tissues ($C_{[parasite]} / C_{[host\ tissue]}$) [7].

3. Studies performed

In the last two decades several studies have been performed worldwide in different habitats and/or conditions (theoretically both polluted and unpolluted terrestrial and mainly aquatic ecosystems, including freshwater, estuarine and marine environments) in order to investigate heavy metal pollution using parasitological models. Different host-parasite models using several groups of vertebrates (mainly fish, mammals and birds) and parasites have been assessed. These models often included acanthocephalans, but also cestodes and nematodes were evaluated. Considering the wide diversity within this type of research, it is possible to categorize studies according to more homogeneous groups. We do not intend this chapter to be a complete revision of the available data on this subject. In fact, it emphasizes some general aspects and reviews the work performed by our research group.

3.1. Aquatic ecosystems

Toxic element pollution has been more extensively investigated in aquatic environments than in terrestrial habitats. Fish and birds inhabiting aquatic ecosystems have been largely used as organisms for monitoring environmental pollution. Marine ecosystems are continuously threatened by contaminants, including heavy metals, which are frequent products of both anthropogenic activities and natural processes. The biomagnification of some metals such as mercury along the food chain is a major concern in marine environments increasing the potential for human exposure [34-36]. The use of bioindicators allows evaluating the risk of exposure, acting as early warning systems for environmental deterioration but firstly the selection of representative species is required [37]. With respect to the marine compartment, seabirds have been intensively used because of their high trophic position and bioaccumulation capacities together with their ability to process certain inorganic elements [38]. In this context, we have studied the concentration of some toxic elements in two seabird species using fresh carcasses obtained from beached bird surveys along the central coast of Portugal: the Atlantic gannet, *Morus bassanus* (Sulidae) and the razorbill

Alca torda (Alcidae) [39, 40]. In addition we are studying the usefulness of the model *Tetrabothrius* sp. / *M. bassanus* as a bioindicator of marine toxic element pollution. When assessing potentially toxic elements in *M. bassanus* it was evidenced that: i) mercury was roughly above the minimum level for adverse effects in birds, ii) there was a higher accumulation of Se and Cd in kidney, of Pb in feathers, and of Mn in liver, and iii) the age of the analyzed birds (juveniles, sub-adults and adults) seems to affect the accumulation of Cd, Co, Hg, Mn, Se and Zn [39]. Preliminary data concerning the model *Tetrabothrius* sp. / *M. bassanus* seem to indicate that it could be proposed as a good bioindicator system to evaluate environmental Cd and Pb pollution in marine environments (*personal data*). In the similar study involving *A. torda* again it was possible to evidence a possible contamination risk by mercury and also by chromium [40]. A higher accumulation of Se and Cd was detected in kidney, of Zn and Pb in feathers, and of As and Mn in liver of *A. torda*. Razorbill's age was also found to affect the accumulation of Cd, Cr, Cu, Hg, and Mn, with juveniles presenting higher levels of Cu and Mn than older individuals [40].

In the polluted estuarine habitat of the Ria de Aveiro (Portugal) we analyzed trace element concentrations in *Proteocephalus macrocephalus* (Cestoda) and *Anguillicola crassus* (Nematoda) in comparison to their fish host, the European eel, *Anguilla anguilla* [41]. Our results confirmed that the consumption of eels collected in this study area represents no risk for humans, because the values of toxic elements quantified in the edible portion of the analyzed eels were below the maximum limits allowed by EU regulations. Consequently, the consumption of these fish by humans should be safe after removing all viscera, which at times presented higher concentrations than those in the edible portion. Nonetheless, since eels are prey to birds and carnivores, eels might represent a real contamination risk for wildlife. The survey also allowed concluding that the model *P. macrocephalus* / *A. anguilla* is a promising bioindicator system to evaluate environmental Cr, Ni, Pb and Zn exposure in aquatic areas where both species are present.

3.2. Terrestrial ecosystems

As mentioned earlier, most studies dealing with environmental terrestrial conditions, using or not parasitic models, have been designed under experimental conditions [27-29] or focused either on theoretically polluted areas such as floodplains, landfills, dumping sites, mine areas and polluted cities, or on areas where major ecological accidents have occurred [e.g. 25, 26, 30, 32]. Contrarily, insufficient attention is devoted to areas not included

in the latter circumstances, where environmental quality is often high because they are usually subjected to low disturbances [13, 15]. However, it should be taken into account that long-term pollutant activities may disturb the more pristine ecosystems.

In this sense, we performed an environmental study in an area of high ecological interest, the El Hierro Island in the Canary Archipelago, declared a reserve of the biosphere by the UNESCO in 2000. Later we compared the obtained insular data (El Hierro Island) on Cd and Pb pollution using the models *Rattus rattus* / *Moniliformis moniliformis* and *Mus domesticus* / *Rodentolepis microstoma* with those previously reported in NE Spain using the model *Apodemus sylvaticus* / *Gallegoides arfaai* also obtained under natural field conditions. We verified that, although lead values were similar in both studies, cadmium tissue levels in the continental area were much higher than those found in El Hierro [13, 31]. In the urban dumping site of Garraf (located only 32 km away from Barcelona, Spain) concentrations of Cd and Pb were also recently tested using another parasitic model (*Apodemus sylvaticus* / *Skrjabinotaenia lobata*) [32]. The reported levels of lead were only slightly higher than those evidenced in El Hierro, but again, cadmium concentrations were much higher than those registered in El Hierro [13, 32]. In general, it could be stated that pollution in El Hierro is lower than in the several areas analyzed in the Iberian Peninsula, corroborating that the environmental quality of zones that have partial or complete protection status is often high. However, as pointed above, long-term pollutant activities (such as tourism-related activities and traffic) might disturb remote or protected ecosystems that require a systematic control using suitable species or helminthological models as bioindicators of pollution by heavy metals.

The model *Apodemus sylvaticus* / *Gallegoides arfaai* was the first terrestrial host-cestode system evaluated for its potential capacity for accumulating cadmium. However, it was found that this model could not be considered an effective system for monitoring environmental cadmium pollution [31]. The same was found for another rodent / cestode model (*A. sylvaticus* / *Skrjabinotaenia lobata*), for a lagomorph / cestode model (*Oryctolagus cuniculus* / *Mosgovoyia ctenoides*), and for a columbiform bird / cestode model (*Columba livia* / *Raillietina micracantha*) [32, 42, 43]. More recently, the same was found for the model *M. domesticus* / *R. microstoma* [13].

Precedents of terrestrial models involving acanthocephalan parasites of vertebrates to study Cd bioaccumulation are scarce. The large acanthocephalan pig's parasite *Macracanthorhynchus hirudinaceus* was considered an important cadmium bioaccumulator on the basis of the study performed in Bolivia under naturally conditions [44]. The acanthocephalan *M. moniliformis* was tested under laboratory conditions on infested *Rattus*

norvegicus and it was concluded that *M. moniliformis* might be used as a highly sensitive bioindicator of cadmium pollution in terrestrial and urban ecosystems [27]. This presumption has recently been corroborated in the above mentioned field study reported from El Hierro although it is necessary to note that natural conditions are much more heterogeneous than experimental premises [13].

As indicated for cadmium, again a reduced number of terrestrial systems involving acanthocephalans parasites of vertebrates were evaluated in respect to their potential for lead bioaccumulation. The species *M. hirudinaceus* was able to bioaccumulate lead at least under the above mentioned conditions in which the study was performed in Bolivia and it was pointed out that *M. hirudinaceus* might be used as an important lead bioaccumulator in terrestrial biotopes especially as it is a very abundant parasite of domestic and wild pigs throughout the world [44]. The model *R. norvegicus* / *M. moniliformis* was tested under experimental conditions and it was considered a useful and promising bioindicator system for lead in urban ecosystems in temperate regions [28]. Despite the variability of natural conditions in contrast to experimental controlled variables, the bioaccumulation factors reported from El Hierro show little difference from those obtained experimentally, and confirm the suitability of this system as a useful and promising tool in environmental monitoring [13, 28].

The first terrestrial model involving a cestode parasite of rodents studied for the possible capacity of lead accumulation was *Rattus norvegicus* / *Hymenolepis diminuta*, probably because the host is widely distributed and easily accessible, meeting some of the criteria set for a good bioindicator [18], either under experimental conditions or in the field [29, 30]. The survey performed in the city of El Cairo (Egypt) allowed considering this model as a promising bioindicator for lead in urban ecosystems [30]. In the same study from El Cairo, the bioaccumulation capacity of the larval stage of *Taenia taeniaeformis* located in cysts in liver tissue of the same rats parasitized by *H. diminuta* was also tested. It was observed that the level of lead in *T. taeniaeformis* was similar to those of the different evaluated tissues. This observation allowed postulating that the cestodes' ability to take up lead is related with the parasite's microhabitat within the host and/or its developmental stage [30]. Furthermore, it was pointed out that heavy metals are not distributed evenly in the cestodes being concentrated especially in the gravid proglottids of the strobila. Thus it was suggested that those parts of strobilae, which are similar in size and have gravid proglottids should be preferred when assessing heavy metals in cestodes [30].

Other studies have also been performed in terrestrial habitats from Spain and Portugal to evaluate the relationship between bioaccumulation in

cestodes of vertebrates and environmental lead exposure. Similar models were used involving rodents, but also lagomorphs and even urban birds (*A. sylvaticus* / *G. arfaai* and *S. lobata*, *O. cuniculus* / *M. ctenoides* and *C. livia* / *R. micracantha*) [13, 31, 32, 42, 43]. The need to use the above mentioned portions of cestodes is easy to fulfill when trying to test entire cestodes smaller than *H. diminuta* such as *R. microstoma*, *G. arfaai* and *S. lobata*.

Table 1 Mean lead bioaccumulation factors [7] calculated for diverse cestode parasitic models in respect to several terrestrial vertebrate tissues.

Model	Parasite Family	Liver	Kidney	Muscle	Ref.
A. sylvaticus *G. arfaai*	Anoplocephalidae	20	6	24	[31]
A. sylvaticus *S. lobata*	Catenotaeniidae	33 – 53	6 – 8	52 -81	[32]
R. rattus *H. diminuta*	Hymenolepidiidae	29 – 87	6 – 11		[30]
M. domesticus *R. microstoma*	Hymenolepidiidae	18	26	28	[13]
O. cuniculus *M. ctenoides*	Anoplocephalidae	1.5	2		[42]
C. livia *R. micracantha*	Davaneidae	10	15	80	[43]

Table 1 presents the lead BFs for models involving cestodes belonging to several families and their vertebrate hosts in terrestrial ecosystems. The *A. sylvaticus* / *G. arfaai* model was proposed as an alternative to evaluate environmental lead exposure, especially outside urban areas where the wood mouse may be readily available [31]. Similarly, the model *A. sylvaticus* / *S. lobata* was tested and proven to be a useful tool for biomonitoring lead pollution in terrestrial habitats [32]. In addition, the BFs reported in the model *M. domesticus* / *R. microstoma* are in agreement with the hypothesis that cestodes with a relatively large tegumental surface in respect to its weight should reach high bioaccumulation factors and therefore they could be considered potentially good bioindicators [13, 43]. It is well-known that cestodes inside the intestine are very efficient taking up bile salts produced in the liver through the hepatic intestinal cycle [10]. This capacity should be increased in the case of *R. microstoma* due to its hepatic (inside the bile ducts) and duodenal location. In fact the BFs reported for this cestode are quite high (see table 1) particularly if we consider that the sample was obtained from

El Hierro Island, an area with very low lead pollution [13]. With respect to models involving parasites of terrestrial birds, only a few have been assessed as bioindicators of environmental pollution. The model *Columba livia* / *Raillietina micracantha* from the city of Santa Cruz de Tenerife (Canary Islands, Spain) was proposed as another promising bioindicator to evaluate environmental toxic element exposure, particularly Pb and Mn, in areas where pollution levels are still relatively low and where both common species are present [43]. The concentration of some toxic elements was also studied in a cestode parasite of lagomorphs, concretely in the model *Oryctolagus cuniculus* / *Mosgovoyia ctenoides* collected from Portugal. Unlike the above mentioned models, the obtained results from *M. ctenoides* and wild-rabbits disproved this model as a promising bioindicator system [42]. The contrasting results obtained in the different models might be due to the large size of *M. ctenoides* in comparison to the other evaluated cestodes and/or due to differences in the tegumental absorption processes between cestode families.

4. Conclusions

The information available to date confirms that several parasitic models are promising bioindicator systems to evaluate environmental cadmium and mainly lead pollution in terrestrial non-urban habitats as it has been widely demonstrated in aquatic environments. In addition, the present knowledge gives a positive answer to the question on whether or not parasites can really reveal environmental impact. In the future, some recommendations for the design of monitoring studies should be taken into question in order to increase the efficiency of environmental monitoring programs.

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The authors wish to thank all personnel at the “Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)” for their support. Over the last years the research group and its work have been supported by the projects HP 2005-0011, 2005SGR00576, CGL2006-04937/BOS, CGL2009-07759/BOS and 2009-SGR-403.

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11. Clinical pharmacokinetics of mycophenolic acid and its metabolites in solid organ transplant recipients

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Abstract. Mycophenolate mofetil (MMF), an ester prodrug of the immunosuppressant mycophenolic acid (MPA), is widely used for maintenance immunosuppressive therapy and prevention of renal allograft rejection in renal transplant recipients.

MPA inhibits inosine monophosphate dehydrogenase (IMPDH), an enzyme involved in the "de novo" synthesis of purine nucleotides, thus suppressing both T-cell and B-cell proliferation. MPA shows a complex pharmacokinetics with considerable inter- and intra- patient by between- and within patient variabilities associated to MPA exposure. Several factors may contribute to it. The pharmacokinetic modeling according to the population pharmacokinetic approach with the non-linear mixed effects

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models has shown to be a powerful tool to describe the relationships between MMF doses and the MPA exposures and also to identify potential predictive patients' demographic and clinical characteristics for dose tailoring during the post-transplant immunosuppresive treatment.

Introduction

Mycophenolic acid based therapies are widely used in combination with calcineurin inhibitors (cyclosporine, tacrolimus, sirolimus) as maintenance immunosuppression in renal transplantation. Mycophenolate mofetil (MMF, brand name cellCept®, Hoffmann-La Roche, Basel, Switzerland) is one of the therapies currently used. In this context, there has been considerable interest in immunosuppressive regimens which permit reduction or elimination of calcineurin inhibitor (CNI)-associated and other chronic toxicities while maintaining adequate immune-suppresion [1]. MPA exposure values show a high variability partly attributable to its complex pharmacokinetics [2,3]. Several factors as albumin concentrations, renal function, co-medication and genetic polymorphism, may contribute to it [2-6]. Recent literature supports the notion that therapeutic drug monitoring (TDM) of MPA improves monitoring of kidney transplant patients on high risk of acute rejection or on calcineurin inhibitor minimization protocols [7,8]. The current review discusses the pharmacokinetics of MPA and its conjugated metabolites as well as the population pharmacokinetic models developed to describe it. Results of an integrated model recently developed by our group including the MPA protein binding, the pharmacokinetics (PK) of both metabolites (7-O-MPA glucuronide (MPAG) and acyl-glucuronide (AcMPAG) and the influence of co-medication and of multidrug resistance associated protein 2 (MRP2) polymorphism on the PK of MPA and its metabolites are also presented.

1. Chemistry of mycophenolic acid and its metabolites

Mycophenolic acid (MPA) is an antibiotic substance derived from *Penicillium stoloniferum*. Mycophenolate mofetil (MMF) is the 2,4-morpholinoethyl esther of MPA [9]. It was developed as a prodrug because the low oral bioavailability of MPA. Molecular structures of mycophenolic acid and its conjugated metabolites, i.e. the phenolic MPA 7-O-glucuronide (MPAG) and the acyl-glucuronide (AcMPAG) are summarized in Fig. 1.

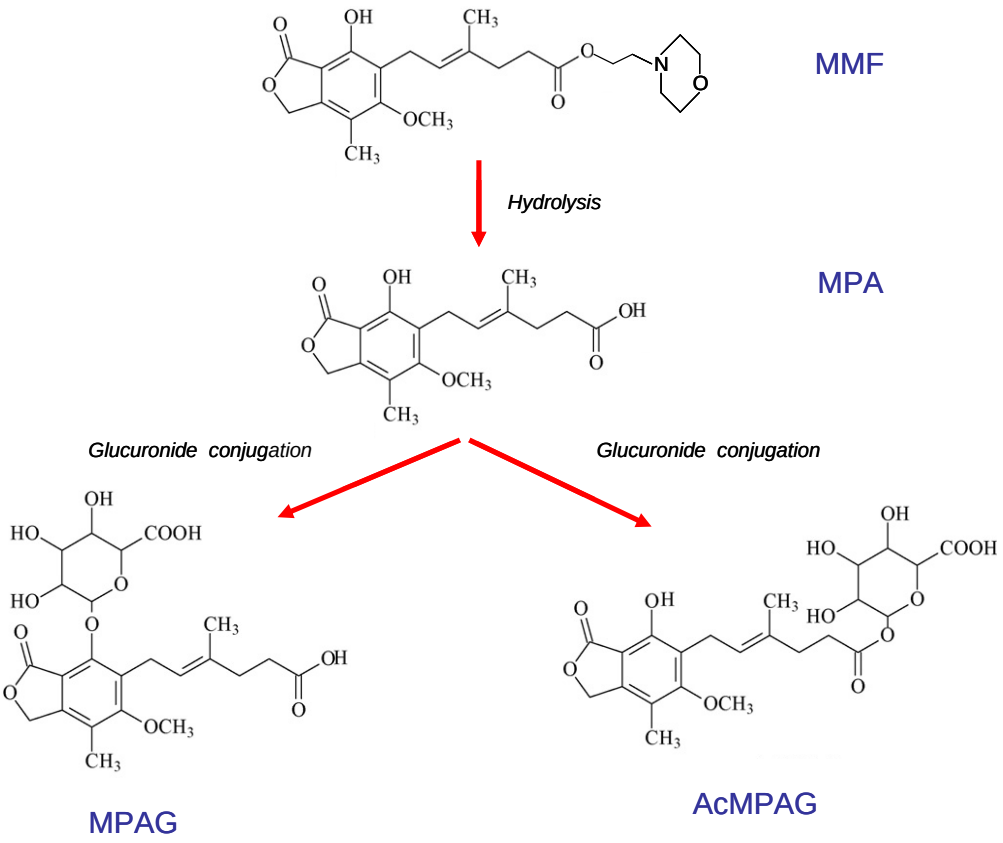

Figure 1. Molecular structures of mycophenolate mofetil (MMF), mycophenolic acid (MPA) and its metabolites, i.e. phenolic MPA 7-O-glucuronide (MPAG) and acyl-glucuronide (AcMPAG).

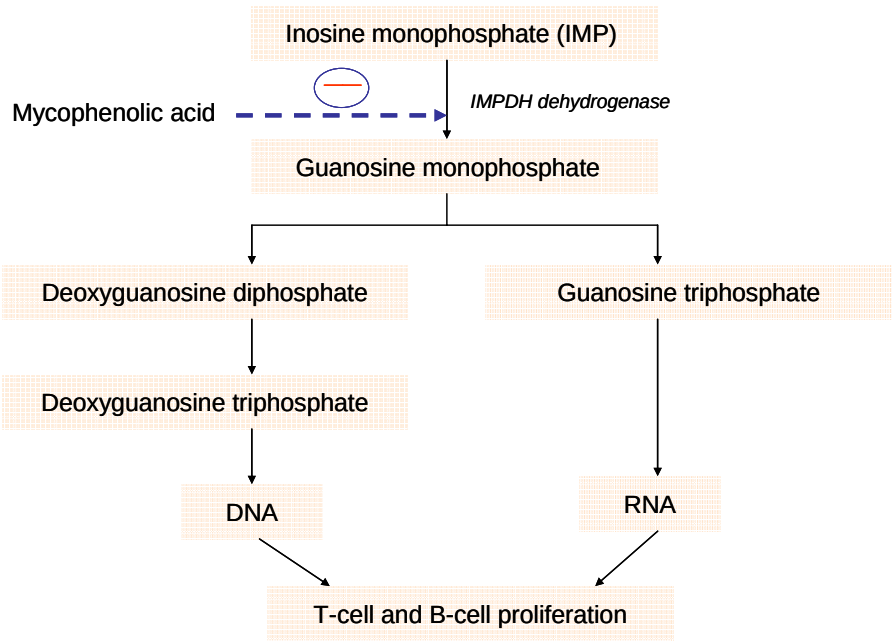

Figure 2. Purine biosynthetic pathways and mycophenolic acid activity.

2. Pharmacodynamics of mycophenolic acid

MPA blocks the “de novo” biosynthesis of purine nucleotides by inhibition of the enzyme inosine monophosphate dehydrogenase [10-12]. Mycophenolic acid is important because of its selective effects on the immune system. As displayed in Fig. 2, it prevents the proliferation of T-cells, lymphocytes, and the formation of antibodies from B-cells. It also may inhibit recruitment of leukocytes to inflammatory sites.

3. Pharmacokinetics of mycophenolic acid

The pharmacokinetic processes that take place when MMF is given orally are summarized in Fig. 3. After its oral administration, MMF is rapidly and essentially, completely absorbed, and then completely hydrolised to MPA, by estearases in the gut wall, blood, liver and tissue [2]. Oral bioavailability of MPA, after MMF administration ranges from 80.7% to 94% [3]. MPA is mainly metabolized by glucuronidation by several uridine diphosphate glucuronosyltransferases (UGTs) in the liver, gastrointestinal tract and kidneys into its inactive MPA 7-O-glucuronide (MPAG) and the pharmacologically active acyl-glucuronide (AcMPAG) [13]. Renal clearance of unchanged MPA is negligible [3] while MPAG and AcMPAG are mainly excreted into urine via active tubular secretion mediated by MRP2 (multidrug resistance-associated protein 2). In the liver MPA is taken up into hepatocytes, glucuronidated to MPAG which is then excreted into bile through MRP2 and then de-conjugated back to MPA by gut bacteria [14]. The formed MPA is then reabsorbed in the colon. Biliary secretion of MPAG, leading to this enterohepatic recirculation (EHC), contributes approximately 40% to the area under the plasma concentration-time curve (AUC) and is considered as the major cause of the secondary peak (observed from 6 to 12 h after oral administration) of MPA in plasma [15]. The EHC is also assumed to occur for the conjugated metabolite AcMPAG [16]. Ciclosporin causes a decrease in the biliary secretion of MPAG and thereby decreases MPA plasma levels and its exposure [14,17,18]. This interaction has been associated with MRP2 inhibition of biliary secretion of MPAG and MPA by ciclosporin [19,20]. On the other hand, MPA is extensively bound to serum albumin [21] and a protein binding ranging from 97% to 99% has been reported in patients with normal renal and liver functions [2]. Although lower, MPAG also displays a high albumin protein binding (82%) in stable patients [3]. Therefore, competition between MPA and MPAG by albumin binding may exist. The interplay between all the processes above described leads to large between-patient and within–patient variabilities associated to

the exposure of MPA and its metabolites in renal transplant patients, as it has been previously reported [2,3].

4. Pharmacokinetic variability

Renal function, serum albumin concentrations, haemoglobin levels, delayed graft function and immunosuppressive co-medication have been described as factors contributing to the variability and time-dependent pharmacokinetics described for MPA [6,22-27]. Previous studies [28] demonstrated that decreased albumin plasma concentrations lead to increased free fractions both of MPA and MPAG. As consequence, relatively more free MPA (fMPA) is available for elimination resulting in decreased total MPA (tMPA) exposure. However, the fMPA exposure is unaffected. This often happens with drugs of low extraction rate [29] as it is the case of MPA [30] and it implies that, in these cases, tMPA exposure could drop below the therapeutic values, so that MMF dose should be increased in order to avoid acute rejection. The influence of renal function on the pharmacokinetics of

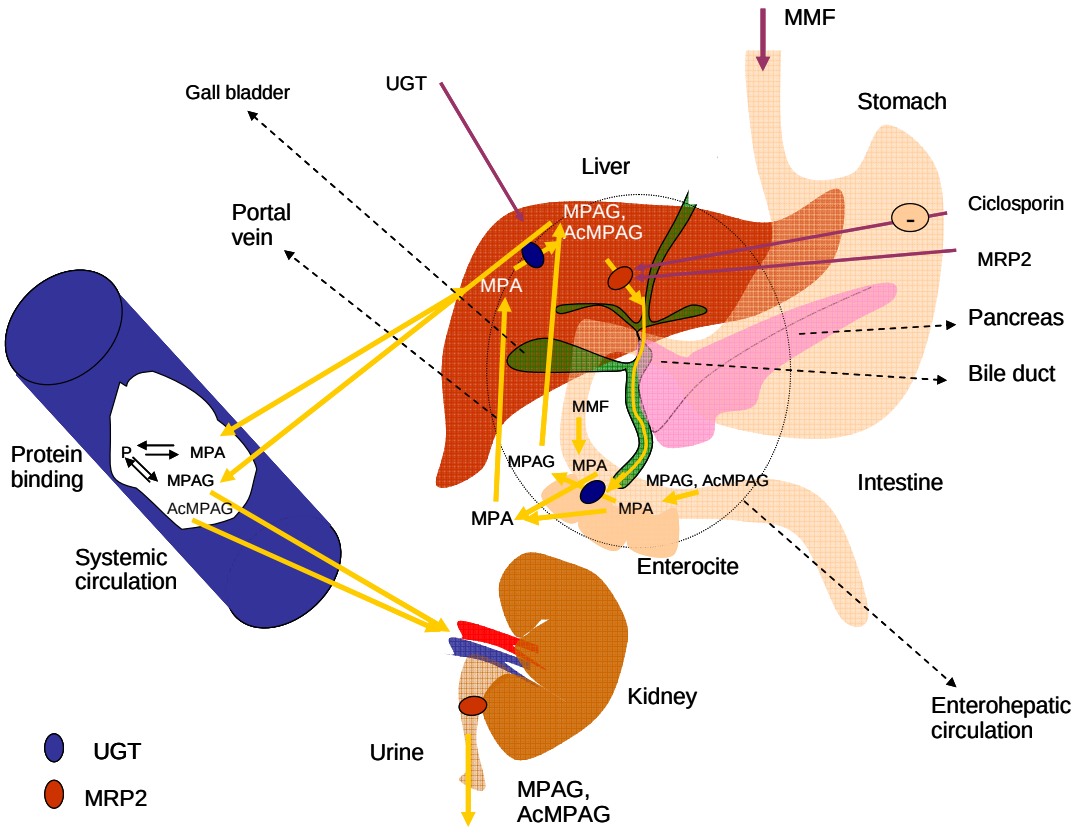

Figure 3. Pharmacokinetics of mycophenolic acid (MPA) and its metabolites, phenolic MPA 7-O-glucuronide (MPAG) and acyl-glucuronide (AcMPAG) after mycophenolate mofetil (MMF) oral administration.

MPA and its conjugates has also been widely reported [22,28]. As it should be expected, a very poor renal allograft function (i.e. CL_{CR} values around 10 mL/min), mainly occurring in the earliest phases after transplantation, have major effects on both MPAG and AcMPAG exposures than on exposures to tMPA or fMPA. According to De Winter et al. [28], depending on the calcineurin inhibitor (CNI) given to patients with impaired renal function, differential effects on tMPA and fMPA exposures can be observed. In the case of ciclosporin tMPA exposure decreases and fMPA exposure remains the same. In patients under tacrolimus an increased exposure to tMPA and a small increase in fMPA exposure are observed.

The justification of this is that under tacrolimus, accumulation of MPAG, as consequence of low renal function, results in increased transport of MPAG to the gallbladder leading to increased recirculation of MPAG to MPA. Because the extra-recirculation, MPAG does not accumulate to an extent where it can displace MPA from its protein binding sites. It results in increased tMPA and fMPA due to extra-recirculation and no change in unbound fraction of MPA. By contrast, in patients under ciclosporin the accumulated MPAG following renal impairment cannot be compensated by increased recirculation because MRP2 transport is inhibited by ciclosporin. As a result MPAG displaces MPA from its protein binding sites, leading to an increased fraction of fMPA. The increased fMPA exposure is immediately compensated for by an increase in MPA glucuronidation according to the theory of restrictively cleared drugs. Therefore, in this case the result is decreased tMPA exposure, unchanged fMPA exposure and an increased fMPA fraction. The genetic factors controlling the level of UGT-mediated MPA metabolism [31-34] and the MRP2-mediated conjugated metabolites transport [35] can also partly explain the observed variability in MPA exposure. The large variability and the MPA narrow therapeutic index lead to optimization of the immunosuppressive regimen in order to avoid the risk of acute rejection (AR), and to prevent adverse-effects associated with long-term immunosuppressive treatment.

5. Relationship between MPA exposure and clinical outcome

In vitro studies have suggested that the fMPA concentrations may more accurately reflect the degree of immunosuppressive action of the drug than does the concentrations of tMPA [21]. The fMPA concentrations have been shown to correlate with the risk of leukopenia and infection [24]. However, although a relationship between fMPA exposure and the risk for acute rejection should be expected, it has not been demonstrated yet. More information is needed about the relationship between fMPA exposure and the

risk for acute rejection and side effects to interpret the clinical effect of changes in protein binding of MPA. Alterations in both albumin concentrations and renal function have little effect on fMPA exposure and thereby little clinical relevance. However special attention must be paid on patients under tacrolimus with very poor renal function, as the increased fMPAG can cause elevated exposure to both tMPA and fMPA. Regarding to tMPA, a correlation between tMPA exposure and the risk of acute rejection has been reported [36]. The proper tMPA exposure (AUC) range to avoid acute rejection has been recommended to be 30-60 μg·h/mL when combined with ciclosporin [36-39]. Under this co-medication there is no further reduction in acute rejection at AUC values >60 (mg/L)·h [36]. Therefore, avoidance of higher exposure would seem prudent on the basis of these results. For patients under tacrolimus, the range of 30-60 μg·h/mL has also been suggested for MPA exposure [39]. Regarding to patients with low albumin concentrations or poor renal function, accompanied by low tMPA exposures, candidates to increased doses of MMF to avoid acute rejection, the dose increase will also increase the fMPA exposure. Therefore, the neutrofil count in these patients should be accurately monitored, as there may be a potential increased risk of leucopenia and infections. In that sense it should be noted that fMPA exposures > 0.14 (mg/L)·h, are expected to increase the risk of adverse events. This cut-off has been established in a pediatric study in the early post-transplant phase [24]. On the other hand, measurement of the pharmacologically active metabolite AcMPAG could also provide information about proper exposures to it in order to avoid the risk for adverse events. The population approach has shown to be a powerful tool for these purposes. The characteristics of this approach are reviewed below.

6. Population pharmacokinetic approach: Non linear mixed effects modeling

According to the FDA Guidance [40], population pharmacokinetics, is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of a drug of interest. It allows to identify the determinants of pharmacokinetic variability, explaining between-patient differences in drug exposure and can provide clinically applicable models for dose tailoring. With this approach, the description of the pharmacokinetics of a given drug in the target population can be performed by determining a*)* what is the mean population pharmacokinetic behaviour, b*)* which factors influence the mean population PK behaviour and, c*)* what is the uncertainty degree associated to the mean population PK behaviour, i.e. the magnitude of between-patient variability and residual error can be quantified. Its advantages when compared

to the classical approach are numerous such as being able to analyze concentration-time data of parent compound and metabolites, data from several doses or plasmatic and urine data, simultaneously. Moreover, apart from analyzing dense data (intensive sampling in each individual) it allows to analyze unbalanced data and sparse data (few samples per individual) coming from observational studies, phase II/III/IV clinical studies or from special populations (neonates, children, elderly, transplantation, HIV) or from therapeutic drug monitoring (TDM).

Non linear mixed effects models implemented in several softwares are the most widely used for these purposes [41]. Once validated, the models developed with this approach will allow, a) description and a better knowledge of the pharmacokinetic profile of a given drug in the target population, b) simulations of new scenarios to predict exposures to different dosing regimens, to evaluate consequences of alternative sampling designs, or to evaluate the impact of changes in patients' characteristics, and c) calculation of the first dose and dose tailoring during the therapeutic drug monitoring, in order to achieve the target value of a given PK parameter (in general, AUC or area under the curve, peak or through concentrations) demonstrated to be the best marker of efficacy. Regarding to the last point, it should be noted that computer programs designed for dose optimisation for individual patients to be used as a part of routine clinical care, exist [42]. These programs require the implementation of the PK parameters estimated through the non linear mixed effects modeling.

In the mixed-effects modeling context [41], the collection of population characteristics is composed of population mean values (derived from fixed-effects parameters) and their variability within the population (generally the variance-covariance values derived from random-effects parameters). This approach allows to estimate directly the parameters of the population from the full set of individual concentration values. The individuality of each subject is maintained and accounted for, even when data are sparse. Fig. 4 summarizes the relationship between fixed and random effects in a population PK model. According to this and taking into account one of the PK parameters, i.e. clearance (CL), it is assumed that the deviations of individual plasmatic clearances of subject 1 (CL_1) or 2 (CL_2) from the mean population value ($\overline{CL}$) are given by η_1^{CL} and η_2^{CL}, respectively (left panel). The set of values of etas (η_i) of all individuals in the target population is assumed to follow a normal distribution of mean equal to zero and variance ω^2. Moreover, the set of deviations of the observed concentrations in each individual at a given sampling time j (open circles), from the individual predictions by the model (continuous lines or $f(CL_1)$, $f(CL_2)$, or f(CLi) for the i subject), given by $\varepsilon_{ij,}$ in general (i denoting the individual, i.e. i=1,2,..n; and

j the sampling time, i.e.,j=1,2,…t) are also assumed to follow a normal distribution of mean equal to zero and variance σ^2 (right panel). f($\overline{CL}$) represents the concentrations predicted for an individual of the target population showing the typical clearance value equal to the mean population value, $\overline{CL}$. In summary, the population parameters estimated by the non linear mixed approach are the following:

a) the fixed effects, i.e. the mean population pharmacokinetic parameters as $\overline{CL}$ or the corresponding regression parameters when there is a stististically significant relationship between the PK parameters and continuous (age, body weight, creatinine clearance, doses or exposure parameters corresponding to co-medication…) or discontinuous (gender…) covariates.
b) the variances of the η and ε distributions, that is ω^2 and σ^2, respectively.

During the estimation process the η and ε values for each individual will be calculated and thus the individual PK parameter values obtained to be used for dose tailoring during the TDM. However, before that, the validation or evaluation of the predictability of the model developed is required. Internal or external validation techniques can be applied for this purpose [40].

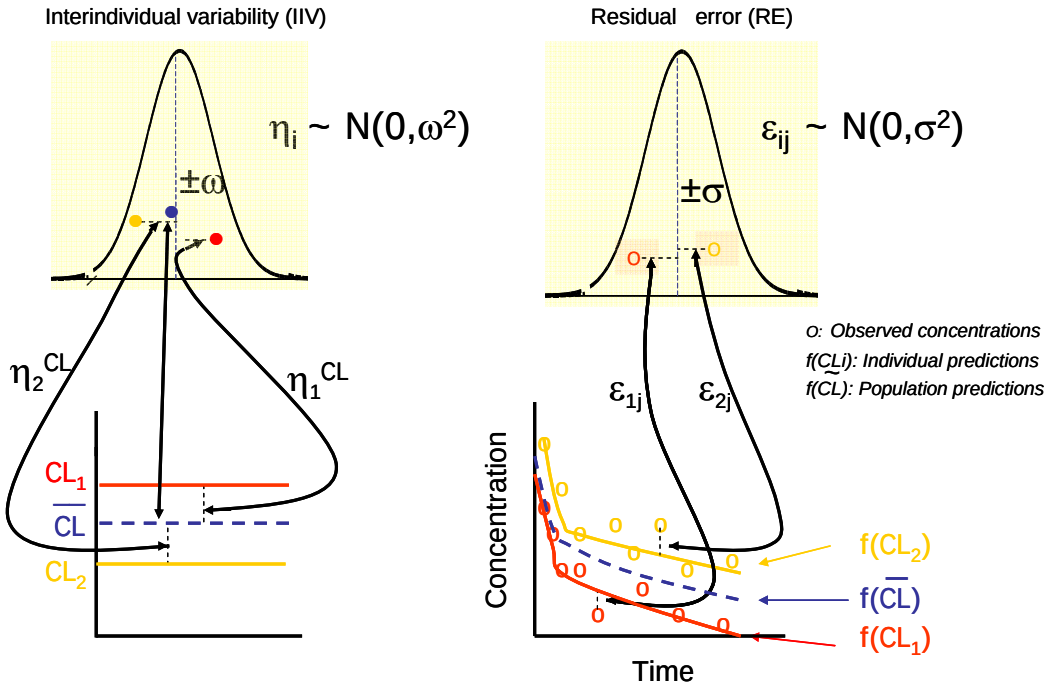

Figure 4. Relationship between fixed effects and random effects in the non linear mixed effects approach.

7. Population pharmacokinetic modeling of mycophenolic acid and its metabolites

Several MPA population pharmacokinetic models have been previously developed trying to identify the sources of variability of MPA pharmacokinetics after MMF oral administration [4,6,28,34,43-48]. Some of them have allowed to describe the second MPA plasma peak by including the EHC in the modeling process [34, 49], as the influence either of changes of protein binding [28,47], or of genetic polymorphism in UGT on MPA exposure [34]. Recently our group has addressed its work on the simultaneous modeling of total and free MPA as total MPAG and AcMPAG concentration vs time data proceeding from the PK sub-study of the Symphony study [50]. In the Symphony PK sub-study the effect of four different immunosuppressive therapies on the PK of MPA was evaluated. Briefly, patients randomized in four groups were given fixed doses of MMF (1 g twice daily) together with either standard doses of ciclosporin (group A), low doses of ciclosporin (group B) or low doses of the immunosuppressive macrolides that is to say tacrolimus (group C) or sirolimus (group D). PK sampling was performed on 5 occasions (on day 7 and at 1, 3, 6 and 12 months after transplantation) during the first year after transplantation and each time tMPA, fMPA, tMPAG and tAcMPAG exposures were measured. Results corresponding to the non-compartmental analysis of this study [50] indicated that tMPA and fMPA exposures were lower in patients receiving ciclosporin compared to those that were given macrolides. In contrast, exposures to the metabolites MPAG and acylMPAG were higher in patients treated with ciclosporin compared to the others (Fig. 5).

Moreover, in general, a trend to increased tMPA and fMPA exposures and decreased tMPAG and tAcMPAG exposures with post-transplant time was observed (Fig. 5). This was attributable to the decreasing and increasing of MPA and MPAG/AcMPAG clearances, respectively, with post-transplant time. On the other hand, Lloberas et al. [35], reported statistically lower tMPA and fMPA exposures in patients treated with macrolides being homozygous (TT) or heterozygous (CT) carriers of the MRP2 C24T single nucleotide polymorphism (SNP) vs non carriers (CC), after having investigated the influence of genetic polymorphism of MRP2-mediated transport on the MPA exposures in all groups of treatment. It should be noted that this effect could not be observed in the group of patients treated with ciclosporin. The time-dependent clearance found in this study was in agreement with results of previous studies [4,6,45]. As previously reported, ciclosporin dose tapering and improvement of the renal function along the post-transplant period can be among others, some of the causes of it. Since

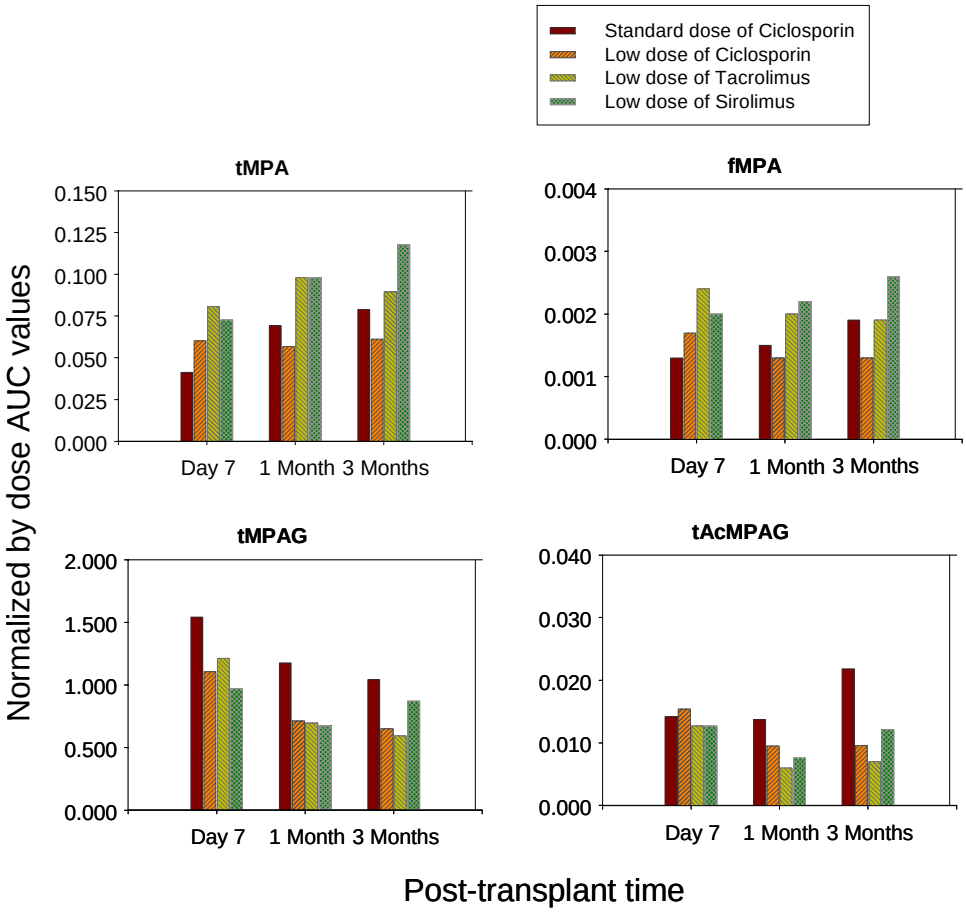

Figure 5. Normalized by dose exposure values given by AUCs (area under the the curve) observed for tMPA, fMPA, tMPAG and tAcMPAG, during the first three post-transplant months of the PK sub-study of the Symphony study [40].

MPA is a low extraction rate drug, the low albumin concentrations associated to a delayed graft function during the early post-transplant stages can also lead to higher MPA clearances values at the early stages vs the late. On the other hand, inhibition of MRP2 transport by ciclosporin, in patients treated with it, leads to decreased MPAG biliar excretion that in turns results in decreased EHC, increased MPAG and AcMPAG exposures and decreased MPA exposures when compared to the others. Regarding to the influence of C24T SNP, results of this study suggested a lower activity of transport of MPAG or AcMPAG through MRP2 in presence of C24T SNP that led to a decreased EHC, followed by lower MPA exposure and subsequently lower MPAG or AcMPAG exposures caused by its decreased formation, when compared to the non carrier (CC) genotype. This was only evidenced under macrolides when the effect of ciclosporin was not masking that of the SNP. Prompted by these results, a population pharmacokinetic model to allow the description of all these processes was developed (Fig. 6) (submitted for

publication). The pharmacokinetics of fMPA, tMPA, tMPAG and tAcMPAG were best described by an integrated model consisting on three linked compartment models; i.e. two two-compartment models for fMPA and tMPAG and a one-compartment model for tAcMPAG (Fig. 6). The model was parameterized in terms of volumes of distribution (V) and plasmatic (CL) and distributional (CL_D)clearances. An albumin compartment was also linked directly to the central compartment of fMPA to describe its binding to this plasmatic protein (K_B=binding rate constant). EHC could not be successfully modeled.

According to this model, after oral administration, MMF was transformed to and absorbed as MPA according to a time lagged first order kinetic process. Once in the systemic circulation MPA was simultaneously bound to albumin (bMPA) and the free fraction (fMPA) distributed and eliminated.

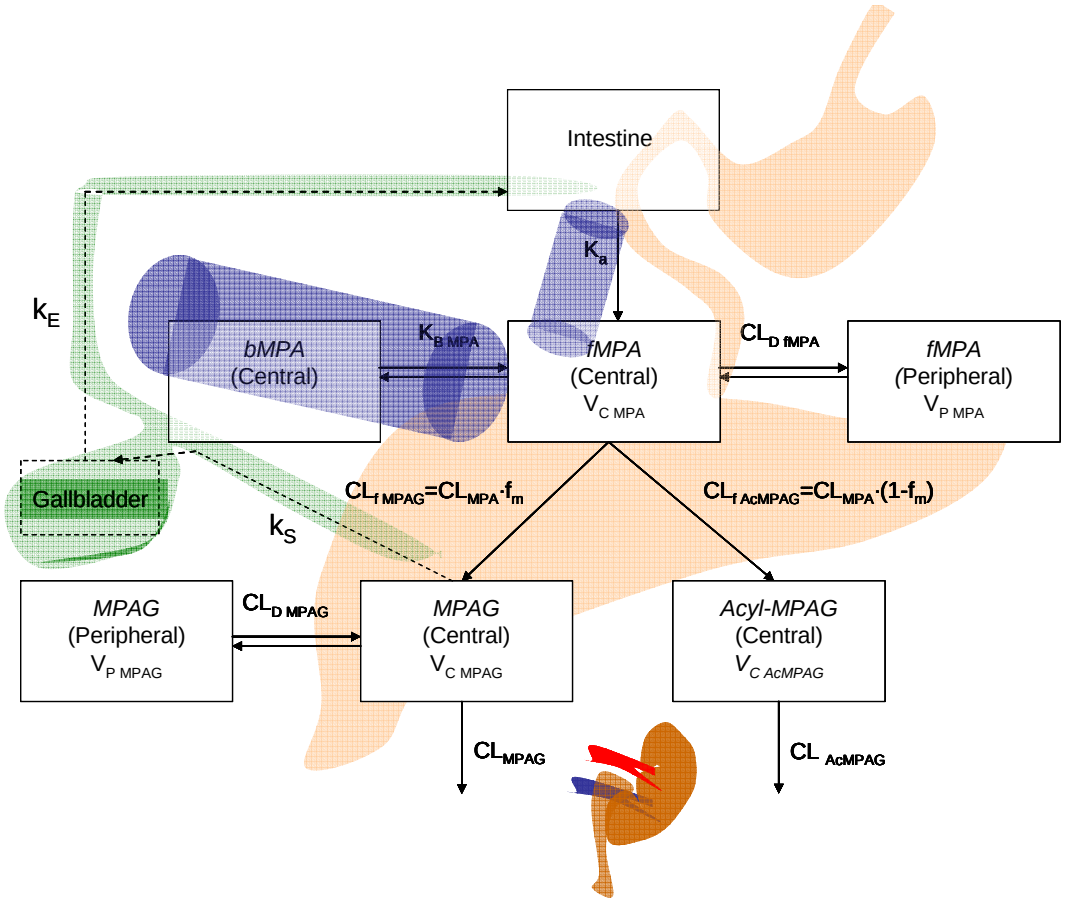

Figure 6. Schematic representation of the final pharmacokinetic model to simultaneously describe the protein binding of free mycophenolic acid (fMPA) and its conversion to its phenolic 7-O-glucuronide (MPAG) and acyl-glucuronide (AcMPAG) conjugates by first order processes, after oral administration of mycophenolate mofetil. Dashed lines correspond to the part that could not be successfully modeled. bMPA: MPA bound to albumin.

The fMPA protein binding was best described by a linear model ($bMPA=K_B \cdot fMPA$) as previously reported by Van Hest et al [46]. This might be due to the fact that the fMPA concentrations found in the current study (from 0.000019 to 0.005573 mmol/L) were far below the median plasma albumin concentrations of the studied population (42 g/L or 0.6087 mmol/L) and no saturation of the binding sites could be achieved. Obviously, taking into account at least one binding site to albumin, the protein binding would be hardly saturated with the current fMPA concentrations. It should be noted that fMPA and tMPA concentrations found in our study were around twice those observed by Van Hest et al [46] in which higher ciclosporin doses were given (around twice those of our study), and nevertheless the linear protein binding still described correctly our data. Effectively, unlike our results, Van Hest et al. [46], found statistically significant correlations between individual K_B values and albumin plasma levels and MPAG concentrations. This could be attributed to the fact that most of the patients included in the current study showed albumin plasma concentrations within the expected range of normal healthy adults (43 g/L, ranging from 35 to 53 g/L), even on day 7 of the study when delayed graft function should be expected to be more likely. Only 8 out of 56 patients and 2 out of 56 patients showed albumin plasma levels less than 35 and greater than 53 g/L, respectively; the albumin concentrations of the remaining patients were around the study population median level (42 g/L). Regarding to MPAG concentrations, these were lower in our study than those found by Van Hest el al. [46]. The stable renal function of patients and also the lower doses of ciclosporin in patients under this co-medication, might be the contributing factors to this fact. According to Bullingham et al. [3], MPA free fractions (fu) can increase as the MPAG concentrations increase to 475 mg/L (957 mmol/L). In our study peak MPAG concentrations were lower than 236 mg/L (479 mmol/L) in most patients, so that no displacement of bound MPA (bMPA) by MPAG should be expected.

Regarding to the elimination process of fMPA it took place by first-order kinetic processes as did the metabolites. According to the f_m value obtained (0.874), MPAG was the major metabolite, while only a 0.126 ($1-f_m$) of fMPA present in the blood stream was transformed to AcMPAG. These values were in agreement with that reported by Shipkova et al [16], The total clearance value of fMPA was the contribution of the clearance of formation MPAG ($f_m \cdot CL_{MPA}$) and the clearance of formation AcMPAG ($(1-f_m) \cdot CL_{MPA}$). Although EHC modeling could not be included, it does not seem to have a relevant impact from a clinical point of view, since the covariates that could affect the recycling rate constant (K_S) could be incorporated in both the MPA and its metabolites clearances. In effect, in agreement with results of the non-compartmental analysis, an statistically significant relationship was found

between CL_{MPA} and ciclosporin through concentrations in patients under ciclosporin, in such a way that CL_{MPA} increased with it and was higher in C24 SNP homozygous (TT) or heterozygous (CT) carriers vs non carriers (CC), in patients under macrolides. The time-dependent clearance of MPA in the target population was also confirmed by the model that suggested that CL_{MPA} on day 7 was higher than at the remaining monitoring days; however, gradual tapering of ciclosporin through concentrations along the post-transplant period was not sufficient to describe these changes over time nor other covariates whose significance could not be demonstrated as low graft function or acidosis or uremia associated to it and consequently low levels of albumin.

Regarding the metabolites, AcMPAG showed around a ten times faster elimination than MPAG, probably due to the highest hydrophilicity of the former. Plasmatic clearances of both metabolites were influenced by renal function through the estimated CL_{CR} according to the Cockcroft-Gault formula, as it should be expected, due to its elimination major pathway by urinary excretion [3, 16, 22]. Explicitly, both CL_{MPAG} and CL_{AcMPAG} were estimated to increase with renal function. Moreover, CL_{MPAG} decreased in a significant statistically way with increasing ciclosporin trough concentrations in patients under ciclosporin and similarly to $CL_{MPA,}$ it was higher in C24 SNP homozygous (TT) or heterozygous (CT) carriers vs non carriers (CC), in patients under macrolides. Regarding the influence of C24T SNP in CL_{AcMPAG}, it was statistically significant, but only in patients under macrolides. By contrast, no statistically significant effect of ciclosporin through concentrations on CL_{AcMPAG} could be evidenced.

Simulations performed once the model had been evaluated (Fig. 7), showed that after multiple fixed doses of MMF (1 g), at one month of the post-transplant period, both fMPA and tMPA exposures decreased significantly with increasing ciclosporin through concentrations from 100 to 300 ng/mL. In patients co-treated with macrolides (sirolimus or tacrolimus), fMPA and tMPA were significantly lower in homozygous and heterozygous variants of the C24T SNP vs wild-type. Among all patients, the highest exposure would be observed in wild-type or no variant alleles co-treated with sirolimus or tacrolimus while the lowest exposure would be found in patients co-treated with standard doses of ciclosporin. Regarding MPAG, exposures increased significantly with increasing ciclosporin through concentrations (from 100 to 300 ng/mL) and in patients co-treated with macrolides, they were significantly lower in homozygous and heterozygous variants of the C24T SNP vs wild-type. In the case of A_CMPAG, significantly lower exposures were observed in homozygous and heterozygous variants of the C24T SNP vs wild-type in patients co-treated with macrolides and also vs the

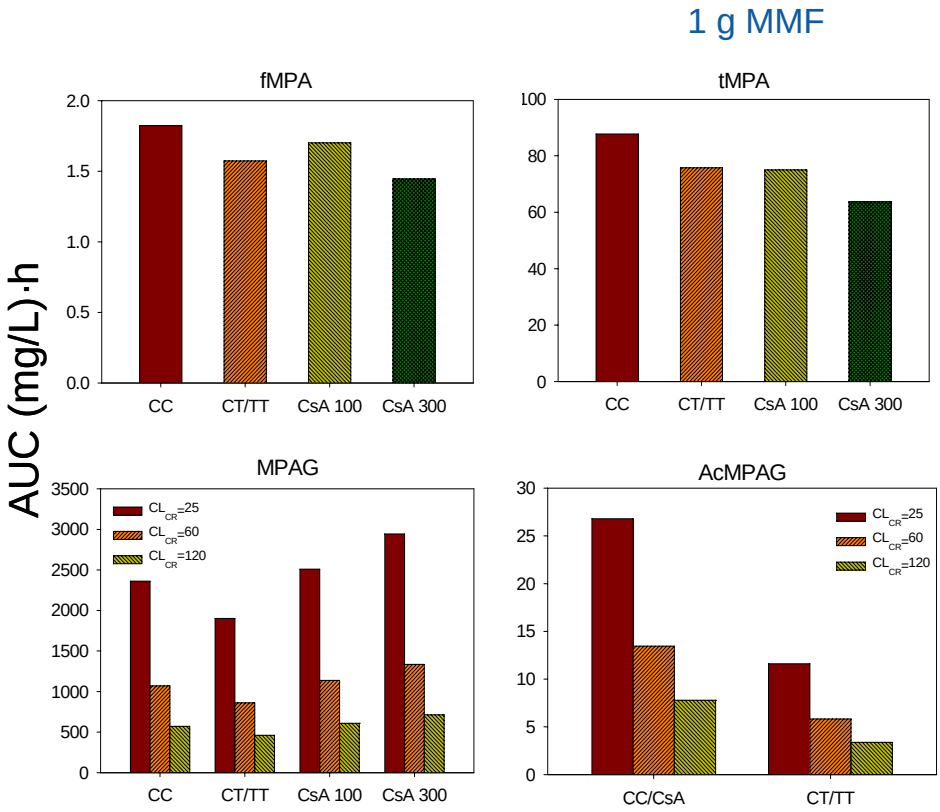

Figure 7. Median values of simulated exposures of fMPA, tMPA and MPAG and AcMPAG, after 1 g MMF given orally to patients under ciclosporin with trough concentrations of either 100 or 300 ng/mL and to patients under macrolides whether non carriers or CT/TT carriers of the C24T SNP of MRP2. The impact of renal function given by CL_{CR} estimated according to cockroft-Gault is also shown for MPAG and AcMPAG.

remaining patients. On the other hand, as expected, the major effects on both tMPAG and tAcMPAG exposures were due to changes in renal function given by CL_{CR} (estimated according to Crokroft-Gault). In effect, independently of the co-medication group, exposures significantly decreased with increasing CLCR values, the effect was higher for MPAG (around 55% from 25 mL/min to 60 mL/min and around 47% from 60 mL/min to 120 mL/min) than for AcMPAG (around 26% from 25 mL/min to 60 mL/min and around 42% from 60 mL/min to 120 mL/min). Then, renal function is the most influential covariate in both cases (CL_{MPAG} and CL_{AcMPAG}) followed by ciclosporin through concentrations and C24T SNP in the case of MPAG. The effect of C24T SNP is more relevant in CL_{AcMPAG} than in CL_{MAPG}. Unfortunately, the developed model did not allow the evaluation of the impact of changes in renal function on tMPA or fMPA exposures. Regarding changes in MPAG exposure with renal function observed in the current work, they are comparable to those found by De Winter et al. [28]. In summary we

built a population PK model to adequately describe plasma data of tMPA, fMPA and its currently known metabolites as a function of co-medication, C24T SNP of Mrp2 and renal function. According to it, patients under macrolides and non carriers of C24T SNP would require lower doses of MMF (around 40% less) than those under standard doses of ciclosporin with the same renal function. Since the model development does not allow evaluation of the impact of changes in albumin plasma levels and renal function on tMPA and fMPA it should be applied to patients with median albumin plasma levels of 42 g/L and median CLCR values of 60 mL/min , as the median of the population of the current study.

8. Conclusion

In the present chapter we summarize the relevance of modeling by using the population approach in order to describe the pharmacokinetics of mycophenolic acid and its metabolites. It allows, not only increasing our knowledge to better understand the clinical PK of this drug, but also it may prove useful in predicting the PK of MPA and all the characterized metabolites after various administration regimens of MMF.

Moreover, based on the protein binding model developed, precise predictions of fMPA concentrations can be made. This can be useful in at least two situations: a) for historical data were only tMPA concentrations are available, fMPA concentrations can be predicted and used in developing PK/PD relationships, and b) as the tMPA assay is considerably simpler than the fMPA assay, measuring only tMPA concentrations may be an alternative to measuring fMPA.

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12. Immunomodulation by conjugated linoleic acid (CLA) in early life

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Abstract. Conjugated linoleic acid (CLA) has been reported to exert beneficial physiological effects on body composition and the immune system. However, little information is available on the influence of CLA on immune function during early life periods. The present study evaluates the effect of feeding an 80:20 mixture of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers during gestation, suckling and early infancy on the systemic and mucosal immune responses of Wistar rats at three different time points: at the end of the suckling period (21-day-old rats), in early infancy (28-day-old rats), and later in life (adulthood). *Cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers were detected in the milk of CLA-fed dams and in the plasma of all CLA-supplemented pups, and the highest content was achieved in those rats supplemented over the longest period. Dietary supplementation with that CLA mix enhances the systemic production of the main *in vivo* and *ex vivo* immunoglobulin (Ig) isotypes in 21- and 28-day-old rats. Moreover, CLA supplementation during suckling and early infancy also enhances the humoral immune defenses at intestinal level, by means of mucosal IgA increase, whereas down-regulates the systemic lymphoproliferative response. Finally, we described

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herein how feeding a diet enriched with the same isomer mix of *cis*9,*trans*11- and *trans*10,*cis*12-CLA from gestation to adulthood improves the capacity of adult rats to achieve a specific systemic and mucosal immune responses. All these data support the immunomodulatory effects of dietary supplementation of CLA, particularly of the *cis*9,*trans*11-CLA isomer, during early stages of life on immune system development, as well as the long-term effects on the specific immune response in adult age.

Introduction

Birth is for the newborn a transition from a sterile environment to a world full of bacteria and viruses, where protection is crucial. During the first stages of life, antibodies from the mother are transferred to the foetus and the child decrease the number of infectious episodes caused by microorganisms to which exist maternal immunological memory [1,2]. The immune system is in constant evolution, and their function is profoundly influenced by maternal, environmental, dietary, and behavioural factors. Although the impact of these factors is greatest during the prenatal and immediate postnatal periods, their influence extends beyond this period. Patterns of development in postnatal life determine many of the immune outcomes in later life [3].

Over the last years, the effect of nutrition on the development of the immune system has acquired great interest and has led to adoption of the term "immunonutrition". Because breast milk is the only natural food for newborns, and dietary contact has a pivotal role in the development of their immune system, significant progress has been made in the characterisation of milk components affecting growth, development and functions of the gastrointestinal tract and the immune system [4]. In this sense, breast milk is rich in, among others, immunoglobulins (Ig) that can bind and neutralise pathogens in the intestinal tract; bactericidal factors such as lactoferrin, lactoperoxidase and lysozyme and growth factors, nucleotides and cytokines that improve immune defence and gut-barrier function [5]. Besides these compounds, the dietary lipids present in breast milk have been studied with special attention. In this sense, it has been suggested that polyunsaturated fatty acids (PUFA), specifically docosahexaenoic acid and arachidonic acid, which constitute a relatively low fraction of the total fatty acids in human breast milk, participate in neonate immune development [6].

Human milk contains measurable quantities of conjugated linoleic acid (CLA), a class of positional and geometric conjugated dienoic isomers of linoleic acid. The predominant CLA isomer in milk and dairy products is *cis*-9,*trans*-11 (*c*9,*t*11)-CLA, also called rumenic acid, which ranges in human milk from 83 to 100% of total CLA [7,8]. The *trans*-10,*cis*-12 (*t*10,*c*12)-CLA isomer is also found in dairy products, although in lower proportion than rumenic acid [8,9], but even very low doses of the *t*10,*c*12-

CLA isomer seem to have large biological effects [10]. The concentration of CLA in milk is influenced by the intake of food of ruminant origin [8].

CLA has been reported to exert beneficial physiological effects on body composition and inhibition of carcinogenesis, atherosclerosis, and diabetes [11-14]. Moreover, CLA isomer mixtures have been shown to modulate immune function *in vitro* and *in vivo* [15-19]. Results from these studies show great variability, partly because of differences in the experimental animal species used and the length of the studies, but also because of differences in the isomer mixtures used for supplementation. However, little work has been done on the immunomodulatory effects of CLA during the early postnatal periods (lactation or infancy), and even less during the prenatal period (gestation).

The present study evaluates the influence of dietary supplementation during gestation, suckling, and early infancy with an 80:20 isomer mix of *c*9,*t*11- and *t*10,*c*12-CLA on the systemic and mucosal immunity in Wistar rats at the end of the suckling period (21-day-old rats), in early infancy (28-day-old rats), and later in life (adulthood).

1. Dietary supplementation with CLA during early life on the development of the immune system in rats

The aim of this part of the study was to evaluate the effect of dietary supplementation during early life with an 80:20 isomer mixture of *c*9,*t*11- and *t*10,*c*12-CLA on the development of the immune system in Wistar rats. CLA supplementation was performed during three life periods: gestation, suckling, and early infancy. For that purpose, the immunomodulatory effects of dietary CLA during gestation and suckling were evaluated in 21-day-old rats (at the end of the suckling period) [20]. And, on the other hand, the influence of CLA supplementation limited to suckling period and to early infancy was evaluated in 28-day-old rats (early infant rats, 1 wk after weaning) [21]. Moreover, as it has been suggested that CLA intake during early stages of development may have effects later in life [22,23], we have also studied whether CLA supplementation limited to suckling produces effects which can last until early infancy [21].

At both ages, their immune system is still in maturation (i.e., antibody production), however, in 28-day-old rats some immune functions are similar to those of adult animals (i.e., lymphoproliferative response). To investigate the immunomodulatory ability of CLA supplementation on the immune system of 21- and 28-day-old rats, both systemic and mucosal immune responses were evaluated [20,21,24].

1.1. Experimental design

Animals were distributed in eight experimental groups according to the total period of CLA supplementation, administration route used, and age at the time immune status was assessed (21- or 28-day-old) (**Fig. 1**).

Day 21 assessment: Pregnant Wistar rats at 7 days' gestation were randomly assigned to one of the following four dietary groups, and pups from these groups were sacrificed at the end of the suckling period (21- day-old):

- 21/5* group: Pups from dams fed a 1% CLA diet during the last 2 weeks of gestation and throughout the suckling period. During suckling, the pups received CLA through the dams' milk. The total period of supplementation was 5 wk.
- 21/5 group: Pups from dams fed a 1% CLA diet during gestation and a standard diet during suckling. During suckling, pups were CLA-supplemented daily by oral (p.o.) administration. The total period of supplementation was 5 wk.
- 21/3 group: Pups from dams fed the standard diet during gestation and suckling. Pups received CLA daily by p.o. administration throughout the suckling period. The total period of supplementation was 3 wk.
- 21/0 group (Ref): Pups from dams fed the standard diet throughout the study. These animals constitute the reference diet group. The total period of supplementation was 0 wk.

Day 28 assessment: On the day of birth, pups from dams fed standard diet during gestation were randomly assigned to one of the following four dietary groups. All dams were fed standard diet throughout the period of study. Pups from these groups were sacrificed 1 week after weaning (28- day-old).

- 28/4 group. Pups received CLA daily by p.o. administration during suckling; after weaning, animals were fed 1% CLA diet from day 21 to 28 (early infancy). The total period of supplementation was 4 wk.
- 28/3 group. Pups received CLA daily by p.o. administration during suckling; after weaning, animals were fed standard diet up to day 28. The total period of supplementation was 3 wk.
- 28/1 group. Pups received 1% CLA diet exclusively for one week after weaning (days 21–28). The total period of supplementation was 1 wk.
- 28/0 group (Ref). Pups fed standard diet during suckling and early infancy. These animals constitute the reference diet group. The total period of supplementation was 0 wk.

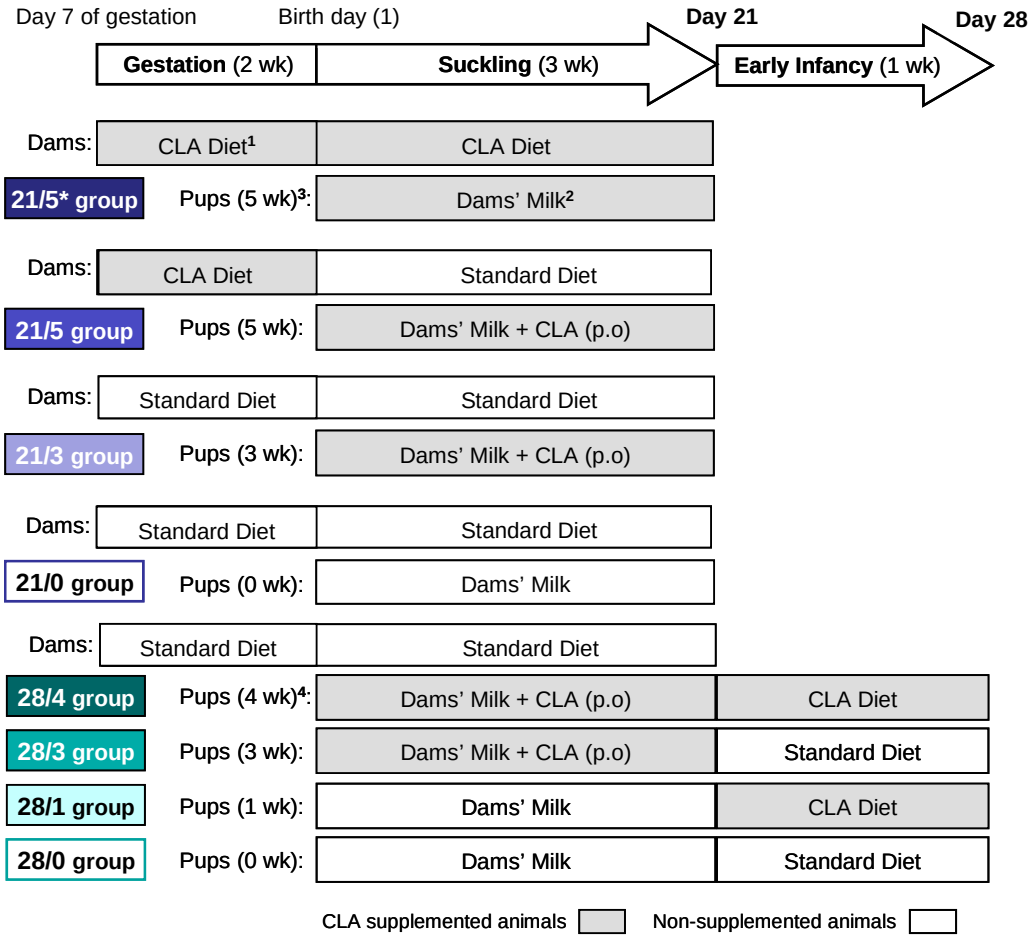

Figure 1. Diagram of the experimental design beginning on day 7 of gestation until day 21 of suckling or day 28 of life. [1] CLA arrives at the foetus by transplacental transfer. [2] CLA arrives at pups through the dams' milk. [3] Total period of CLA supplementation from gestation until the end of suckling. [4] Total period of CLA supplementation from the day of birth until 1 wk after weaning.

1.2. Dietary CLA supplementation

The standard diet used in this study corresponded to the American Institute of Nutrition (AIN)-93G formulation, containing 7% soybean oil. The 1% CLA diet was obtained from modified standard flour AIN-513 (Harlan) containing 10 g CLA/kg [20]. Thus, the supplemented diet contained 6% soybean oil plus 1% CLA oil. The CLA isomer mixture was approximately 80% *c*9,*t*11 and 20% *t*10,*c*12 from the total CLA isomers in oil. This proportion has been chosen due to its resemblance to that one present in breast milk [7]. CLA oil was kindly supplied by Loders Croklaan (Lipid Nutrition, Wormerveer, The Netherlands). The 1% CLA diet in suckling animals corresponded to a daily administration of 1.5 mg CLA oil provided/g of rat body weight from day 1 to 21.

1.3. Relative CLA concentration in dams' milk

In order to confirm the CLA transfer from dams to pups through milk, we collected milk from dams on day 21 post-partum [20] and evaluated the content of *c*9,*t*11- and *t*10,*c*12-CLA isomers by fast gas chromatography, as previously described [25] (**Table 1**). The results showed that, at the end of suckling (day 21), milk from dams fed the CLA diet during gestation and suckling showed higher concentrations of the *c*9,*t*11- and *t*10,*c*12-CLA isomers than rats fed the standard diet. Moreover, the presence of a small quantity of *c*9,*t*11- and not of *t*10,*c*12-CLA in the milk of non-supplemented rats supports the concept that rats produce rumenic acid (*c*9,*t*11-CLA), as has been suggested by other authors, by conversion of free linoleic acid by the intestinal bacterial flora [26] or by the endogenous conversion of trans-vaccenic acid, present in vegetable oils contained in standard diets [27].

On the other hand, the proportion of these two isomers in milk, 86:14, varied from that supplemented to dams, 80:20 (**Table 1**). The lower proportion of *t*10,*c*12-CLA than that initially supplemented is probably due to the faster metabolism of this isomer. It has been reported that *t*10,*c*12-CLA activates the β-oxidation system more strongly than *c*9,*t*11-CLA in rats; therefore, the former could easily become oxidised [28].

Table 1. Relative content of *c*9,*t*11- and *t*10,*c*12-CLA isomers in dams' milk and in the plasma of 21- and 28-day-old pups (% total fatty acids).

	***c*9,*t*11-CLA**[1]	***t*10,*c*12-CLA**[1]
Dams milk: **standard diet**	0.02 ± 0.00	0.00 ± 0.00
Dams milk: **CLA diet**	2.93 ± 0.11**	0.46 ± 0.02**
Plasma: **21/5* group**	1.34 ± 0.01*$^{\sigma\varpi}$	0.21 ± 0.01*$^{\sigma\varpi}$
Plasma: **21/5 group**	1.78 ± 0.05*$^{\sigma}$	0.13 ± 0.01*$^{\sigma}$
Plasma: **21/3 group**	0.90 ± 0.01*	0.05 ± 0.00*
Plasma: **21/0 group**	0.15 ± 0.01	N.D.
Plasma: **28/4 group**	1.31 ± 0.03*$^{\phi\Psi}$	0.13 ± 0.01*$^{\phi\Psi}$
Plasma: **28/3 group**	0.44 ± 0.03*$^{\phi}$	0.05 ± 0.00*$^{\phi}$
Plasma: **28/1 group**	1.23 ± 0.03*	0.08 ± 0.00*
Plasma: **28/0 group**	0.15 ± 0.01	N.D.

[1] Values are expressed as mean ± SEM (n= 4-7 dams/group; n= 10 pups/group). N.D., non-detectable. Significant differences: **P<0.001 *vs.* standard diet group; *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3; $^{\varpi}$P<0.05 *vs.* 21/5; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

1.4. Relative CLA concentration in rat plasma

Once confirmed that the CLA from the dams' diet was able to be transferred through dams' milk to pups, the efficiency in the incorporation of such PUFA from milk or directly by p.o. was also evaluated in the pups [20,21]. Therefore, the content of *c*9,*t*11- and *t*10,*c*12-CLA isomers in the plasma of 21- and 28-day-old pups was quantified by fast gas chromatography (**Table 1**). Regarding the concentration of CLA in the plasma of pups determined on the day of weaning, pups from group 21/0 (Ref) showed a low plasma content of *c*9,*t*11-CLA and no *t*10,*c*12-CLA. Groups 21/5*, 21/5 and 21/3 had approximately nine, twelve and six times higher levels of *c*9,*t*11-CLA than group 21/0, respectively. Moreover, the plasma CLA content in the groups supplemented with the 80:20 CLA mix during gestation and suckling (21/5* and 21/5 groups; 5 wk) was ~ 1.5 and 2 times higher, respectively, than that of animals supplemented only during suckling (21/3 group; 3 wk) (**Table 1**).

These results demonstrate that CLA was absorbed by all the supplemented animals since *c*9,*t*11- and *t*10,*c*12-CLA isomers were detected in the plasma of all 21-day-old rats fed CLA. They also indicate that CLA, besides being incorporated after CLA p.o. supplementation, was also able to be transferred from dams to pups during suckling. However, these results cannot demonstrate that CLA transfers through the placenta, since the plasma of newborns was not analysed. Nevertheless, Chin *et al.* [29] found a 20-fold increase of CLA in the liver of foetuses (at day 20 of gestation) from dams fed a 0.5% CLA diet during gestation than in foetal livers coming from dams fed a control diet. This, together with our findings of a higher content of *c*9,*t*11- and *t*10,*c*12-CLA in the milk of rats fed CLA than in those fed the standard diet and the higher content of this isomer in 21/5 group plasma than in 21/3 group, confirms that CLA is transferred through the placenta to the foetus, as well as through the milk to the pup. The highest plasma CLA values in pups from 21/5 group may be due to CLA transfer during suckling from the maternal stores accumulated during gestation, besides supplementation by p.o. administration.

Similarly to that occurred in dams' milk after CLA intake, the supplemented proportion of *c*9,*t*11- and *t*10,*c*12-CLA (80:20) changed in all the groups (**Table 1**). The proportion of CLA isomers was 86:14, 93:7 and 94:6 for groups 21/5*, 21/5 and 21/3, respectively. Hence, group 21/5 presented a higher content of *c*9,*t*11-CLA, but a lower content of *t*10,*c*12-CLA than group 21/5*. Moreover, differences between 21/5* and 21/5 groups in the proportion of CLA isomers in pups' plasma may be due to the influence of the food matrix in 21/5* group, as milk is one of the major factors that affect PUFA bioavailability [30].

Regarding the concentration of CLA isomers quantified in plasma from 28-day-old animals (**Table 1**), plasma from reference rats (28/0 group) had no *t*10,*c*12-CLA and a low content of *c*9,*t*11-CLA, which were lower than those found in the rest of the groups. The animals who received CLA supplementation continuously during suckling and early infancy (28/4 group; 4 wk) showed the highest content of both CLA isomers among the groups. However, rats from the 28/3 group (3 wk) presented lower content of both CLA isomers in plasma than rats from the 28/1 group (1 wk). And, again, as showed for dams' milk and 21-day-old pups' plasma, the relative proportion between *c*9,*t*11- and *t*10,*c*12-CLA was higher in all CLA-supplemented groups (28/4, 91:9; 28/3, 90:10; 28/1, 94:6) than that of the original mixture (80:20).

These results match with the above data concerning 21-day-old animals and with other PUFA studies using similar experimental designs [31]. The highest content of CLA in plasma was achieved in the longest and continuous CLA supplementation (28/4 group), whereas the group that received CLA for 3 wk and then nothing for 1 wk (28/3 group) exhibited lower CLA plasma content than the 28/1 group which only received CLA for the last week. Previous interventional studies have shown that the proportion of fatty acids in the dietary supplement is highly correlated to that one found in plasma and membrane [32]. Therefore, we can suggest that in our study, just finishing the supplementation period, CLA plasma levels are an indicative measure of the CLA incorporated in the cell membranes, in our case for 28/4 and 28/1 groups. But this is not the case for the 28/3 group due to the time lapse between the last CLA intake and the day of quantification of CLA plasma content. The CLA membrane incorporation suggested here agrees with the study of Subbaiah *et al.* [33], which demonstrates that CLA is incorporated into cell membranes to varying extent, depending upon the experimental conditions.

On the other hand, the relative proportion of *c*9,*t*11/*t*10,*c*12 in the plasma of CLA-supplemented animals (~90:10) is higher than in the original isomer mixture (80:20) administered to the animals. These changes in proportions could be attributed to the isomer-differentially β-oxidation induction above commented *(see section 1.3)*. However, although it has been described that both isomers have similar absorption rates in adult rat intestine [34], it is possible that young rodents preferentially absorb the *c*9,*t*11-CLA isomer. Additionally, although no *t*10,*c*12-CLA was found in the plasma of reference animals (28/0), small quantities of *c*9,*t*11-CLA isomer were detected, which would indicate an endogenous production of *c*9,*t*11-CLA, as previously described [26], and, therefore, its influence on the *c*9,*t*11/*t*10,*c*12 ratio observed in plasma.

1.5. Immunoglobulin concentration in dams' milk

One of our objectives was to evaluate the modulation on the Ig response in pups due to CLA, either coming through the milk or directly by p.o. administration. However, it has been described that IgA and IgG have a local mammary gland production [35], and are absorbed by pups from the ingested milk through the intestinal mucosa and extended beyond this compartment. Therefore, to better dissect the direct effect of CLA on Ig production by pups, we first evaluated the CLA influence on IgA, IgG and IgM concentrations in dam's milk whey at the end of the suckling period [20].

As can be shown in **Figure 2**, the predominant Ig present in rat milk was IgG (~280 μg/mL), followed by IgA (~30 μg/mL) and finally IgM (~5 μg/mL). Dams had a milk concentration pattern of IgG > IgA > IgM, which agrees with that described by Dahlgren *et al.* [35]. This pattern was maintained in dams fed the CLA diet, although IgG and IgA concentrations, the main Ig isotypes in rat milk, were much higher in these rats. Specifically, dams fed the CLA diet during gestation and suckling increased the concentration of IgG and IgA, about 6- and 2-fold, respectively. Thus, rat milk from CLA fed dams not only transfers CLA to pups, but also high concentrations of antibodies.

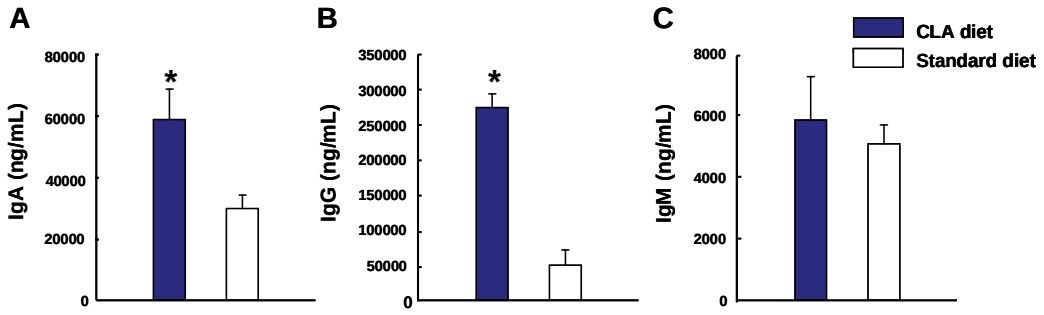

Figure 2. CLA effects on IgA (**A**), IgG (**B**) and IgM (**C**) of rat milk collected on day 21 of the suckling period. IgA, IgG and IgM concentrations were quantified by ELISA in milk whey supernatant fractions after centrifugation and fat layer discarding. Values are expressed as mean ± SEM (n= 4-7). Significant differences: *P<0.05 *vs.* standard diet. Modified of Ramírez-Santana *et al.* [20].

1.6. Effect of CLA on the development of the systemic immunity in rats

As had been mentioned before, this study evaluated the effect of CLA on the systemic immune response in Wistar rats. For that purpose, we quantified serum Ig concentrations, spleen cell proliferation and cytokine secretion ability and *ex vivo* splenocyte Ig production as biomarkers of systemic immune development [20,21].

1.6.1. Serum immunoglobulin concentration

This study evaluated the effect of feeding an 80:20 CLA isomer mixture during gestation and/or suckling and/or early infancy in the incipient antibody production of suckling and early infant rats. For that, serum IgG, IgM and IgA concentrations were quantified in 21- and 28-day-old animals from all the groups included in the exhaustive experimental design (**Fig. 3**).

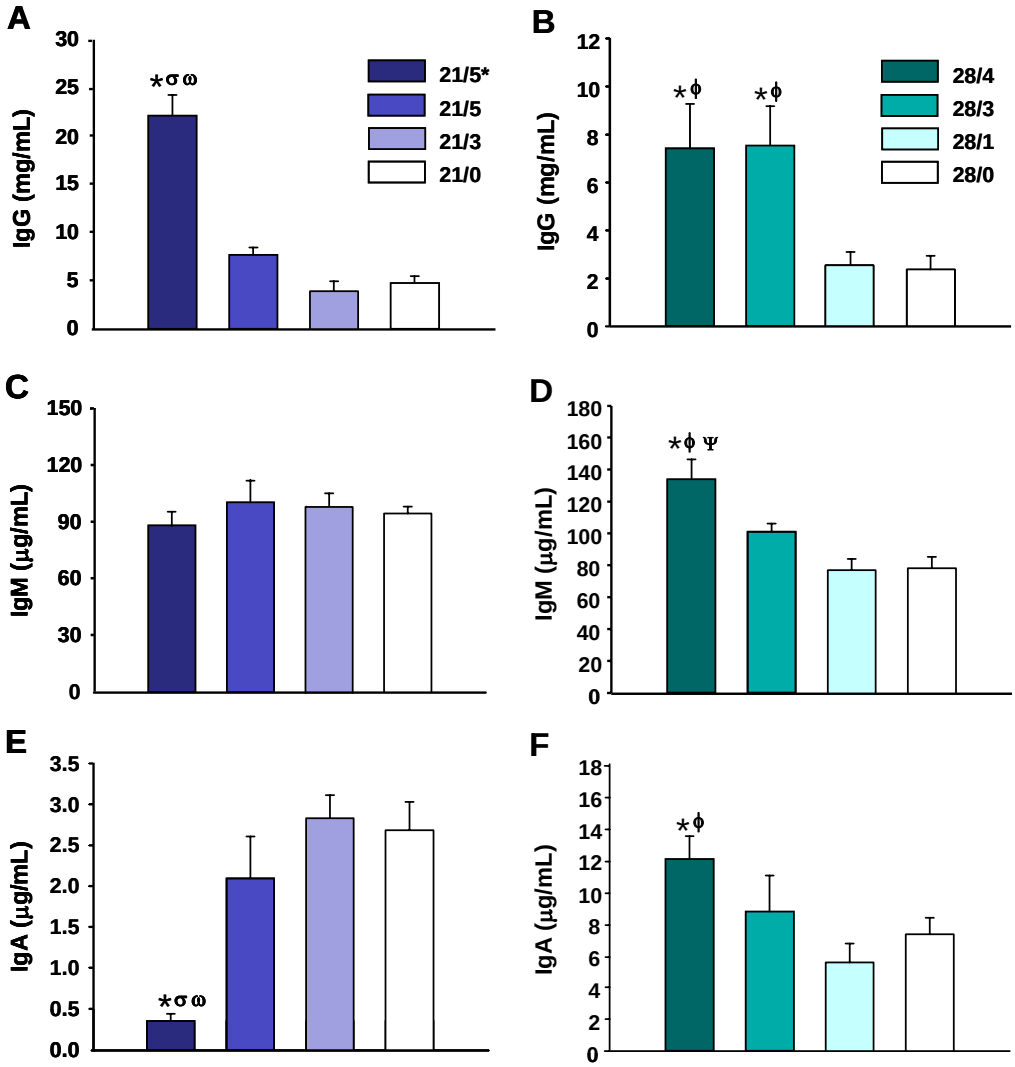

Figure 3. CLA effects on serum IgG, IgM and IgA from 21- and 28-day-old animals. Serum IgG (**A**, **B**), IgM (**C**, **D**) and IgA (**E**, **F**) concentrations were quantified by ELISA at the end of the suckling period (day 21; **A**, **C**, **E**) and 1 week after weaning (day 28; **B**, **D**, **F**). Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3; $^{\varpi}$P<0.05 *vs.* 21/5; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

Regarding the results obtained from the 21-day-old animals, those from the group 21/0 (Ref) showed ~5 mg/mL of IgG (**Fig. 3A**), ~95 μg/mL of IgM (**Fig. 3C**) and ~2.7 μg/mL of IgA (**Fig. 3E**). CLA supplementation for 5 wk, 2 wk during gestation and 3 wk during suckling through the dams' milk (21/5* group), increased the total Ig serum concentration almost 4-fold, mainly by enhancement of IgG. At this age, there were no differences in serum IgM concentration among the groups. However, 21/5* group exhibited a lower IgA serum concentration than those of the other groups.

With respect to 28-day-old animals, rats from the 28/0 group (Ref) had ~2 mg/mL of IgG (**Fig. 3B**), ~80 μg/mL of IgM (**Fig. 3D**) and ~8 μg/mL of IgA (**Fig. 3F**). Similarly to that observed in younger animals, those that received CLA diet during and after suckling (28/4 group) showed higher serum IgG, IgM and IgA concentrations than those receiving CLA only for 1 week after suckling (28/1) and rats from the 28/0 group. Moreover, rats from the 28/3 group, i.e., those receiving CLA diet only during suckling, showed 3 times the IgG concentration than those from the 28/1 and 28/0 groups. Considering total serum Ig, CLA supplementation during suckling (28/4 and 28/3 groups) increased the Ig concentration 4-fold compared to the 28/1 and 28/0 groups, mainly by increasing IgG.

All these results show the enhancing properties of CLA on the main *in vivo* serum Ig isotype, IgG. However, this effect was not observed in all the supplemented groups, a fact that underlines the importance of continuous CLA supplementation during gestation and suckling (21 day assessment), or during suckling and early infancy (28 day assessment). This immuno-enhancing effect has already been reported in older animals, as by Sugano *et al.* [15] in 7-week-old rats receiving 1% CLA (50:50 isomer mix), showing an increase in serum IgA, IgG and IgM concentrations and a decrease in IgE. Song *et al.* [36] reported a similar effect in human subjects following supplementation with a 50:50 isomer mixture for 12 wk. Nevertheless, Yamasaki *et al.* [37] found no significant effect on serum IgA, IgG or IgM concentrations after feeding 5-week-old rats for 3 wk with a 50:50 CLA isomer mixture at doses ranging from 0.05 to 0.5%. Discrepancies with the study reported by Yamasaki *et al.* [37] might be due to the low doses of CLA tested in that study. Studies carried out in other species during gestation and lactation periods have also reported serum IgG increases [38,39], in keeping with the present results.

With respect to the serum IgA decrease observed in the present study after CLA supplementation during gestation and suckling, Turpeinen *et al.* [40] also detected an IgA reduction in subjects with birch pollen allergy supplemented for 12 wk with a CLA mixture containing 63,5% of *c*9,*t*11.

Yamasaki *et al.* [37] found a slightly lower concentration of serum IgA after feeding the 0.5% CLA dose, although the reduction was not significant. Considering that IgA is the main Ig in the gut surface (80–90%) and the fact that systemic IgA-plasma cells continuously migrate to the intestinal wall [41], the serum IgA decrease should not be interpreted as harmful. In fact, this serum IgA decrease is accompanied by an increase in intestinal IgA in CLA-supplemented weaned rats [24; *see section 1.7.1.*]. We also have reported an increase in mucosal IgA after a specific challenge in adult rats following this CLA diet through life [42; *see section 2.3.*].

1.6.2. *Ex vivo* spleen immunoglobulin production

The CLA immunoenhancing effects on antibody synthesis, observed upon the supplementation of *c*9,*t*11- and *t*10,*c*12-CLA isomers at 80:20 ratio *in vivo* in suckling and infant rats, were also studied *ex vivo.* With this purpose, IgM and IgG production by unstimulated spleen cells, from animals corresponding of all groups, were quantified after 7 days of culture [20,21].

The spontaneous IgM and IgG production by splenocytes from 21-day-old rats showed that IgM was the main isotype found in supernatant fractions (**Fig. 4A**), being forty times higher than IgG (**Fig. 4B**). Regarding CLA supplementation, IgM production from both groups supplemented for 5 wk (21/5* and 21/5 groups, ~900–1200 ng/mL) was higher than that of the groups supplemented for 3 wk (21/3 group) and 0 wk (21/0 group) (both ~500 ng/mL). However, this increase was only significant when pups received CLA during gestation and suckling by p.o. administration (21/5 group) (**Fig. 4A**). Otherwise, IgG production was very low (~10 ng/mL) and no differences were found among groups (**Fig. 4B**). Thus, continuous CLA supplementation during gestation and suckling enhances splenocyte IgM production, increasing total Ig concentration by almost 2-fold that of non-supplemented animals.

With respect to 28-day-old assessment, IgM was also the predominant isotype found in supernatants (**Fig. 4C**), being ~100 times higher than IgG production (**Fig. 4D**). The groups supplemented with CLA during suckling (28/4 and 28/3 groups) synthesised higher IgM and IgG levels than the reference group (28/0), but it was only significant in the 28/3 group. This fact indicates the lasting of the CLA effect on the Ig synthesis later in life, or at least 1 week after finishing the CLA intake. Spleen Ig production from the 28/1 and 28/0 groups was similar.

Overall, in the present study, CLA supplementation in early life enhanced spleen IgM production. These results agree with other studies carried out in older rats, which reported enhancement of splenocyte Ig

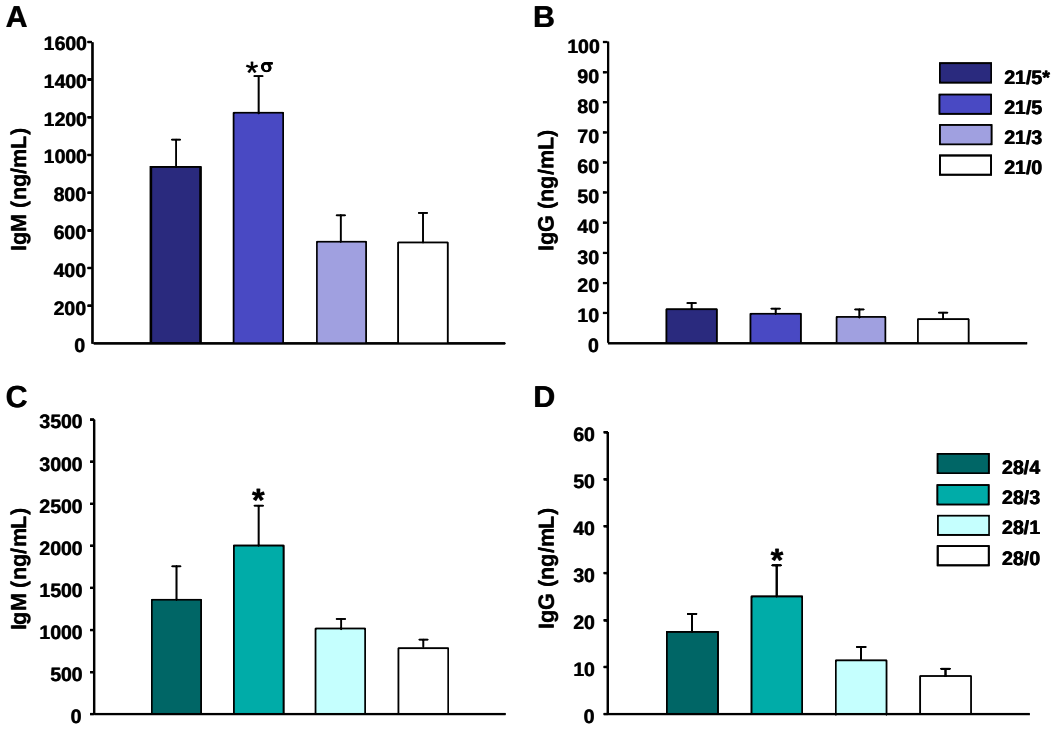

Figure 4. CLA effects on spleen cell Ig production from 21- and 28-day-old animals. IgM (**A**, **C**) and IgG (**B**, **D**) concentrations in supernatants after 7 days of spleen cell culture were quantified by ELISA at the end of the suckling period (day 21; **A**, **C**) and 1 week after weaning (day 28; **B**, **D**). Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3. Modified of Ramírez-Santana *et al.* [20,21].

production after feeding 50:50 CLA isomer mixtures [15,37]. In addition, some authors have reported this increase in old mice after feeding the pure *t*10,*c*12-CLA isomer [43]. Although the present results confirmed the immunoenhancing effect of the *c*9,*t*11 isomer, we cannot rule out immune functions for *t*10,*c*12-CLA.

1.6.3. Spleen lymphocyte functionality: Proliferation and cytokine secretion

Besides the capacity of *in vivo* Ig production, the influence of dietary CLA supplementation on the functionality of *ex vivo* isolated spleen lymphocytes was also evaluated by their ability to proliferate and to produce cytokines.

At the end of suckling (21-day-old assessment), the CLA diet did not modify the lymphoproliferative capacity in any group (21/5*, 21/5, 21/3), measured 72 h after mitogen-stimulation when compared to reference animals (21/0) (**Fig. 5A**). However, in the assessment of older animals (1 wk after weaning), CLA supplementation during suckling (28/4 and 28/3 groups)

reduced *ex vivo* spleen lymphoproliferative ability compared to that in the 28/1 and 28/0 groups, but it was only significant for the 28/4 group (**Fig. 5B**). Cell viability was slightly reduced after mitogen-stimulation, but, this decrease did not differ among groups in 21-day-old evaluation, indicating that CLA dietary supplementation had no effect on splenocyte viability. On the other hand, CLA supplementation effect on 28-day-old animals conferred more resistance to the mitogen toxic effects, as is demonstrated by the lowest mortality of 28/4 group among the groups. Thus, the decrease in the proliferation rate cannot be attributed to a lower viability caused by CLA diet.

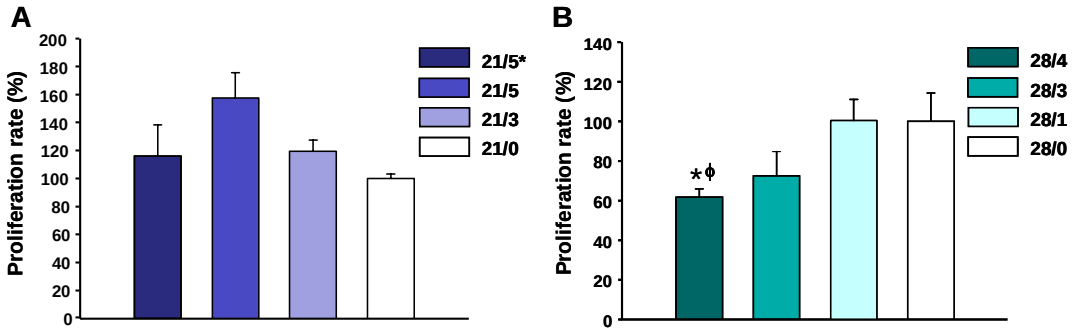

Figure 5. CLA effects on proliferation rate in mitogen-stimulated spleen cells from 21- and 28-day-old animals. Lymphoproliferative response was determined after stimulating with phorbol myristate acetate (PMA) plus ionomycin (Io) for 72 h at the end of the suckling period (day 21; **A**) and 1 week after weaning (day 28; **B**). The proliferation rate (%) are expressed relative to the age-matched reference group (21/0 or 28/0), which was set at 100%. Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\phi}$P<0.05 *vs.* 28/1. Modified of Ramírez-Santana *et al.* [20,21].

Moreover, interleukin (IL)-2 production, the main proliferative signal for lymphocytes, was measured in supernatant fractions obtained after 24 h of spleen cell stimulation and was not modified by CLA supplementation either in 21- or 28-day-old assessment (**Table 2**).

The lack of CLA dietary effect on lymphocyte proliferation found in 21-day-old rats is in agreement with the results reported by Kelley *et al.* [16] in 8-week-old mice after feeding pure isomers of CLA for 56 days, and in human subjects either by ingestion of pure isomers [44] or 50:50 and 80:20 isomer mixtures [16,45]. Since IL-2 plays a central role in the cell-mediated immune response by regulating proliferative abilities, it could be expected that if CLA does not modify splenocyte proliferation, it will not affect IL-2 production. The present results are consistent with most other studies in animals and human subjects, which have found no significant effects on IL-2 splenocyte secretion among the dietary groups [43,44,46].

Table 2. Content of cytokines in 24 h splenocyte supernatants after mitogen (PMA/Io) stimulation[1].

Cytokines (day 21)	21/5* group	21/5 group	21/3 group	21/0 group
IL-2 (ng/mL)	4.1 ± 1.1	4.1 ± 1.0	5.6 ± 0.9	4.1 ± 0.8
IFNγ (ng/mL)	4.6 ± 0.8	5.9 ± 1.0	6.4 ± 1.4	3.7 ± 0.8
IL-4 (pg/mL)	29.3 ± 7.9	32.4 ± 6.0 $^{(*)}$	15.9 ± 1.3	12.4 ± 1.8
IL-10 (pg/mL)	181.7 ± 47.2	176.3 ± 43.8	90.6 ± 8.5	108.0 ± 19.6
Cytokines (day 28)	**28/4 group**	**28/3 group**	**28/1 group**	**28/0 group**
IL-2 (ng/mL)	6.2 ± 0.8	6.1 ± 0.8	7.2 ± 1.1	6.8 ± 1.2
IFNγ (ng/mL)	10.9 ± 1.4	7.1 ± 0.9	7.3 ± 0.8	6.0 ± 1.3
IL-4 (pg/mL)	27.8 ± 4.0	18.8 ± 2.8	28.7 ± 7.4	17.3 ± 2.4
IL-10 (pg/mL)	244.4 ± 41.0	224.9 ± 29.2	222.4 ± 34.5	191.6 ± 24.2
IL-6 (pg/mL)	219.0 ± 38.0*$^{\phi\Psi}$	40.1 ± 12.9	45.7 ± 15.1	N.D.

[1] Values are expressed as mean ± SEM (n= 10). N.D., non-detectable. Significant differences: $^{(*)}$ P=0.06 *vs.* reference group (21/0); *P<0.05 *vs.* reference group (28/0); $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

Despite the above results, a wide range of PUFA, including CLA, have been found to reduce the mitogen-stimulated proliferation of lymphocytes isolated from several species [47,48]. Thus, the lack of effect observed in 21-day-old animals, may be probably due to the immaturity of this function (which is acquired later in life) at a very early age [49]. In contrast, the mitogen-induced splenocyte proliferation on 28-day-old animals was down-modulated in the longer-lasting CLA supplementation, similarly to that described in adults [42,50,51]. Thus, the age of 28 days is optimal to evaluate CLA effects, since at this age CLA still increases antibody production and is also able to down-modulate lymphoproliferation. Calder and Newsholme [52] reported that some PUFA inhibited lymphocyte proliferation without decreasing IL-2 concentration, a fact that is consistent with the present results showing that CLA supplementation did not modify IL-2 production. In this sense, many studies have described conformation changes in IL-2 receptors by PUFA, specifically modifying lipid rafts [53,54]. Thus, CLA, even with trans double bonds, could potentially alter membrane structure, including lipid rafts, by preventing IL-2Rα migration to soluble membranes, where IL-2 signalling occurs and T-cell activation and proliferation are consequently induced [53,55,56]. In addition, the anti-proliferative lymphocyte effect exhibited by dietary CLA in the present study could be mediated by the nuclear peroxisome proliferator-activated receptor (PPAR)γ,

because intestinal PPARγ gene expression has been found up-regulated after CLA supplementation *(see section 1.7.2.)* [24]. The PPARγ-dependent CLA effect was first described by Bassaganya-Riera and Hontecillas [57] in a pig--inflammatory bowel disease model, where dietary CLA resulted in intestinal disease amelioration and PPARγ gene expression up-regulation.

Besides IL-2 quantification, in the present study other cytokines were also analyzed in splenocyte supernatants after mitogen-stimulation (**Table 2**). Interferon (IFN)-γ, a T helper 1 (Th1) cytokine, was also secreted in similar amounts in all experimental groups. T helper 2 (Th2) cytokines, IL-4 and IL-10, were also quantified in the same splenocyte supernatant fractions and although no statistical differences were found among groups, due to the large intra-group variability, rats from 21/5* and 21/5 groups showed almost 2-fold higher values than those observed in 21/3 and 21/0 groups (**Table 2**). This IL-10 increase is in line with studies showing higher IL-10 production by dendritic cells incubated with *c*9,*t*11-CLA after stimulating with lipopolysaccharide [58]. This effect may be related to the anti-inflammatory properties attributed to CLA [22,59]. Moreover, by increasing IL-4, CLA might be promoting T helper 2 (Th2) responses, such as modulating antibody production and inhibiting several cellular functions, which is in agreement with the present results regarding CLA enhancement of the principal *in vivo* and *in vitro* Ig.

However, in 28-day-old animals, the concentration of IL-6, which was also included in the study, was increased in the 28/4 group compared to the 28/3 and 28/1 groups, while IL-6 was not detected in the 28/0 group (**Table 2**). This result is of interest because IL-6 is clearly defined as a prominent regulator of T-cell proliferation and differentiation of Ig-secreting B cells [60]. Therefore, it might be suggested that the effects on proliferation -in early infant rats- and antibody production -in both suckling and early infant rats- induced by CLA could be due to an up-regulation of IL-6 production. Further studies should confirm this hypothesis and ascertain the mechanism involved.

1.7. Effect of CLA on the development of the mucosal immunity in rats

As it had been mentioned before, this study also evaluated the effect of CLA-supplementation during gestation, suckling, and/or early infancy on mucosal immunity (small intestine and colon) in Wistar rats. In these periods, their mucosal immune response is still in development. In fact, the mucosal immune system of the rat continues developing during the suckling period and early infancy, and, as occurs in humans, mucosal Ig production is poor. Previous studies have shown that IgM production by lamina propia cells

begins during the second week of life in parallel to the phenotypic development of B cells in rat intestine, and later, weaker production of IgA initiates [61]. For that reason, in this study we quantified intestinal IgA at both the gene and protein levels as a biomarker of mucosal immune development, and TGFβ and PPARγ gene expression as possible mediators of CLA's immunomodulatory effects [24]. Finally, a broad array of genes was studied in that compartment to elucidate other mediators in CLA action.

1.7.1. Effect of CLA on IgA gene expression and IgA protein secretion in intestinal mucosa

IgA gene expression in small intestine and colon was assessed in 21- and 28-day-old animals from all experimental groups. Dietary CLA did not modify IgA gene expression in small intestine or colon at the end of the suckling period. However, IgA gene expression in animals continuously CLA-supplemented during suckling and early infancy (28/4 group; 4 wk) was up-regulated almost 5-fold (**Fig. 6A, 6B**). This increase was seen in both tissues analyzed as compared with the reference group (28/0). Supplementation limited to the suckling or early infancy periods failed to produce this immunoenhancing effect (**Fig. 6A, 6B**).

In addition to detection of changes in gene expression, IgA protein concentration was quantified in intestinal washes of 28-day-old animals (**Fig. 7**). IgA content was statistically higher in animals CLA-supplemented for 4 weeks (28/4 group) than in animals in the 28/3, 28/1 or 28/0. These results demonstrate that continuous CLA dietary supplementation for a longer period, during suckling and early infancy, increases expression of the IgA

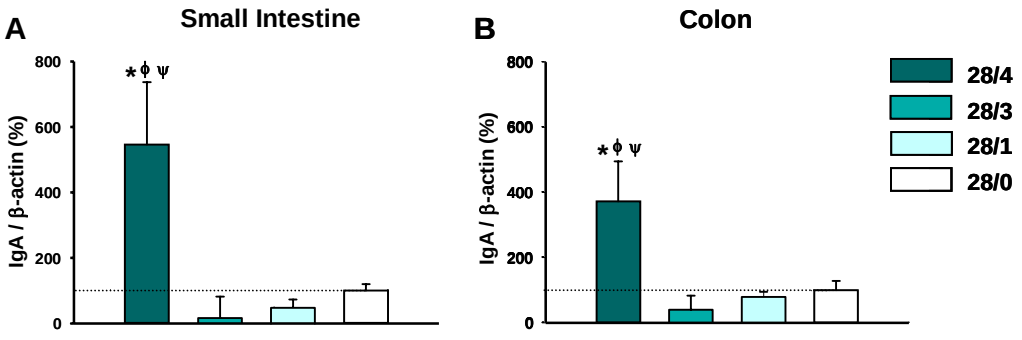

Figure 6. Effect of CLA on IgA gene expression in 28-day-old animals. Gene expression was evaluated in small intestine (**A**) and colon (**B**) by Real Time PCR. IgA gene expression was normalized using β-actin and are showed as percentage relative to values in age-matched reference animals (21/0 or 28/0). Values are expressed as mean ± SEM (n= 5). Significant differences: *P<0.05 *vs.* reference group (28/0); $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Pérez-Cano *et al.* [24].

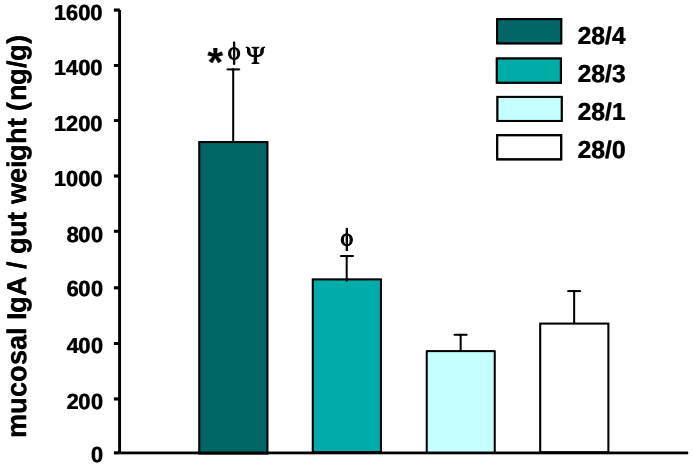

Figure 7. Effect of CLA on IgA in intestinal washes from 28-day-old rats. Secretory IgA was quantified in intestinal washes of small intestine by ELISA. Results are expressed as IgA protein (ng) referred to intestinal weight (g) used for the wash. Values are expressed as mean ± SEM (n= 9-10). Significant differences: *P<0.05 *vs.* 28/0; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Pérez-Cano *et al.* [24].

gene and protein, thereby enhancing development of the rat's mucosal defense system. This is the first *in vivo* report, to our knowledge, showing an increase of mucosal IgA after feeding CLA during early life [24]. In line with our data, Sugano *et al.* [15] reported an increase in IgA secretion from cultured mesenteric lymph nodes of 7-week-old rats, fed a 1% CLA 50:50 isomer mix.

The specific mechanism by which CLA enhances IgA levels at mucosal sites remains unknown. But since CLA has been shown to suppress IL-4 production *in vitro* [62], attenuate Th2 responses in challenged animals [63], and regulate the number and effectors functions of several lymphocytes [64], further studies should be addressed to elucidate whether it exist a direct enhancer mechanism of CLA on IgA-producing cells.

1.7.2. Effect of CLA on TGF-β and PPARγ gene expression in small intestine and colon

Because IgA gene expression and intestinal production were increased after feeding CLA, we also studied molecules which could have a role in this process. In this sense, transforming growth factor (TGF)β gene expression was a candidate due to its involvement in the isotype switching process from IgM to IgA [65,66]. However, intestinal TGFβ gene expression was not modified in any of the CLA-supplemented groups during gestation, suckling and/or early infancy. Nonetheless, an influence of CLA on TGFβ cannot be

ruled out. If CLA is modulating the effects of TGFβ on IgA production, it is probably due to posttranscriptional and/or translational regulation, which are important in this cytokine, because it has been suggested that TGFβ mRNA levels do not completely correlate with the quantity of protein produced [67]. On the other hand, the increase of IgA as result of CLA supplementation might be independent of the isotype switching mechanism produced by TGFβ, which has been described, but is not completely defined [68].

On the other hand, two main mechanistic theories have been proposed to explain the immunoenhancing effects of dietary CLA: a PPARγ-dependent and a PPARγ-independent pathway [69]. The present study investigates PPARγ gene expression in the small intestine and colon of 21- and 28-day-old animals fed standard and CLA-supplemented diets (**Fig. 8**). At both ages, there were no differences in PPARγ gene expression in small intestine between CLA and non-supplemented groups. However, PPARγ was up-regulated in colon tissue, particularly in 21-day-old animals fed CLA (21/5*, 21/5, and 21/3 groups), when compared with reference animals (21/0) (**Fig. 8A**). This 2-fold up-regulation was only significant in the 21/5* group. Similar to the results found in 21-day-old animals, colon PPARγ gene expression was also up-regulated in 28-day-old animals fed CLA (28/4, 28/3, and 28/1), when compared with reference animals (28/0) (**Fig. 8B**). Nevertheless, only the 28/3 and 28/1 groups showed statistical differences, since 28/4 group had a great variability. Overall, CLA-supplemented rats showed higher PPARγ expression than non-supplemented animals. Specifically, the effects seem to be related to a

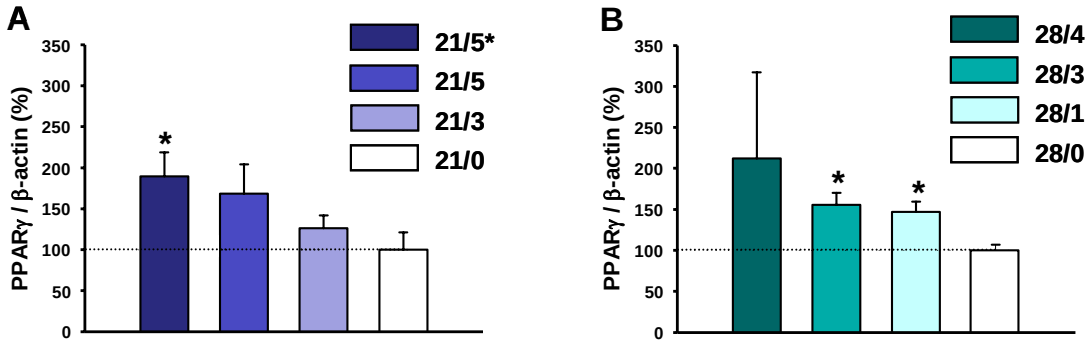

Figure 8. Effect of CLA on colon PPARγ gene expression in 21- and 28-day-old animals. Gene expression was evaluated by Real Time PCR at the end of the suckling period (day 21; **A**) and 1 week after weaning (day 28; **B**). PPARγ gene levels were normalized using the β-actin gene and are expressed as percentage relative to values from age-matched reference animals (21/0 or 28/0). Values are expressed as mean ± SEM (n= 5). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0). Modified of Pérez-Cano *et al.* [24].

dose-dependent manner that was proportional to the duration of supplementation over gestation, suckling, or early infancy. Thus, CLA modulated PPARγ expression in all the dietary conditions examined, even when animals were supplemented for only 1 week.

These results concur with findings from studies showing an increase of PPARγ mRNA expression associated with CLA supplementation in colon of healthy and ill mice and pigs [22,57,63,69]. Moreover, PPARγ up-regulation by CLA is in line with the results of Takamura *et al.* [70], who showed that specific natural or synthetic ligands of PPARγ can induce a mean 2- to 3-fold expression of this receptor in a positive feedback loop. *In vitro* studies have also indicated that the PPARγ activating capabilities of CLA are cell type-dependent and isomer specific [69].

PPARγ comprises two isoforms, PPARγ1 and PPARγ2. Both are expressed in adipocytes, but PPARγ1 is expressed in T and B cells, monocytes, dendritic cells, and epithelial cells [71,72]. Hence, the effects of CLA found in the present study may be due to the interaction of CLA with PPARγ1. There are several possible options through which CLA might act. First, although a direct relationship between PPARγ increase and IgA gene expression has not been described, Ponferrada *et al.* [73] reported that PPARγ agonists can revert stress-induced decrease of IgA production in the colon mucosa, even beyond the IgA-controlled basal concentration. Moreover, it seems that this nuclear receptor acts through modulation of transcriptional factors such as NF-κB, AP1, and STAT1 [74,75], which are involved in B-cell regulatory processes. Second, recent research has also indicated close links between intestinal-microbial interactions and regulation of PPARγ expression by epithelial cells of colon tissue [76], suggesting that CLA may influence the natural mechanisms involved in intestinal homeostasis regulation. Lastly, it has been demonstrated that PPARγ regulates the epithelial differentiation process [77]. Thus, CLA may be modulating the entry of luminal antigen, the capacity for direct antigenic presentation, or even the transmission of antigen to dendritic cells from the intestinal mucosa. These hypotheses are supported by the fact that dendritic cell immunogenicity is regulated by PPARγ [78].

1.7.3. Effect of CLA on rat mesenteric lymph nodes gene expression profiles

The data reported in above *sections 1.7.1.* and *1.7.2.* provide scientific evidence of the impact of lipid nutrition, particularly the influence of the *c*9,*t*11-CLA isomer, on mucosal immunomodulation. On the other hand, it is known that food components play a role in influencing, either directly or

indirectly (through hormonal regulation), the expression of genes involved in immune responses [79]. Thus, further studies were developed to define the mechanism of action CLA at genic level in the intestinal compartment [80].

For that purpose, the gene expression profile in animals supplemented with CLA during gestation and suckling of mesenteric lymph nodes, sites of activation and proliferation of lymphocytes coming from the intestinal tissue (i.e. an inductor site), was determined. The specific GeneChip® Rat Genome 230 2.0 (Affymettrix) and bioinformatic analyses (GeneSpring GX software) were used. It led to the identification of 123 genes differentially expressed in all CLA dietary approaches with respect to the reference group. Generation of a biological association network evidenced several genes, such as connective tissue growth factor (Ctgf), tissue inhibitor of metalloproteinase 1 (Timp1), galanin (Gal), synaptotagmin 1 (Syt1), growth factor receptor bound protein 2 (Grb2), actin gamma 2 (Actg2) and smooth muscle alpha actin (Acta2), as highly interconnected nodes of the resulting network [80]. All these genes modulated by CLA supplementation may have a role on lymphoproliferation and mucosal immune responses in early life.

2. Long-term feeding of CLA from gestation to adulthood: Effect on the immune system in adult rats

As previous studies have suggested that CLA intake during developmental phases may have effects later in life [22,23], this second part of the study was performed from gestation to adulthood. The aim was to ascertain whether the capacity to produce a specific immune response in ovalbumin (OVA)-sensitized adult rats is influenced by long-term feeding of an enriched diet containing an 80:20 CLA isomer mix of *c*9,*t*11- and *t*10,*c*12-CLA, respectively [42].

2.1. Experimental design

Pregnant Wistar rats at 7 days' gestation were assigned to 1 of the 2 dietary groups and after delivery, litters were kept with their dams until weaning (day 21). Thereafter, pups consumed the same diet as their mothers. The 2 dietary groups were the CLA group and the reference (REF) group (**Fig. 9**). The CLA group was constituted by rats whose dams were fed a 1% CLA diet *(see section 1.2.)* during gestation (2 wk) and suckling (3 wk); pups received CLA through the placenta and milk, respectively. From weaning until the end of the study (15-wk-old rats), animals were also fed 1% CLA diet. The total period of supplementation was 17 wk. Rats from the reference group were fed a standard diet throughout the 17 wk of study. Nine-week-old

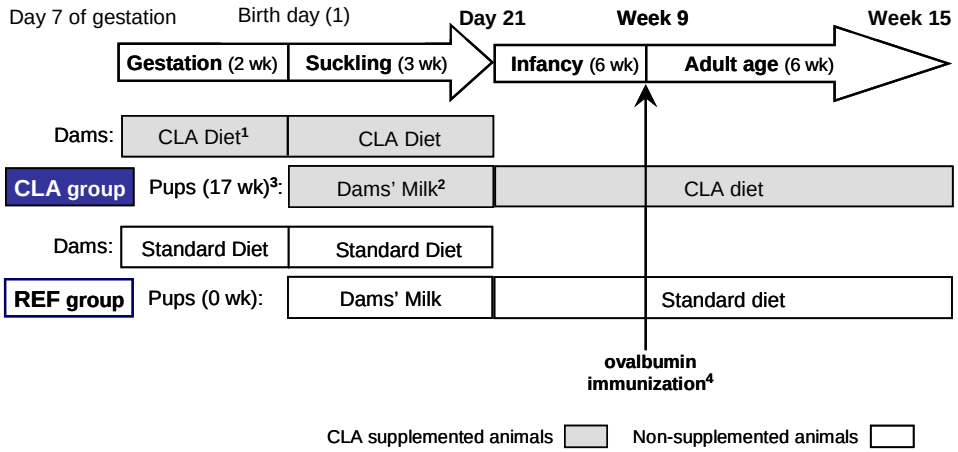

Figure 9. Diagram of the experimental design beginning on day 7 of gestation until week 15 of life. [1] CLA arrives at the foetus by transplacental transfer. [2] CLA arrives at pups through the dams' milk of dams. [3] Total period of CLA supplementation from gestation until 15 weeks of age. [4] Ovalbumin immunization by i.p. injection to 9-week-old rats.

rats from both groups were immunized with ovalbumin (OVA) emulsified with alum adjuvant by intraperitoneal (i.p.) injection. Six weeks after immunization, 15-wk-old rats were sacrificed and immune status was assessed (**Fig. 9**).

2.2. Effect of CLA on the polyclonal and antigen-specific cell immune response

Although the main goal was to examine whether a long-term CLA diet was able to modulate the capacity to generate an antigen (Ag)-specific cell immune response, the *ex vivo* capacity to generate a mitogen-induced immune response was also evaluated in isolated spleen lymphocytes by means of their ability to proliferate and to secrete IL-2 [42]. The **Figure 10A** shows that spleen cells from rats fed CLA throughout the study (17 wk) had a ~10% lower proliferative response than reference rats after mitogen-stimulation. This down-regulatory effect by dietary CLA was not due to cell viability loss, because viability from the CLA after mitogen addition was comparable to that of reference cells. Secretion of IL-2 was also lower in cell cultures of CLA-fed rats than in those of rats fed the standard diet (**Fig. 10B**). These results agree with those found in the 28-day-old assessment *(see section 1.6.3.)* and those of Tricon *et al.* [44], who showed that peripheral blood mononuclear cells from subjects fed either *c*9,*t*11- or *t*10,*c*12-CLA isomers, after mitogen-stimulation, decreased CD69 expression, which strongly correlates with lymphocyte proliferation. However, there are other studies using diverse CLA isomer mixtures that described either increased splenocyte proliferation or no effect after stimulus addition [45,47,81].

Regarding the long-term effects of dietary CLA supplementation on Ag-specific immune response, the lymphoproliferative capacity after OVA addition was evaluated. Reference and CLA groups had a higher (2- to 3--fold) splenocyte proliferation after OVA stimulation than unstimulated cells and after control protein addition (**Fig. 11**). In terms of specific proliferative response, splenocytes recovered from OVA-immunized rats fed CLA had

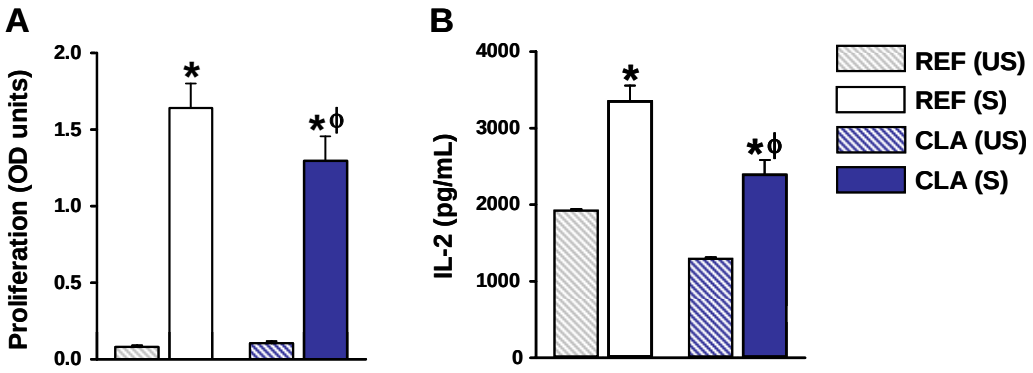

Figure 10. Proliferative response (**A**), and IL-2 production (**B**) of unstimulated (US) and mitogen-stimulated (S) spleen cells from rats fed a CLA or standard diet (CLA or REF groups). Polyclonal proliferative response was determined after stimulating with PMA/Io for 72 h. IL-2 was quantified by ELISA in 24-h supernatant cultures. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* US cells within the same dietary group; $^{\phi}$P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

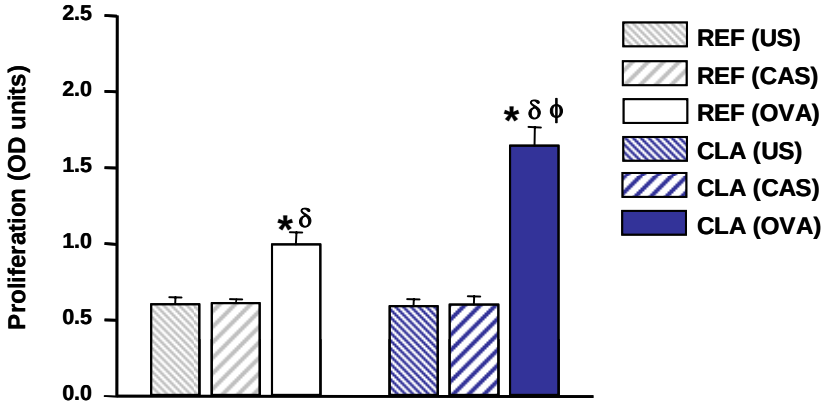

Figure 11. OVA-specific proliferative response of spleen cells from rats fed the CLA or the standard diet (CLA or REF groups). Specific response was determined after OVA stimulation for 96 h. Control protein (casein, CAS) and only medium (unstimulated cells, US) were used as negative reference controls. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* US cells within a diet group; $^{\delta}$P<0.05 *vs.* CAS-stimulated cells within a diet group. $^{\phi}$P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

higher (~275%) lymphoproliferative response to OVA than splenocytes recovered from OVA-immunized rats fed the standard diet (~165%). The OVA-specific splenocyte proliferation enhancement by CLA found here agrees with that reporting a higher specific proliferative response of T CD8+ lymphocytes from pigs fed a CLA diet (~50:50 isomers mix) [50,51]. In addition, following hepatitis B vaccination, specific lymphocyte proliferation was higher in humans fed CLA 50:50 than in the control group [45]. Conversely, Kelley *et al.* [82] showed no effect on influenza-specific proliferation in humans after feeding CLA, but in this case, the two main isomers used contributed only 40% of total CLA isomers, whereas in most of the studies affecting proliferative response, the main isomers made up ~80% of all CLA isomers.

2.3. Effect of CLA on the Ag-specific systemic and mucosal humoral immune responses

To ascertain long-term CLA diet effects on systemic humoral immune response, we have quantified serum OVA-specific antibody concentration, *ex vivo* spleen anti-OVA antibody production, and spleen anti-OVA-antibody-secreting cells (SC) number. Long-term dietary CLA did not modify the humoral immune response against the OVA-specific challenge either in serum or in splenocyte supernatants. However, using enzyme-linked immunosorbent spot technique (ELISPOT) we counted spontaneous anti-OVA IgG-, IgM-, and IgA-SC in spleens and we found that OVA-immunized rats had more spleen anti-OVA IgA-SC in CLA-fed rats (15.6 ± 3.5; mean ± SEM) than in reference group (11.9 ± 1.9). These results show overall that, although OVA-primed spleen B cells produced specific anti-OVA antibodies after later OVA contact, rats fed a CLA diet did not generate a higher systemic (serum and spleen) humoral response against OVA. This might suggest that the presence of 1% CLA in the diet increased neither the number of primed memory B cells nor their ability to produce specific antibodies. Our results agree with others carried out in humans and animals fed CLA [51,82,83], although Albers *et al.* [45] showed a higher concentration of anti-B hepatitis antibodies in subjects consuming CLA 50:50 capsules. On the other hand, CLA feeding did not modify total serum Ig concentrations. This result agrees with many others [16,81,82] but disagrees with a human study that reported increased IgM and IgA plasma concentrations after consuming CLA [36]. Nevertheless, better humoral enhancing effects were observed in developing states *(see section 1.6.)*, although specific adaptive responses were not addressed in such studies [20,21].

Regarding mucosal sites, we found interesting CLA results in this particular immune compartment: the dietary CLA modulated mucosal IgA production. Long-term dietary CLA increased the anti-OVA IgA synthesis in the intestinal mucosa ~75% (**Fig. 12**), although CLA did not modify total gut IgA. These data suggest that the CLA diet had a restricted enhancement effect on OVA-specific IgA intestinal production but not a general effect on humoral immunity.

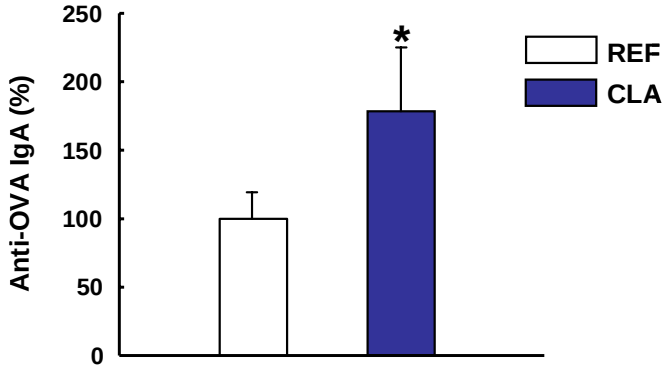

Figure 12. OVA-specific IgA in intestinal washes from rats fed the CLA or the standard diet (CLA or REF groups). Six weeks after immunization, secretory IgA was quantified in intestinal washes of small intestine by ELISA. Results are expressed relative to the reference group, which was set at 100%. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

The boost of specific intestinal IgA is of great importance, because this Ig is the main isotype present in all mucosa and confers high protection against foreign substances and microbe entry through the intestine, as well as by other mucosal compartments, due to specific secretory IgA homing among mucosal sites [84]. Thus, to our knowledge, this is the first time that a CLA supplementation enhancement of Ag-specific mucosal responses has been reported.

Because the CLA diet increased only intestinal-specific IgA, but not spleen or serum antibodies, it is plausible to suggest that CLA may be enhancing B cells present in the lamina propria or even promoting the IgA-secreting cell migration to the intestine from other immune compartments. This particular type of immunoenhancement induced by CLA, acting on a specific cell subset, is likely, because Bassaganya-Riera *et al.* [64] reported a higher percentage of a particular immune cell subset, but not of others, in swine fed CLA.

3. Conclusions

The data reported in this chapter contribute to the scientific evidence pointing to the potential impact of lipid nutrition on immune system development during early life, particularly the effect of the *c*9,*t*11-CLA isomer, the main CLA isomer present in breast milk. Although further studies should be carried out to elucidate CLA signalling mechanisms, the results presented herein by the dietary supplementation with an 80:20 *c*9,*t*11:*t*10,*c*12 CLA mix demonstrate that:

- CLA is transferred from the dams' diet to breast milk and later is efficiently incorporated by their pups. This supplementation also increases dam's milk IgG and IgA concentrations.
- The capacity of immune cells to synthesise antibodies, i.e. humoral immune response, is enhanced by CLA during gestation, suckling and/or early infancy. It is demonstrated, *in vivo* and *ex vivo*, by the increase of serum IgG concentration and spleen IgM production, respectively, in CLA fed animals.
- CLA supplementation during suckling and early infancy down-regulates the systemic lymphoproliferative response and increases the secretion of some Th2 cytokines during early age.
- CLA increases the intestinal immune defenses of rats during the first stages of life. CLA-dependent enhancement of humoral mucosal immune response was demonstrated by the striking increase of intestinal IgA expression in early infant rats fed CLA along life. Moreover, PPARγ gene expression levels were up-regulated in a supplementation period-dependent manner.
- It is clearly shown that the effects of CLA are more pronounced the earlier and more long-lasting CLA dietary supplementation. Specifically, the importance of the continuous supplementation during gestation-suckling or suckling-early infancy is evidenced by the observation of some immunomodulatory effects only produced when CLA is received during these periods.
- CLA diet administered from gestation to adulthood enhances specific systemic cell-mediated immunity as well as the mucosal IgA immune response, whereas it down-regulates the polyclonal activation of the immune system. These data support the long-term effects of dietary CLA on the immune system.

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