Heparin: new life for an old drug

Carlos Raúl Aláez[1], Elena Lantero[2,3] and Xavier Fernàndez-Busquets[2,3,4]

[1]BIOIBERICA, Polígon Industrial "Mas Puigvert", Ctra. N-II, km. 680.6, ES-08389 Palafolls, Spain

[2]Nanomalaria Group, Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 10-12, ES-08028 Barcelona, Spain

[3]Barcelona Institute for Global Health (ISGlobal), Barcelona Center for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Rosselló 149-153, ES-08036 Barcelona, Spain

[4]Nanoscience and Nanotechnology Institute (IN2UB), University of Barcelona, Martí i Franquès 1, ES-08028 Barcelona, Spain

Summary

Heparin is one of the oldest drugs, which nevertheless remains in widespread clinical use as an inhibitor of blood coagulation. The history of its identification a century ago unfolded amid one of the most fascinating scientific controversies turning around the distribution of credit for its discovery. The composition, purification, and structure-function relationship of this naturally occurring glycosaminoglycan regarding its classical role as anticoagulant will be dealt with before proceeding to discuss its therapeutic potential in, among other, inflammatory and infectious disease, cancer treatment, cystic fibrosis, and Alzheimer's disease. The first bibliographic reference hit using the words "nanomedicine" and "heparin" is as recent as 2008. Since then, nanomedical applications of heparin have experienced an exponential growth that will be discussed in detail, with particular emphasis on its antimalarial activity. Some of the most intriguing potential applications of heparin nanomedicines will be exposed, such as those contemplating the delivery of drugs to the mosquito stages of malaria parasites.

KEYWORDS: *Anopheles*; antimalarial drugs; heparin; malaria; mosquitoes; nanomedicine; nanotechnology; *Plasmodium*; targeted drug delivery

Discovery of heparin

The history of heparin discovery dates back to 1915, when a second-year medical student, Jay McLean, arrived at Johns Hopkins Medical School in Baltimore to work under the supervision of Prof. William Henry Howell. Howell was a renowned physiologist that had developed a blood coagulation theory, according to which coagulation was produced by the binding of the phosphatide thromboplastin (also called cephalin) to antithrombin, and the subsequent conversion of prothrombin to thrombin in presence of calcium [1]. As cephalin was a key element in Howell's theory, he assigned McLean the task of studying its purity and demonstrate that it was the phosphatide itself and not a contaminant the responsible for cephalin clotting action [2]. McLean, who had limited financial resources to pay for the expenses related to the university, agreed with his tutor a one-year deadline to reach this goal. By 1916, not only McLean had studied cephalin but also the properties of another related phospholipid extracts. It was precisely this additional work which led to the discovery of heparin, when the retest of stored extracts from ox heart and dog liver unexpectedly showed *in vitro* anticoagulant properties [3]. Though initially skeptical, Howell soon realized the importance of this finding and continued working on the subject once McLean left Baltimore with a fellowship for the university of Pennsylvania. Between 1918 and 1924 Howell

substituted the original isolation protocol, which used organic solvents, by a more efficient aqueous extraction. He named the new substance "heparin" (from the Greek word *hepar* that means liver) and studied its chemical composition. Howell stated that the water-soluble extract prepared by him was not a phosphatide but a carbohydrate containing glucuronic acid. This different chemical composition convinced him that this substance was different from McLean's material. Consequently, although Howell recognized McLean's pioneering studies on the anticoagulant phosphatides, he did not credit his student for the heparin discovery.

Except for occasional attempts during his years as a medical student, McLean did not further investigate on heparin until after his graduation in 1919. He performed some unsuccessful preclinical studies and made two clinical trials in humans [4], whose results were published in scientific journals in the early 1940s. These reports were part of a campaign that McLean had started to change the widespread perception about Howell being the heparin discoverer. McLean was convinced that Howell's carbohydrate was already present in the phosphatide preparations he made during 1916; therefore, he claimed for priority. His claim, which also included letters to researchers, lectures and even an autobiography, found some opponents among the scientific community but was finally successful [5]. As J.A. Marcum commented in his article about heparin discovery [1], scientific findings are the result of the dynamic interplay between different ideas, events, and people. From this perspective, both McLean's and Howell's work were complementary. McLean's selection of alternative tissues to extract cephalin allowed the observation of the anticoagulant action of phosphatides. However, Howell's theory gave McLean a scientific frame to recognize the importance of his finding and the unexpected character of it stimulated Howell to improve the extraction process.

Chemistry and clinical use of heparin

Heparin belongs to the family of glycosaminoglycans (GAGs), a group of linear negatively charged polysaccharides that also includes heparan sulfate, dermatan sulfate, chondroitin sulfate, hyaluronic acid and keratan sulfate. Heparin chains are biosynthesized in the Golgi complex of connective tissue-type mast cells as part of a serglycin glycoprotein. Its biosynthesis involves the activity of several enzymes that participate in a multi-step process initiated with the synthesis of heparosan, a polysaccharide composed of alternating glucuronic acid and N-acetyl glucosamine residues. This polymer is further modified by N-deacetylation/N-sulfation of the glucosamine units, C-5 epimerization of the glucuronic acid and O-sulfation at different sites of the chain. Most of these transformations do not proceed to completion, leading to high structural heterogeneity [6,7]. The cellular mechanisms that regulate the expression and activity of the enzymes at each biosynthetic step are currently unknown [8,9]. Once heparin chains are formed, they are randomly cleaved by an endo-β-D-glucuronidase generating shorter fragments that are stored, together with histamine and mast cell basic proteases, in the cytoplasmic secretory granules. These fragments constitute the commercial unfractionated heparin (UFH) recovered during extraction [10]. Chains of heparin active pharmaceutical ingredient are in the range of 5,000 to 50,000 Da [11].

The manufacturing process of clinical grade heparin starts with a proteolytic digestion of the tissue source, followed by recovery of the heparin-like material using binding to anion-exchange resins or complexation with quaternary ammonium. After release from resins or complex, crude heparin is precipitated with organic solvents and dried. Components of crude heparin are heparin and dermatan sulfate, besides minor

impurities such as proteins, nucleic acids, other GAGs and endotoxins [12]. Purification of crude heparin is performed in different ways depending on the manufacturer. This stage usually involves a treatment with an oxidative agent aimed at reducing color, endotoxin content and the microbial and viral contaminations. Small and low-sulfated heparin chains are removed during purification through selective precipitations with organic solvents [13]. Nowadays clinical grade UFH is sourced almost exclusively from porcine intestinal mucosa. Nevertheless, some Islamic and Latin-American countries use ovine or bovine intestinal heparin for religious or economic reasons. In addition, the Food and Drug Administration is currently considering the reintroduction of bovine heparin into the US market to avoid possible shortages or contamination issues of the global supply chain, currently highly dependent on China [14,15].

Around 70% of the heparin chain is composed of a repeating trisulfated disaccharide (Figure 1A) that forms highly sulfated and regular domains [16]. In addition, other short and less sulfated sequences are present containing disaccharides with different substitution patterns such as: 2-O-desulfated iduronic acid, 6-O desulfated glucosamine, N-acetylglucosamine instead of N-sulfated glucosamine and glucuronic acid instead of iduronic acid (Figure 1B).

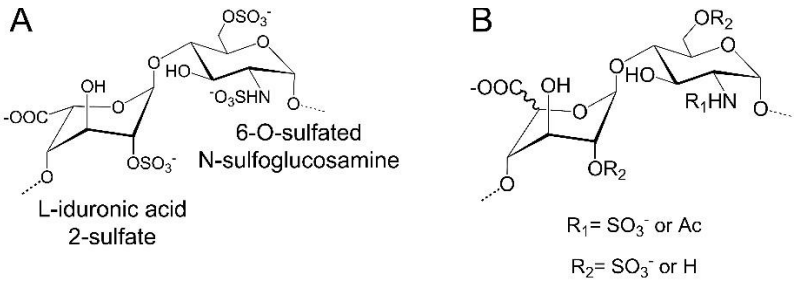

Figure 1. Repeating disaccharides in the heparin chain. Main trisulfated disaccharide (A) and less abundant disaccharides (B).

The extension of these substitutions varies with the tissue source for heparin extraction. For example, bovine lung heparin has the lowest content in N-acetylglucosamine units and the highest degree of 6-O-sulfation in comparison to porcine and bovine intestinal heparins. The latter is the less sulfated material with the lowest level of 6-O-sulfation [15]. In addition, heparins from different sources also differ in their average molecular weight. Source-dependent structural modifications could have clinical impact in terms of required therapeutic dose, amount of protamine to neutralize the anticoagulant effect, and formation of complexes with platelet factor 4 [14,17,18]. Approximately 1/3 of the heparin chains in UFH contain a specific sequence of five sugars with a central 3-O-sulfated glucosamine residue (Figure 2).

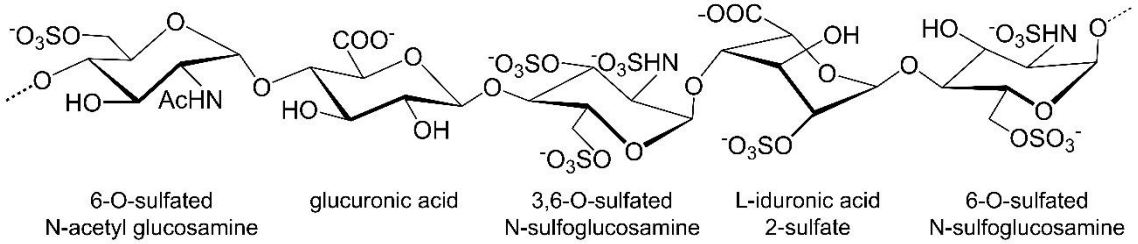

Figure 2. Pentasaccharidic antithrombin-binding domain.

This pentasaccharide is responsible for the anticoagulant activity of heparin mainly through its strong binding to antithrombin (AT), a key plasma coagulation inhibitor. The binding induces a conformational change in AT, potentiating the rate of its inhibitory action on serine proteases of the coagulation cascade, such as factor Xa and factor IIa. This catalytic action of heparin is size-dependent. Factor Xa requires the presence of the AT-binding pentasaccharide only, while inhibition of factor IIa additionally needs a minimum chain length of 18 residues (Mw > 5,400 Da) to form a ternary complex [AT-heparin-factor IIa] [19,20]. The development of powerful separation and analytical techniques has allowed the identification of some structural variations of the pentasaccharide sequence [15,21-23] as well as oligosaccharide fragments bearing more than one antithrombin-binding domain [24,25]. In addition, it was recently demonstrated that the structure of sequences flanking the pentasaccharide can modulate AT affinity [26]. Altogether these variations highlight the structural complexity of the heparin molecule, which can be further increased by the conditions of the extraction process, in particular during the purification steps. Strong alkaline or oxidative treatments induce the formation of structural artifacts (2,3-epoxides, galacturonic acid [27], acetylated uronic acid [28] or oxidated reducing-end N-acetylglucosamine units [29]) that constitute fingerprints of the type of purification performed.

The discovery of the molecular mechanism underlying the anticoagulant and antithrombotic activities of heparin led to the development of the low molecular weight heparins (LMWHs), which are characterized by having at least 60% of the chains with an average molecular weight below 8,000 Da. Consequently, they partially inhibit factor IIa and their anti-factor Xa/anti-factor IIa ratio is greater than 1.0 [30]. LMWHs are prepared by incomplete depolymerization of UFH using chemical or enzymatic reactions. Commonly used depolymerization methods for commercial production are: deaminative cleavage, perioxidative cleavage and chemical or enzymatic β-elimination [31]. Alternative methods to produce new LMWHs are being continuously reported [32-35]. The type of depolymerization reaction or changes in the reaction conditions for the same depolymerization method can induce variation in the molecular weight of the resulting product, its sulfation degree, the content and position of the AT-binding domain in the oligosaccharide fragments, and the type and content of terminal residues at the cleavage site. Purification conditions can also contribute to further structural variability. Altogether these process-related structural features make each LMWH a unique and non-interchangeably therapeutic entity with a distinct pharmacological and biomedical profile [16,36,37].

UFH and LMWHs are indicated for the treatment and prophylaxis of venous thromboembolism (VTE), a lethal disorder including both deep vein thrombosis and pulmonary embolism. Due to their predictable pharmacokinetic behavior, LMWHs can be administered at a fixed or body-weight dose, usually without monitorization. This, together with their reduced risk of side-effects, makes LMWHs preferred to UFH. LMWHs have shown great efficacy in the treatment and prevention of VTE in trauma and in major orthopedic surgery of hips and legs. They are also preferred for the treatment and prophylaxis of VTE during pregnancy and in cancer patients [38]. In combination with thrombolytic agents, UFH and LMWHs are used in the treatment of patients with ST segment elevation myocardial infarction. Additionally, UFH is the most commonly used antithrombin agent for percutaneous coronary intervention [39,40]. LMWHs have also been approved as anticoagulant in extracorporeal circulation system for patients undergoing hemodialysis due to acute renal failure or chronic renal

insufficiency [41]. Heparin solutions of different concentrations are commonly used as a flush solution to prevent clotting in intravascular catheters [42].

Novel potential therapeutic applications of heparin

Besides its original therapeutic use as an anticoagulant, other potential applications of heparin for a number of human diseases have been identified [43]. Since heparin itself is found in mast cell granules complexed with basic proteases [44], anticoagulant activity is unlikely to be its major natural function, and indeed it is active in other systems due to its structural similarity to the cell surface and extracellular matrix GAG heparan sulfate [45]. Heparin interacts non-specifically with proteins such as cytokines, growth factors, adhesion molecules and proteases. Many of these molecules are associated to inflammation processes [46], which are attenuated when heparin or similar compounds are administered. Because inflammation, atherogenesis, thrombogenesis and cell proliferation are related with each other, and heparin influences all of them, its increased therapeutic activity might be due to such simultaneous activities [47]. Based on these properties, several studies on the effect of heparin and modified heparin derivatives have been performed in some inflammation-involving disorders such as asthma, cystic fibrosis, ulcerative colitis, Alzheimer's disease and cancer [48]. Structural tailoring to these novel applications is usually limited to reduction of anticoagulant activity, which may be achieved by mild chemical treatment such as glycol-splitting, more comprehensive chemical modification such as selective desulfation, or by extensive depolymerisation into small oligosaccharides.

When mast cell granules are released, their contents change tissue morphology, allowing the infiltration of granulocytes and starting the inflammatory process [49]. Different mechanisms have been suggested for the heparin-mediated inhibition of inflammatory response [50]. The anti-asthmatic activity of heparin [51], which is inversely proportional to its molecular weight and independent of its anticoagulant activity, is related to its capacity to inhibit the degranulation of mast cells by interacting with the intracellular receptor of inositoltriphosphate [9]. LMWHs smaller than 2,500 Da had no effect on such release but nevertheless could diminish inflammation of airways in allergic sheep [52]. In guinea pigs, airway hiperresponse normalization induced by heparin could be reversed by nitric oxide synthase (NOS) inhibitors [53]. Because thrombin stimulates mucus secretion and induces goblet cell metaplasia, it has been suggested that inflammation could also be controlled through the heparin-mediated inhibition of thrombin [54]. In sepsis, heparin has been shown to decrease endotoxin-stimulated gene expression and the production and release of pro-inflammatory cytokines [55]. For other applications in airway inflammatory processes like cystic fibrosis, heparin has exhibited mucolytic activity [56,57], although in separate studies with cystic fibrosis patients [58] and in prophylaxis treatments against pneumonia [59], heparin inhalation had no influence on the symptoms.

Heparin plays a significant role in gastric ulcer healing by virtue of its ability to activate NOS and facilitate mucosal cell proliferation by stimulating growth factors [60]. In colitis-related diseases, both in animal models [61] and clinical trials [62], administration of heparin helped to restore normal conditions. However, a systematic review [47] highlighted that the studies on the effects of heparin in inflammatory bowel disease offered conflicting results, as inflammatory factors remained similar in heparin-treated subjects and controls.

Heparin may also possess antineoplastic properties. In cell-based and animal models, non-anticoagulant heparin significantly slowed down the migration of breast cancer cells and reduced their metastatic spread [63]. Data evaluation from preclinical

studies in anticancer therapy indicated that heparin seemed to affect the formation of metastases rather than the growth of primary tumours [64]. Chemically modified heparins with no or limited anticoagulant activity also showed anti-metastatic properties. Possible mechanisms to explain these observeations include inhibition of blood coagulation, of cancer cell-platelet and -endothelial interactions by selectin blocking, and of cell invasion and angiogenesis [65].

GAGs have been considered of interest in Alzheimer's disease (AD) ever since they were first demonstrated in amyloid plaques and neurofibrillary tangles. AD also has an inflammatory process involved, and it has been hypothesized that LMWH could cross the blood-brain barrier and attenuate this inflammation [66]. Heparin oligosaccharides have also been found to inhibit amyloid-β peptide (Aβ) precursor protein (APP) secretion in the brain [67] and Aβ production by cortical neurons [68]. In an AD murine model LMWHs have been described to reduce the ability of Aβ to activate complement and contact systems as well as lowering Aβ deposits in the brain [69], and to inhibit experimental amyloidosis in clinically relevant doses [70]. It is known that heparin and heparan sulfate regulate the activity of a number of proteases, and it has been reported that heparin inhibits β-site APP-cleaving enzyme 1 (BACE-1) activity *in vitro* [71]. A low concentration of heparin can stimulate recombinant human BACE-1, leading to increased autocatalytic cleavage of the protease domain and a subsequent loss of enzyme activity [72]. GAGs and other sulfate-containing compounds significantly attenuated the toxicity of Aβ on human neuroblastoma SH-SY5Y cells [73] and on neuronal differentiated PC12 cells [74,75]. To avoid anticoagulant effects, a mixture of heparin oligosaccharides showed neuroprotective capacity in AD experimental models, also by the oral route [76]. Heparin has been observed to accelerate aggregation and amyloid formation by the model protein muscle acylphosphatase [77], although the actual role of amyloidogenesis in AD is still a matter of dispute. *In vitro*, heparin and other GAGs have been shown to enhance Aβ fibrillogenesis [78], but they may also prevent the persistence of the toxic forms of Aβ oligomers or protofibrils by transforming them into harmless aggregates [79].

The observation that heparin-containing tissues are in direct contact with the external environment suggests also a role in host defense. The potential application of heparin derivatives in the field of infectious disease is less studied compared to inflammatory and oncologic conditions. However, they are also under investigation for use as antimicrobial agents due to their inhibitory effects on pathogen binding to cell surfaces. The pathogenesis of most infectious diseases involves an adherence of microbes to cell membranes in which GAGs play a key role [80]. Adhesion is usually followed by internalization of the organism into the cell, which, in turn, leads to downstream effects of the infectious process on the cellular level. The successful use of 3-O-sulfated octasaccharides to inhibit entry of herpes simplex virus type 1 into corneal fibroblasts indicated the possibility of using heparin-based compounds in anti-viral therapy [81]. Heparin has also been described to have a clear potential for the treatment of malaria, as it will be discussed in the next section.

Heparin and malaria

The malaria infectious cycle [82] starts when a parasitized female *Anopheles* mosquito, while taking a blood meal, inoculates sporozoites of the malaria parasite, the protist *Plasmodium spp*. In the liver, sporozoites develop into merozoites [83], which enter the circulation, invade red blood cells (RBCs) [84], and replicate asexually through trophozoite and schizont stages to produce daughter cells that invade new RBCs to perpetuate the blood-stage cycle. Some parasites eventually differentiate into sexual

stages, female or male gametocytes that are ingested by a mosquito from peripheral blood. Following fertilization in the insect's midgut, the zygote differentiates into an ookinete that moves through the midgut epithelium and forms an oocyst, which releases sporozoites. The malaria transmission cycle is restarted when sporozoites migrate to the salivary glands and are injected into a human with the mosquito's next bite. The current trend of global warming and generalised transcontinental travel, added to the growing number of displaced populations in endemic areas due to political, economic, and environmental reasons, threatens with expanding the geographic distribution of the disease. Despite the undeniable importance of malaria elimination on the global research agenda, current vaccines in development do not offer prospects of complete protection [85] and the available front-line drugs are rapidly losing efficacy [86]. Thus, alternative strategies [87] working through radically new mechanisms are urgently needed.

GAGs are one of the main molecules associating with *Plasmodium*-parasitized RBCs (pRBCs). Binding to the GAG chondroitin 4-sulfate (CSA) is thought to cause pRBC sequestration in the microvasculature [88] and the placenta [89], which has been linked to the severe disease outcome of pregnancy-associated malaria [90,91]. Negatively charged polysaccharides, such as heparin, chondroitin and dextran sulfates, fucoidan, and the nonsulfated GAG hyaluronan, block the interaction of pRBCs with various host cell surface receptors [91-94] and disrupt *P. falciparum* adhesion to RBCs to form rosettes [95,96]. Heparan sulfate (HS), or a HS-like molecule exposed on RBCs is the ligand responsible for rosetting [97]. The potential use of heparin as drug in malaria therapy [98-102] has been hindered by its high anticoagulation and bleeding properties [103] and by the potential risk of infection since some GAGs are obtained from mammals. However, depolymerized heparin lacking anticoagulant activity has been found to disrupt rosette formation and pRBC cytoadherence *in vitro* and *in vivo* in animal models and in fresh parasite isolates [104]. Nevertheless, shorter heparin fragments consisting of hexa- and octasaccharides having insignificant anticoagulant activity [105] exhibited a much smaller antimalarial activity *in vitro* than the native polymer, with respective IC50s of 174 and 134 μg/mL [106], compared to around 4 μg/mL for heparin. As an interesting alternative approach, non-mammalian marine organisms are a rich source of unique sulfated polysaccharides, some of them with structures resembling pRBC-binding GAGs [107-109]. Some of these marine sulfated glycans inhibit *P. falciparum* cytoadhesion and *in vitro* growth as efficiently as heparin at concentrations where their anticoagulant activity is very low [110,111], and might therefore offer interesting alternatives for future antimalarial therapies. Remarkably, efforts to select for heparin-resistant parasites have proven unsuccessful [112], which places sulfated polysaccharides as interesting candidates in the race for finding efficient long-lasting antimalarials. GAG antimalarial activity unfolds by inhibition of merozoite invasion [92,93,110,112-118]. Naturally acquired immunity to malaria is largely directed against extracellular merozoites [119] but currently there are no drugs targeting erythrocyte invasion by *Plasmodium* [120], although some candidates have been proposed [121]. Heparin has been shown to bind merozoites inside late-stage pRBCs (Figure 3), a finding that, as it will be discussed below, opened interesting perspectives for the incorporation of heparin to future antimalarial nanomedicines.

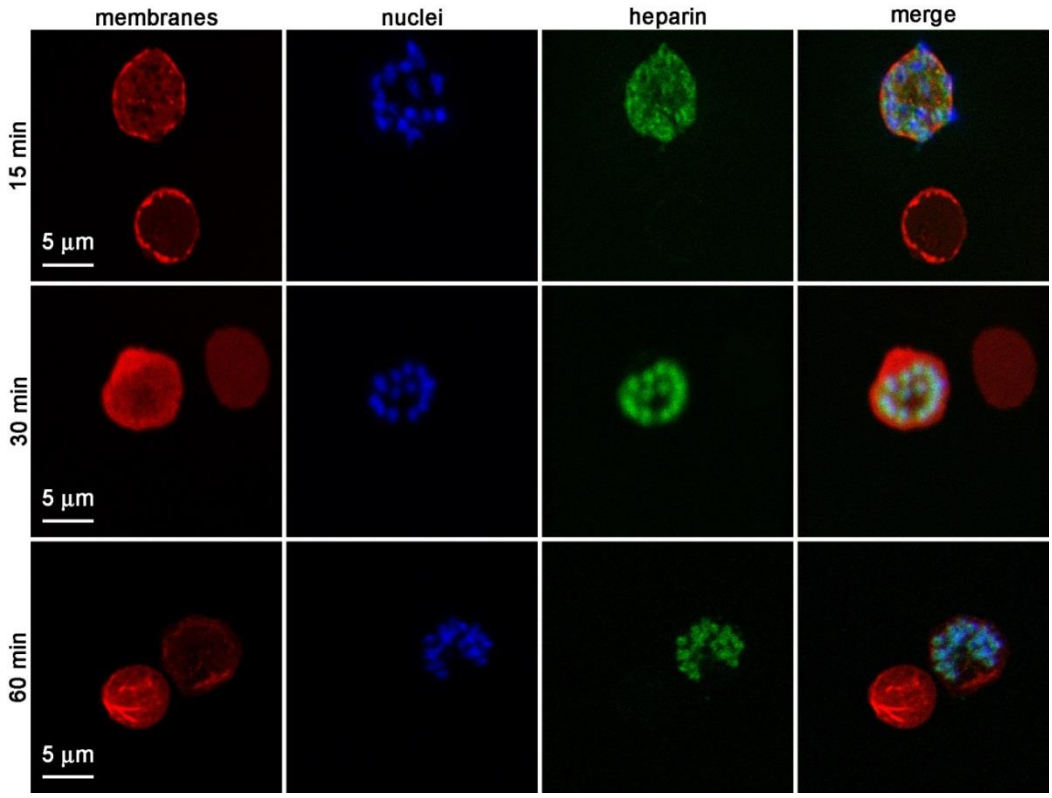

Figure 3. Specific targeting of heparin to pRBCs *vs.* RBCs. Heparin-FITC was added to living cocultures of *P. falciparum*-infected RBCs and non-infected RBCs and incubated for the indicated times before sample preparation for microscopic analysis. Each series shows a pRBC and a non-infected erythrocyte as a control of the specificty of heparin targeting. Free FITC used at the same concentration according to fluorescence intensity was not observed to stain any cells. Reproduced from Ref. [122], with permission.

Heparin in nanomedicine

The first bibliographic reference hit using the words "nanomedicine" and "heparin" dates from 2008 [123], describing iron-heparin complexed hollow capsules displaying a more prolonged anticoagulant activity than free heparin. Since then, nanomedical applications of heparin have experienced an exponential growth. Functionalization of nanoparticles with heparin is accomplished through the formation of covalent bonds or through electrostatic interactions. Heparin confers novel properties to nanoparticles such as stealth, which contributes to bypass clearance by the reticuloendothelial system [124], improved targeting of molecules with enhanced uptake and accumulation [122,125], and increased stability and solubility [126]. A potential nanomedicine consisting of heparin-loaded chitosan/carboxymethyl-β-cyclodextrin nanocarriers has been proposed for the treatment of asthma [127]. Self-assembled stable heparin-containing nanoparticles [128-130] have been described to possess a high growth factor loading capacity allowing cytokines to maintain their bioactivity for longer periods and representing a promising delivery system for tissue regeneration [131,132]. Vascular endothelial growth factor (VEGF) encapsulated in chitosan-heparin nanostructures stimulated proliferation of endothelial cells *in vitro*, increased fibroblast infiltration and extracellular matrix production, and accelerated vascularization in a murine subcutaneous implant model *in vivo* [132]. Nanoparticle-coated decellularized bovine jugular vein scaffolds exhibited highly effective localization and sustained release of

VEGF for several weeks; the physical adsorption of heparin could prevent early degradation of growth factors, thereby preserving their biological activity. Heparin-chitosan nanocomplexes loaded with placental growth factor and bone morphogenic protein have been described to provide a sustained dual release of both molecules which provided greater potential for bone tissue regeneration than the delivery of either growth factor alone [133]. LMWH-protamine nanoparticles have been used as carriers for heparin-binding growth factors [134], which in this way were protected from inactivation by heat and proteolysis thus substantially prolonging their biological half-life. Heparin-nanomodified acellular bovine jugular vein scaffolds showed significantly improved biomechanical stability and biocompatibility, allowing the sustained release of heparin for several weeks [135]. The modified scaffolds had reduced platelet adhesion and stimulated proliferation of endothelial cells *in vitro*, and exhibited low *in vivo* calcification in a subcutaneous implantation rat model. Heparin incorporated into poly(L-lactide-co-ε-caprolactone) nanofibers was released in a controlled manner and this system was suggested to be a potential substitute for natural small-diameter vessels in planned vascular bypass surgery [136]. The intercalation of LMWH into layered double hydroxide (LDH) nanoparticles also resulted in an enhanced pharmaceutical efficacy of heparin, whereby cellular uptake of LMWH-LDH conjugates into cultured rat vascular smooth muscle cells was more than ten times greater than that of LMWH alone [137].

Sugar-based biopolymers, including heparin, might open a new emerging nanomedicine era for cancer imaging and therapy [138]. *In vitro* cell tests revealed marked phototoxicity and high intracellular uptake of pheophorbide a (PhA)-conjugated heparin/gold nanoparticles in contrast with free PhA [139]. The heparin-containing nanostructures also exhibited prolonged circulation, enhanced tumor specificity, and improved photodynamic therapeutic efficacy in tumor-bearing mice. As outlined above, LMWH exerts its anticancer activity by affecting the proliferation, adhesion, angiogenesis, migration, and invasion of cancer cells. Nanoparticle conjugates of LMWH with stearylamine [140] and with chitosan [141] have been shown to be promising delivery systems of the anticancer agents docetaxel and cytolethal distending toxin, respectively. The intracellular delivery of doxorubicin encapsulated in pH-responsive chitosan-heparin nanocapsules fabricated by the layer-by-layer technique on silica nanoparticles followed by dissolution of the silica core resulted in an enhanced bioavailability when compared to the free drug [142]. Heparin-polyethyleneimine nanoparticles have been assayed as nonviral gene carrier and successfully used to deliver plasmids expressing mouse survivin-T34A to treat C-26 carcinoma *in vitro* and *in vivo* [143]. Heparin nanocomplexes have also been proposed for improved magnetic labeling of stem cells in clinical translational studies [144], and heparin-conjugated quantum dots (QDs) exhibit superior imaging properties compared to their native counterparts [145]: heparin binding to the CD11b receptor facilitated internalization of QDs into the nucleus of THP-1 cells whereas the heparin layer may reduce the unfavourable thrombogenic nature of QDs observed *in vivo*. One potential drawback of using heparin is the development of heparin-induced thrombocytopenia (HIT), an immune complication of heparin therapy caused by antibodies to complexes of platelet factor 4 (PF4) and heparin [146]. Nanoformulated heparin, if specifically targeted to its site of action, will require minimal amounts; because PF4-heparin interactions are exclusively charge-dependent [147], changes in molar amounts leading to excess of either compound results in charge imbalance and increased repulsive forces that affect complex assembly, and in this way the incorporation of heparin in nanoparticles will minimize HIT. In addition, heparin-containing nanomedicines designed to be active in

the blood will be often functionalized with poly(ethylene glycol) in order to reduce reticuloendothelial system uptake and increase circulation time; the resulting poor recognition of such nanocarriers by antibody-producing cells will also contribute to limit the risk of HIT.

Various methods have been reported over the last two decades for the synthesis of silver and gold nanoparticles. These involve the reduction of metal salts with a chemical reducing agent, such as sodium citrate, sodium borohydride or other organic compounds that introduce contaminants, which are often toxic. Using as reducing agents natural carbohydrates such as glucose, chitosan or heparin has been proposed as a clean method for the production of metal nanoparticles for medical applications [148]. Finally, heparin sensing has been proposed as the basis for future biomedical sensors having potential applications in the bedside detection of heparin levels in human blood during surgery [149], although the difficulties in achieving selective high-affinity molecular recognition in the complex environment of human blood represents a challenging obstacle. However, preliminary results obtained with biotinylated heparin arrays have verified the feasibility of implementing a heparin microarray to selectively sort pRBCs from non-infected RBCs [150]. Other nanotechnological applications of heparin to malaria are starting to assert themselves as the basis of potential future medical tools.

Heparin for future antimalarial nanomedicines

The concept of antimalarial therapy has been locked for over 100 years on the administration of drugs against which *Plasmodium* has evolved resistance shortly after their deployment. Because malaria pathophysiology is so complex and the disease is so widespread, it is generally accepted that to achieve eradication a combination of tools targeting the parasite and/or mosquito will be needed [151]. These include the improvement of existing approaches and the development of new ones, with drug therapy remaining the mainstay of treatment and prevention to target the parasite reservoir [152], and nanotechnology being able to provide innovative useful strategies. Encapsulation of drugs in targeted nanovectors is a rapidly growing area with a clear applicability to infectious disease treatment [153], and pharmaceutical nanotechnology has been identified as a potentially essential tool in the future fight against malaria [154]. Because malaria is a disease prominent in countries with limited resources, new treatments need to consider such economic landscape. In this regard, the use of molecular elements combining several antimalarial activities, whether drug, targeting, carrier, or booster of immune reactions, will contribute to reduce the cost of their development [155].

Rather than focusing all efforts on identifying new drugs whose efficacy is rapidly diminished by the parasite's evolution of resistance, an important and often disregarded battlefront is the implementation of targeted delivery methods capable of increasing the doses reaching the pathogen up to local levels sufficiently high to minimize this resistance emergence. Regrettably, the search for this long sought-after *magic bullet* against malaria has not taken off in earnest yet. However, recent data outline the feasibility of some such potential novel approaches, among which we can count new types of combination therapies where one of the activities does not act on individual *Plasmodium* gene products [155]. Despite the lack of economic incentives for research in nanomedicine applications to malaria a number of liposome- and polymer-based nanocarriers engineered for the targeted delivery of antimalarial drugs have been developed [122,154,156-161]. Although successful efforts have been made to obtain new nanostructures having affordable synthesis costs while still exhibiting good performance in lowering the IC50 of drugs [122,161], new approaches are required to

further optimize these scarce resources. The implementation of novel delivery approaches is less expensive than finding new antimalarial drugs and may optimize the rate of release of current and future compounds [162].

The potential hemorrhagic activity of sulfated polysaccharides in future antimalarial clinical applications can possibly be averted by encapsulating them in pRBC-targeted nanocapsules as it has been reported for other antimalarial agents [154,160,161]. Liposome-bound heparin has been demonstrated to be capable of substituting for antibodies as targeting molecule of drug-loaded nanocarriers [106,122], thus adding to its own antiparasitic action and potentiating therapeutic activity (Figure 4). 1 μg/mL heparin electrostatically adsorbed onto positively charged liposomes containing the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane and loaded with the antimalarial drug primaquine was capable of increasing three-fold the activity of encapsulated drug in *P. falciparum* cultures [122]. At concentrations below those inducing anticoagulation of mouse blood *in vivo*, parasiticidal activity was found to be the additive result of the separate activities of free heparin as antimalarial and of liposome-bound heparin as targeting element for encapsulated primaquine. Surface plasmon resonance biosensor studies showed that covalent binding through its carboxyl groups dramatically reduced the interaction of heparin with antithrombin [163], and there is evidence of a significantly diminished anticoagulant activity of heparin when covalently immobilized on a substrate [164]. Indeed, the anticoagulant activity of heparin covalently bound to liposomes has been found to be significantly smaller than similar amounts electrostatically bound [106], whereas such primaquine-loaded liposomes had a clearly improved antimalarial activity. In contrast, covalently linked heparin hexa- and octasaccharides did not improve the activity of the liposomized antimalarial drug [106], suggesting that also the pRBC targeting capacity of heparin is lost upon depolymerization. Single-molecule force spectroscopy data have revealed a complete specificity of adhesion of heparin to late-form pRBCs vs. RBCs (Figure 5), with a binding strength matching that of antibody-antigen interactions [165]. Heparin conjugated to the surface of different types of nanoparticles has been found to be more active against *Plasmodium* than in free form [115], highlighting the importance of nanostructured heparin for increased antimalarial activity.

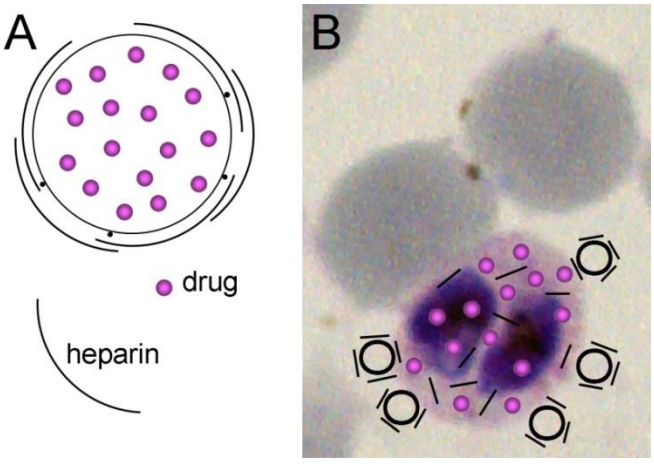

Figure 4. Additive activity of heparin as antimalarial and as targeting agent towards pRBCs. Scheme of heparin-functionalized, drug-containing liposomes (A), and expected outcome when they specifically deliver their contents (both drug and heparin) to pRBCs (B), lowering the drug IC50 and adding to it the antimalarial activity of heparin itself. Reproduced from Ref. [122], with permission.

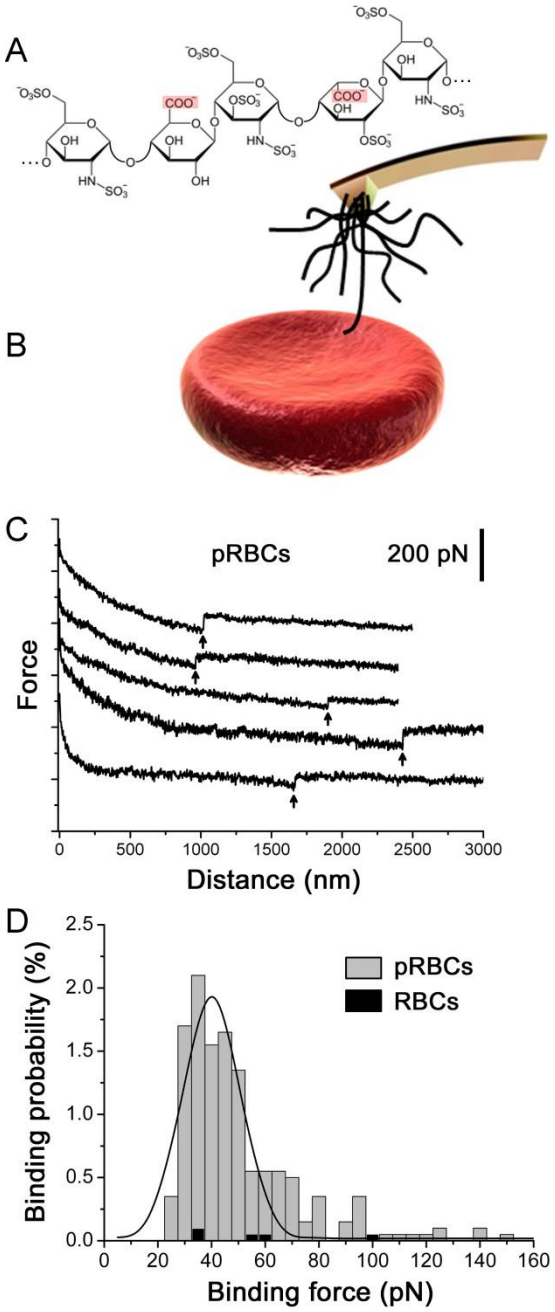

Figure 5. Single-molecule force spectroscopy analysis of heparin-pRBC interaction. (A) Heparin sequence region corresponding to the antithrombin pentasaccharide binding site; the carboxyl groups used for the covalent crosslinking of heparin to atomic force microscope (AFM) cantilevers are shaded. (B) Cartoon of a binding event between an erythrocyte and one of the heparin chains crosslinked to the AFM cantilever tip. (C,D) AFM force spectroscopy results. (C) Typical force curves from independent experiments obtained when retracting heparin-functionalized cantilever tips from pRBCs. Arrows indicate individual heparin-pRBC unbinding events. For the sake of clarity, the force curves were shifted vertically to avoid overlapping. (D) Representative force histograms for the binding of heparin to pRBCs (grey) and RBCs (black) at a loading rate of 17.5 nN s^{-1}. Heparin-pRBC binding data were fitted to a Gaussian curve. Reproduced from Ref. [165] with permission from The Royal Society of Chemistry.

Since resistance of *Plasmodium* to heparin has not been shown so far [112], heparin-based targeting will predictably be more long-lasting than pRBC recognition relying on antibodies, which typically are raised against highly variable exposed antigens whose expression is constantly varied by successive generations of the parasite [166]. The specific binding of CSA to pRBCs infected by the *P. falciparum* CS2 strain, which sequester in the maternal circulation of the placenta [167], suggests that future nanovectors functionalized with CSA can be foreseen to be adapted to target drugs to pRBCs for the treatment of placental malaria. Such nanocarriers will bypass the concerns discussed above regarding the hemorrhagic risks of administering heparin to humans, since CSA has been shown to be devoid of anticoagulant activity [111].

In the past, malaria parasites have developed resistance to widely used drugs [168]. This threat of resistance-driven treatment failure is prompting research oriented to target the transmission stages of the pathogen between humans and mosquitoes [169], represented by smaller populations less likely to contain resistant individuals that would benefit from the removal of susceptible parasites [170]. Although the innate immune system of mosquitoes is capable of completely clearing a malaria infection [171], it is far from the sophisticated arsenal providing long-term protection in mammalian adaptive immunity. In addition, the richness of biological processes required for development in the mosquito likely withdraws from the parasite metabolic resources otherwise allocated to drug resistance. During initial malaria infection in the liver, heparin and HS are hepatocyte receptors for sporozoite attachment [172]. CS proteoglycans in the mosquito midgut and a synthetic polysulfonated polymer that mimics the structure of GAGs present in the midgut epithelium have been described to bind *Plasmodium* ookinetes during host epithelial cell invasion [173,174], whereas ookinete-secreted proteins possess significant binding to heparin [106,175] (Figure 6). Thus, GAGs might be adequate to direct antimalarial-loaded nanovectors to *Plasmodium* stages exclusive to the insect.

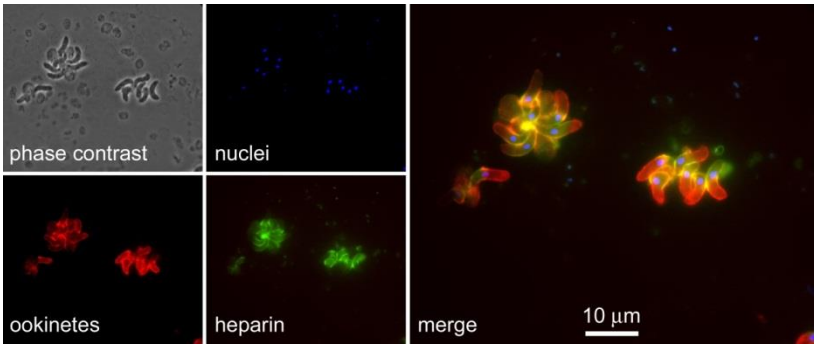

Figure 6. Fluorescence confocal microscopy analysis of the binding of heparin-FITC to *Plasmodium berghei* ookinetes *in vitro*. Ookinete fluorescence is shown by mCherry and parasite nuclei were stained with 4',6-diamidino-2-phenylindole. Reproduced from Ref. [106], with permission (doi: 10.1016/j.nano.2016.09.010; https://creativecommons.org).

A largely unexplored avenue, however, is the development of strategies for blocking the mosquito stages of *Plasmodium* by directly targeting parasites in the insect vector [176]. The engineering of antimalarial nanomedicines designed to be delivered to mosquitoes might spectacularly reduce costs because the clinical trials otherwise required for therapies to be administered to people could be significantly simplified. Strategies that control malaria using direct action against *Anopheles* are not new, but

most of them aim at eliminating the vector, either by killing it with pesticides [177] or through the release of sterile males [178,179]. Since eradicating an insect species might have as a consequence unpredictable disruptions of ecosystems with potential undesirable side effects (e.g. crop failure if pollinators were inadvertently affected), mosquito-friendly antimalarial strategies should be favored whenever possible. Administration of drugs encapsulated in heparin-containing nanovectors to mosquitoes to free them of malaria with the objective of blocking transmission of the disease is a challenging, yes, but promising alternative worth exploring.

Conclusion

Despite existing as drug for over a century, heparin continues being the anticoagulant of choice in the vast majority of clinical applications where blood clotting has to be kept under control. As a wave of new anticoagulants is approaching [180], heparin does not resign itself to being pushed aside, and this enduring drug is finding new and unexpected biomedical uses in several devastating diseases.

Future perspective

The endogenous nature of heparin makes it highly biocompatible and biosafe, especially in the case of low molecular weight heparin. It has an affordable production cost and its purification from animal tissues does not require sophisticated protocols or expensive equipment. Finally, it exhibits an astonishing curative capacity in a plethora of human diseases. These three characteristics place heparin as the basis of potentially key medicines in the threshold of an era where medical care has to be universalized to the farthest corners of the Earth. Developing regions of the world suffer massively of malaria and other infectious diseases, but as their economies grow and their population ages, other pathologies like cancer and Alzheimer's disease will appear with force. Foreseeingly, heparin and its nanomedical derivatives will not only be increasingly used in the wealthiest areas of the planet, but they will also become essential medicines for the welfare and progress of poverty-stricken nations.

Executive summary

Discovery of heparin

• Heparin was identified a century ago amid a fascinating controversy regarding the distribution of credit for its discovery.

Chemistry and clinical use of heparin

• Around 70% of the heparin chain is composed of a repeating trisulfated disaccharide that forms highly sulfated and regular domains.

• Unfractionated heparin and low molecular weight heparin are indicated for the treatment and prophylaxis of venous thromboembolism.

Novel potential therapeutic applications of heparin

• Besides its original therapeutic use as an anticoagulant, other potential applications of heparin for a number of human diseases have been identified.

• Heparin is being currently tested for the treatment of inflammation-related disorders such as asthma, cystic fibrosis, ulcerative colitis, Alzheimer's disease and cancer.

• Heparin is under investigation for use as antimicrobial agent due to its inhibitory effect on pathogen binding to cell surfaces.

Heparin and malaria

• Glycosaminoglycans are one of the main *Plasmodium*-infected red blood cell-binding molecules.

Heparin in nanomedicine

• Nanomedical applications of heparin have experienced an exponential growth since the first published report in 2008.
• Heparin confers novel properties to nanoparticles such as stealth, improved targeting of molecules with enhanced uptake and accumulation, and increased stability and solubility.
• In cancer therapy, heparin-containing nanostructures exhibit prolonged circulation and enhanced tumor specificity.
• Using as reducing agents natural carbohydrates such as glucose, chitosan or heparin has been proposed as a clean method for the production of metal nanoparticles for medical applications.

Heparin for future antimalarial nanomedicines

• Despite the lack of economic incentives for research in nanomedicine applications to malaria a number of liposome- and polymer-based nanocarriers engineered for the targeted delivery of antimalarial drugs have been developed.
• Liposome-bound heparin has been demonstrated to be capable of substituting for antibodies as targeting molecule of drug-loaded nanocarriers.
• The engineering of antimalarial nanomedicines designed to be delivered to mosquitoes might spectacularly reduce costs because the clinical trials otherwise required for therapies to be administered to people could be significantly simplified.
• The administration of drugs encapsulated in heparin-containing nanovectors to mosquitoes to free them of malaria is a promising alternative worth exploring.

Financial & competing interests disclosure

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