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Grau de Química

Treball Final de Grau

Screening of compounds affecting the interaction between domains of Src protein

Cribatge de compostos que alterin la interacció entre dominis de la proteïna Src

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M’agradaria agrair a aquelles persones que han fet possible la realització d’aquest projecte, que d’una manera o altra m’han ajudat, aconsellat o bé hi ha col·laborat.

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REPORT

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1. SUMMARY

The c-Src protein is the leading member of the Src family non-receptor tyrosine kinases (SFKs) involved in many signalling pathways. The SH3, SH2 and kinase domains of SFK members display large sequence and structural similarity. But each SFKs has unique sequences called them as Unique domain (UD). This is an intrinsically disordered region and it is not clear its function. However, several studies demonstrated that UD is crucial for c-Src regulatory activity.

The aim of this study is discovering drugs that can bind to Unique or adjacent domains of c-Src. To do that, a Förster resonance energy transfer (FRET) biosensor composed with SH3, SH4 and Unique domain of c-Src has been obtained by plasmid transformation, protein expression and chromatography purification. FRET pair fluorophores for the biosensor were mClover3 (green) and mRuby3 (red). The binding of some drugs caused a change between the distance between the two fluorophores in FRET-biosensor leading to measure changes in fluorescence spectra. Drugs were classified according to their FRET's effect. Only two drugs presented a pronounced FRET signal (L13B-C2 and L10B-C9). Finally, binding assays were performed with the best drugs in order to determine the binding association constant assuming that they followed a model of binding 1:1 of protein:ligand. Based on the values obtained, L10B-C9 presents a higher affinity for the FRET-biosensor than L13B-C2 (mean of 0,0075 vs 0,006μM^{-1}, respectively).

Keywords: Src kinase, drug screening, Förster Resonance Energy Transfer (FRET), fluorescence, binding constant.

2. RESUM

La proteïna c-Src és el membre principal de la família de Src de tirosina-quinases no receptores (SFK) involucrades en moltes vies de senyalització. Els dominis SH3, SH2 i quinases dels membres de SFK presenten una gran seqüència i similitud estructural, però cada SFK té seqüències úniques que les anomenem Unique domain (UD). Aquesta és una regió intrínsecament desordenada i la seva funció no està clara. No obstant això, diversos estudis van demostrar que el UD és crucial per a l'activitat reguladora de c-Src.

L'objectiu d'aquest estudi és descobrir medicaments que poden unir-se al UD o adjacents de la proteïna c-Src. Per fer-ho, s'ha obtingut un biosensor de transferència d'energia de ressonància de Förster (FRET) compost amb el domini SH3, SH4 i UD de la proteïna c-Src mitjançant un procés d'expressió en bactèries i de purificació cromatogràfica. Els dos cromòfors de FRET pel biosensor van ser mClover3 (verd) i mRuby3 (vermell). Es van analitzar trenta fàrmacs utilitzant el biosensor de FRET sintetitzat. La unió d'alguns dels fàrmacs va provocar canvis en la distància entre els dos cromòfors del biosensor de FRET, donant lloc a la observació de canvis en espectres de fluorescència. Els fàrmacs es van classificar segons el seu efecte de FRET. Només dos fàrmacs van presentar una senyal de FRET pronunciada (L13B-C2 i L10B-C9). Finalment, es van realitzar assajos d'unió amb els dos millors fàrmacs per tal de determinar la seva constant d'unió suposant que aquests seguien un model d'unió 1:1 proteina:lligand. A partir dels resultats obtinguts, veiem que L10B-C9 presenta una afinitat superior per al FRET-biosensor que L13B-C2 (mitjana de 0.0075 vs 0,006μM^{-1}, respectivament).

Paraules clau: Src quinasa, cribatge de fàrmacs, Förster Resonance Energy Transfer (FRET), fluorescència, constant de binding.

3. INTRODUCTION

3.1. SFK (SRC FAMILY KINASES) AND C-SRC

Proteins kinases are enzymes which transfer ATP's terminal phosphate group to specific protein substrates such as residues of serine, threonine or tyrosine[1]. Thus, these proteins carry out important roles in cell signalling and, as a consequence, many of them are involved in various diseases.

The Src family kinases (SFK) that are non-receptor tyrosine kinase is formed by ten proteins: c-Src, Frk, Lck, Lyn, Blk, Hck, Fyn, Yrk, Fgr and c-Yes[2][3][4]. These proteins participate in a wide range of processes such as cell division, survival, differentiation and vesicular traffic[5]. The structure of SFK presents four Src homology domains: SH1, SH2, SH3 and SH4 in this order from the C terminus to N terminus. Also, there is an intrinsically disordered region (IDR) called Unique domain (UD), which is different for each member of the SFK[2]. IDR can maintain their functionality despite the lack of a well-defined structure even under physiological conditions. Intrinsically disordered proteins (IDP) are important in regulatory and cell signal pathways[5].

In this project we work with a construct derived from c-Src, the leading member of SFK. The construct studied contains the SH4, Unique and SH3 domains of c-Src[2]. In addition, the complete protein has the following intramolecular interactions:

- SH3 interacts with UD and a polyproline sequence which work as a linker between SH2 and SH1 domain[2] (figure 1).
- SH2 interacts with the Tyr530 of the C-terminal when it is phosphorylated leading to a closure of kinase domain and, as a result, the inactivation of c-Src takes place[2] (figure 1).
- c-Src presents a myristylation site by which can bind to lipid membrane. Also, SH3, Unique and positive charge of some amino acids of SH4 work out of well for lipid interaction[2].

- Moreover, residues 60-67 of the unique lipid binding region (ULBR) from the UD can also anchor to lipid membrane[6] (figure 1).

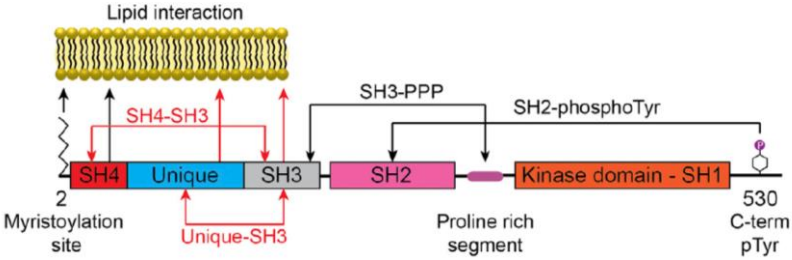


Figure 1. Src structure *(extracted image from ref.1)*

3.2. FLUORESCENCE AND FRET

3.2.1. Fluorescence

In 1852 G. G. Stokes described fluorescence as a physical phenomenon of emission of light by a chemical substance which has absorbed photons or electromagnetic radiation. The light is emitted in a longer wavelength compared to the absorbed light because of the loss of energy by vibrational relaxations or heat[7].

In particular, the mechanism of fluorescence is based on the absorption of a photon by a fluorophore causing the excitation of an electron from its ground state S_0, to an excited energy level S_1. The electron can rise to different vibrational levels on its excited state depending on the wavelength of the absorbed photon. As the excited state is very unstable, the electron relaxes to its ground state in nanoseconds emitting a photon in a longer wavelength[7] (figure 2).

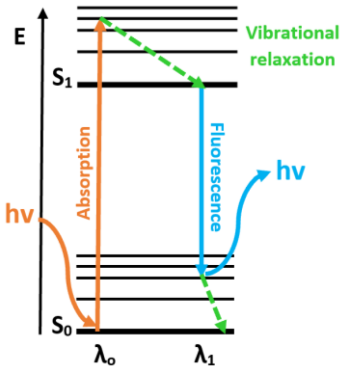


Figure 2. Jablonski's diagram explaining the effect of fluorescence

The substances that can emit light when they are excited are called fluorophores or chromophores.

3.2.2. FRET

Förster resonance energy transfer (FRET) is a phenomenon that describes the energy transfer between two light-sensitive substances known as chromophores. Specifically, part of the energy of the excited fluorophore is transferred to a second fluorophore in a non-radiative dipole-dipole coupling[7]. In particular, FRET is our main technique to study the interactions between our protein and a library of compounds.

The effect of FRET is visualized by Jablonski's diagram (figure 3). First, the donor (mClover3 in our study) absorbs energy and one electron raises from its ground state S_0 to the excitation level S_1. Then, the energy that is emitted from the donor is used to excite the second fluorophore (mRuby3 in our study). Finally, the second fluorophore emits electromagnetic radiation at a different wavelength. It is important to highlight that the origin of FRET is not the absorption by acceptor of a photon emitted by the donor[7][8].

As a result, a reduction in donor's fluorescence and an increase of acceptor emission intensity is observed[8].

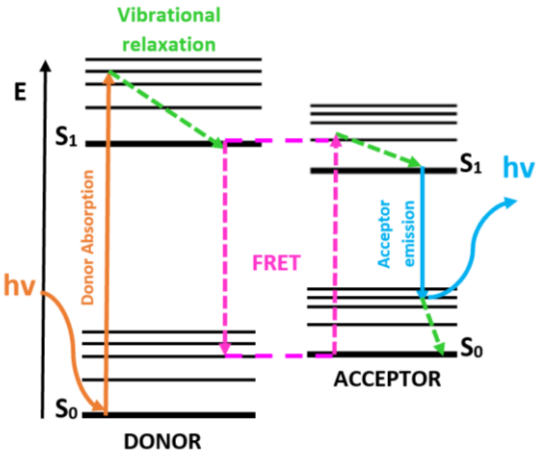


Figure 3. Jablonski's diagram explaining the effect of FRET

FRET has some conditions in order to occur[7]:

1. Overlap of the emission spectrum of the donor and acceptor excitation spectrum in order to have a sufficient energy coupling between fluorophores. An overlap of 30% is enough to have a reliable detection (figure 4).
2. The distance between the donor and acceptor must be in the range of 1-10 nm.
3. Right orientation of the dipole vectors of the donor and acceptor.
4. High quantum yield of the donor. This means that a higher response from the donor with the excitation signal, the higher quantum yield.

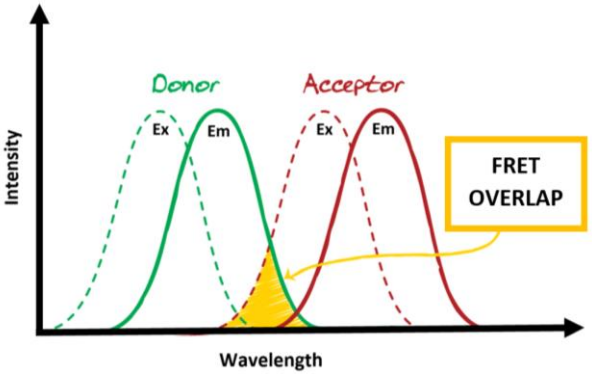


Figure 4. FRET overlap spectrum

The distance between the donor and acceptor is an important parameter because the efficiency of FRET (Eq.1) depends on R_0, the Förster distance, which describes the distance of this pair donor-acceptor when the efficiency is 50% and R is the actual distance between the fluorophores[9].

$$\boldsymbol{Efficiency} = \frac{\mathbf{1}}{\mathbf{1} + \left(\frac{\boldsymbol{R}}{\boldsymbol{R_o}}\right)^{\mathbf{6}}}$$

(Eq.1)

3.3. FRET- BIOSENSOR

FRET is highly sensitive to the distance between donor and acceptor within the 1-10nm range, using this characteristic, FRET-biosensors were designed. They are composed of fluorophores and sensing domains and have been widely adopted as spectroscopic rules to monitor a variety of biochemical activities that produce changes in molecular proximity, such as

protein-protein interactions, conformational changes, intracellular ion concentrations, and enzymes activities[10]. The major advantage of them is that they are non-destructive and minimally invasive for cells. Depending on whether the two fluorophores are conjoined to the same molecules, FRET-biosensors can be classified as intramolecular (donor and acceptor are in the same molecule) or intermolecular type (donor and acceptor are in different molecules). It is crucial in FRET-biosensor choose optimal FRET pair (donor and acceptor fluorophores)[11].

An intramolecular FRET-biosensor has been designed by Biomolecular NMR group to study the intrinsically disordered region of c-Src. In particular, it includes the SH4, Unique and SH3 domains flanked by two fluorescent proteins mClover3 (green fluorescent protein) and mRuby3 (red fluorescent protein) forming a FRET pair (figure 5). These fluorescent proteins are characterized by their brightness, photostability and wide range of distance measurable by FRET[12][8][13].

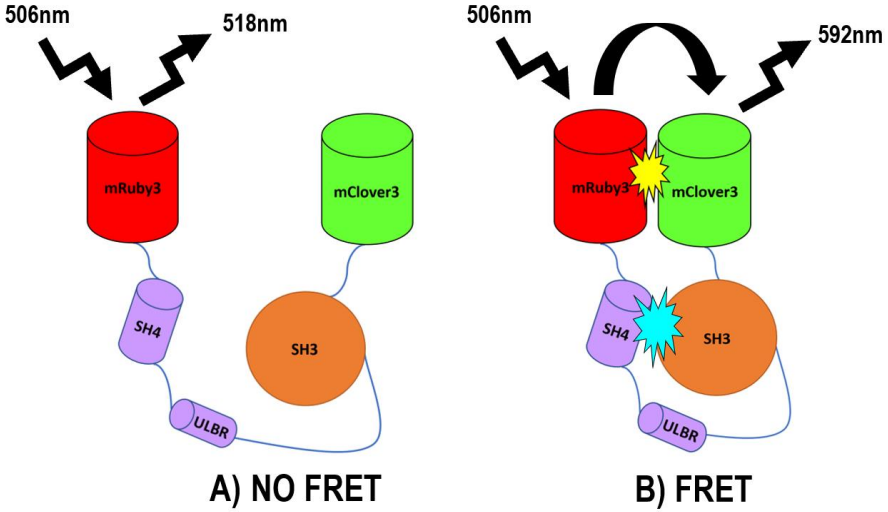


Figure 5. Intramolecular FRET-biosensor. **(A)** No FRET effect **(B)** FRET effect due to a conformational change caused by internal alterations (blue star) and both fluorescent proteins approach (yellow star).

The FRET-biosensor is called CLOBY from **Clo**ver and Ru**by** and its sequence is:

mClover3 - linker - SH3 - Unique - SH4 - linker - mRuby3

GHHHHHHHHHSSGHIDDDDKHMVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEG
DATNGKLTLKFICTTGKLPVPWPTLVTTFGYGVACFSRYPDHMKQHDFFKSAMPEGYV
QERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHYVY
ITADKQKNCIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSHQSKLSK
DPNEKRDHMVLLEFVTAAGITHGMDELYK GGGENLYFQGS NKSKPKDASQRRRSLE
PAENVHGAGGGAFPASQTPSKPASADGHRGPSAAFAPAAAEPKLFGGFNSSDTVTSP
QRAGPLAGG VTTFVALYDYESRTETDLSFKKGERLQIVNNTEGDWWLAHSLSTGQTG
YIPSNYVAPSDS GSAGGANA MVSKGEELIKENMRMKVVMEGSVNGHQFKCTGEGEG
RPYEGVQVMRIKVIEGGPLPFAFDILATSFMYGSRTFIKYPADIPDFFKQSFPEGFTW
ERVTRYEDGGVVTVTQDTSLEDGELVYNVKVRGVNFPSNGPVMQKKTKGWEPNTEMMY
PADGGLRGYTDIALKVDGGGHLHCNFVTTYRSKKTVGNIKMPGVHAVDHRLERIEESD
NETYVVQREVAVAKYSNLGGGMDELYK

The green sequence is mClover3 and the red one is mRuby3. Moreover, there's a His-tag at the beginning, the blue sequence indicates the SH3 domain, the pink one the UD and the purple SH4 domain. The c-Src sequence is connected with the pair of fluorophores through two linkers (yellow sequence).

The chromophores (figure 6) are originated from a spontaneous post-translational modification of three amino acids residues. In figure 6 is observed the structure of each chromophore after a post-translational process:

- **mRuby3:** Gln66-Tyr67-Gly68
- **mClover3:** Ser5-Tyr66-Gly67

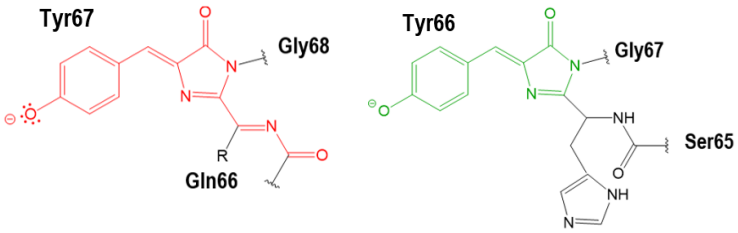
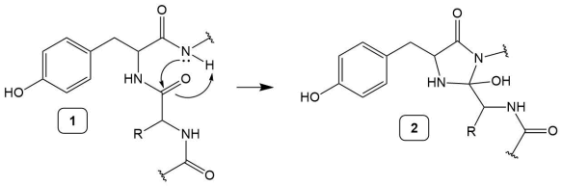


Figure 6. Chromophore structure of mRuby3 (red chromophore) and mClover3 (green chromophore)

3.3.1. Maturation time for fluorescent proteins (Chromophores)

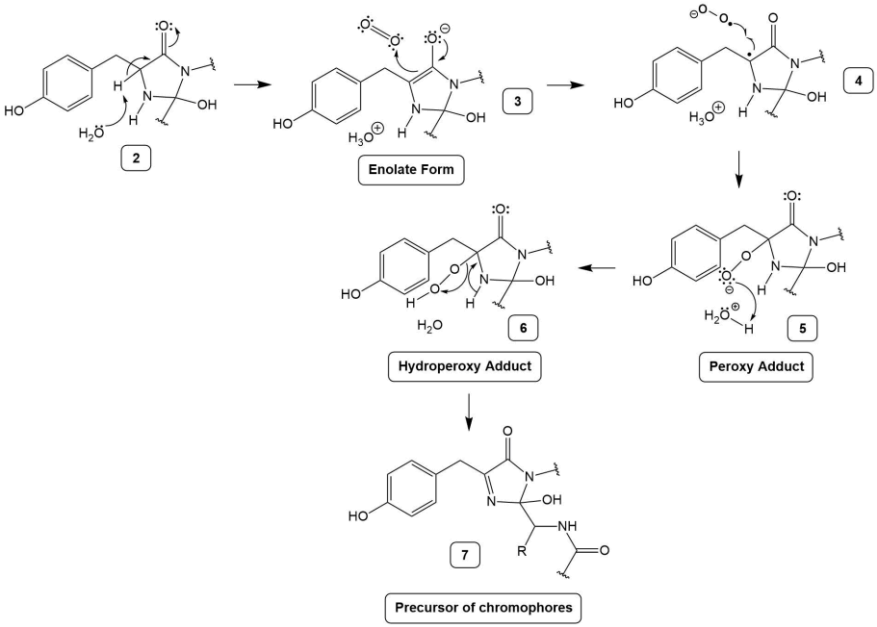
Fluorescent proteins have become a dominant tool for the exploration of the dynamics and localization of the macromolecular contents of living cells. They are used in several biological molecular applications[14]. They are fundamental for FRET-biosensor structure. However, fluorescent proteins have both advantages and disadvantages. Once a fluorescent protein is expressed it has to go through several stages until it becomes functional. These processes are known as "maturation". Until completion of maturation process, the protein, even though already synthesized, is not fluorescent. For this reason, it is important to consider the maturation process of fluorescent protein to obtain a functional FRET-biosensor[15]. Maturation process for mRuby3 and mClover3 has been studied and proposed mechanism has been described to produce red or green signals respectively.

The first steps in the maturation process are common for the two chromophores. Firstly, the formation of the chromophore starts with the cyclization of the main chain to form a five-membered ring intermediate (scheme 1). There is a nucleophilic attack by the pair of electrons present on the nitrogen of the amino acid to the carbonyl of the amide (-CONH-) so as to form a cycle of five. Moreover, there is a protonation in order to form an alcohol[16].



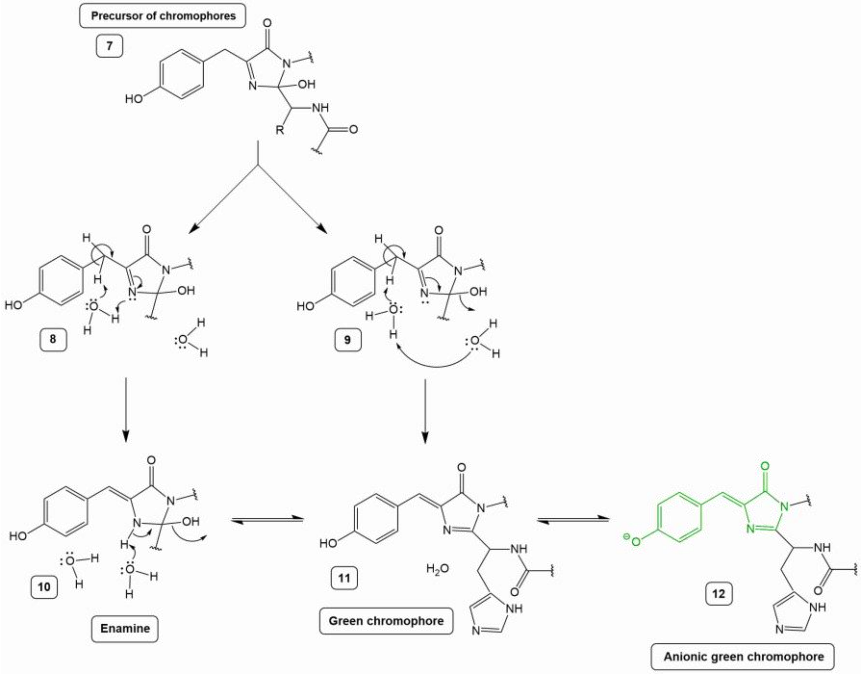
Scheme 1. Mechanism of maturation of chromophores (cyclation)

Then, it takes place an oxidation in order to obtain the precursor chromophore (scheme 2). Specifically, the hydrogen of a β-carbon is attacked by the oxygen of a water molecule which acts as a base in order to form an enolate. The enolate transfers an electron to molecular oxygen present in the medium starting a radical reaction which results on the formation of peroxy adduct. The peroxy adduct reacts with hydronium ions present in the medium to form the hydroperoxy adduct which is unstable. Finally, the hydroperoxy takes a proton from the nitrogen present in the ring giving the mature precursor chromophore[17].



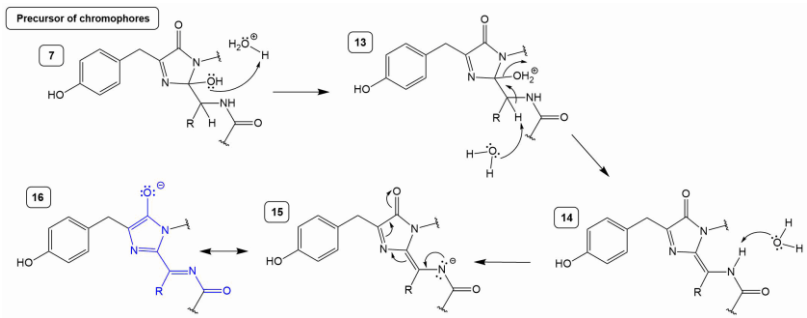
Scheme 2. Mechanism of maturation of chromophores (oxidation)

The green chromophore is obtained by an alcohol removal reaction (scheme 3). Specifically, an electron pair of oxygen's water attacks to a hydrogen forming a double bound. Then, the alcohol is removed by water's attack to a hydrogen of the ring. Alcohol is not a good leaving group so as to be removed it has to protonated in some step, for instance by hydronium ion. Finally, a conjugate structure that represents the green chromophore is obtained. The final colour of the chromophore depends on which amino acid is in the chain (R), in the case of green chromophore is histidine[17].



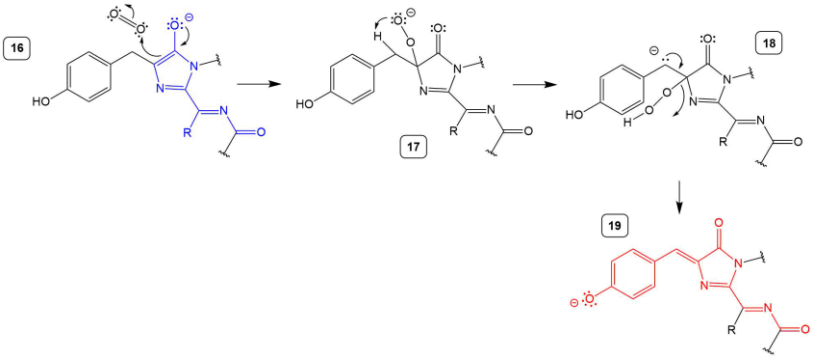
Scheme 3. Mechanism of maturation of chromophores (formation of green chromophore)

The red chromophore is obtained by a dehydration on α-hydrogen respect the alcohol (scheme 4). First, the alcohol is protonated to become a good outgoing group. Then, an elimination reaction takes place when an electron pair of oxygen's water attacks to the α-hydrogen respect the alcohol so as to eliminate -OH_2^+. Finally, the oxygen of a water molecule, which acts as a base, removes a proton from the amide, so that the conjugation is obtained and the blue intermediate is formed.



Scheme 4. Mechanism of maturation of chromophores (formation of blue intermediate)

Finally, an oxidation from the hydrogen of β-carbon results as the formation of the red chromophore (scheme 5). Specifically, a nucleophile attack takes place to oxygen. Then, the peroxy adduct (17) acts as a base in order to form an hydroperoxy adduct (18) which is very unstable and promote the formation of the red chromophore.



Scheme 5. Mechanism of maturation of chromophores (formation of red chromophore)

All the reactions take place in biochemical conditions where some reactions that are not normally spontaneous can take place.

3.4. DRUG BINDING TO FRET-BIOSENSOR

The accurate determination of binding interactions plays an important role in fields such as pharmacology. Binding is often determined by monitoring the response caused by varying one of the binding partners in the experiment, the drug (L) in our assay[18]. The binding between a protein and its ligand can be represented quantitatively by the flowing expression:

$$\mathbf{P + L \rightleftharpoons PL} \qquad K_a = \frac{[PL]}{[P][L]} \qquad \textbf{(Eq. 2)}$$

Where **P** represents the free protein, **L** the free ligand and **PL** the complex ligand-protein. From this equilibrium, it is possible to establish a constant that describes the relation between the quantity of products and the number of reagents (Eq. 2). K_a represents the association constant of the complex protein-ligand and it is used as a measure of the affinity of the protein for the ligand. A higher value of K_a is synonymous of more affinity from protein to the ligand[19].

A graph with the concentration of the free ligand in the X axis versus this relation [PL] /[P_T] is represented so as to obtain the concentration of the complex protein-ligand by equation 4. Once [PL] is known, K_a can be determined by equation 2[19].

So that to plot fluorescence measures this relation is used:

$$\frac{[PL]}{[P_T]} = \frac{F_\infty - F}{F_\infty - F_o} \qquad \textbf{(Eq. 3)}$$

Equation 4 is deduced from the combination of the following relations[19]:

$$K_a = \frac{[PL]}{[P][L]} \qquad C_P = [PL] + [P] \qquad C_L = [PL] + [L]$$

$$\boldsymbol{[PL]^2 K + [PL] K(-C_L - C_P - 1) + C_L C_P K = 0} \qquad \textbf{(Eq. 4)}$$

Abstract nomenclature:

- **[PL]** → concentration of complex protein-ligand.
- **[P]** → concentration of free protein.
- **[P_T]** → total concentration of protein.
- **[L]** → concentration of free ligand.
- **F** → fluorescence at a determinant point.
- **F_o** → minimum value of fluorescence.
- **F∞** → highest value of fluorescence.
- **C_P** → initial protein concentration.
- **C_L** → initial ligand concentration.

3.5. STATE OF ART

High levels of c-Src activity have been related with breast, prostate and colorectal cancer. Previous studies achieved by the Biomolecular NMR group managed by Dr. Miquel Pons at University of Barcelona have demonstrated the importance of Unique domain as a regulator of c-Src activity. This group has proved that the invasive potential effect of a type of colon's cancer's cells can be reduced by 50% changing a single amino acid in UD[6]. These results demonstrate the importance of regulatory roles of disordered domains as well as organized regions such as SH1, SH2 and SH3.

Previous studies conducted by Biomolecular NMR group allowed to design a FRET-biosensor called CLOBY in order to study the intrinsically disordered region of c-Src. Understanding the regulation of these proteins is important in order to use them as therapeutic targets. Specifically, in this project a screening of compounds is done with CLOBY (FRET-biosensor) in order to know if any of them can interact with SH4, SH3 or the intrinsically disordered region of c-Src.

4. OBJECTIVES

The main objective of this final degree project is to screen a library of proposed anti-cancer compounds on a FRET-biosensor of c-Src and study changes produced between SH4, Unique and SH3 domain by FRET variation of two fluorescent proteins anchored to its C-terminal and N-terminal. In order to answer this question, a set of specific objectives are defined, as follows:

- Expression and purification of the construct mClover3-linker-SH3-Unique-SH4-linker-mRuby3 (FRET-biosensor of c-Src).
- Test thirty compounds obtained from a library of compounds which was approved by the food and drug administration (FDA) and assets for cancer.
    - Classification of compounds according to their FRET's effect.
    - For FRET's effect positive compounds, calculation of their binding association constant.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Materials

Plasmid pET-19 and the bacterial cell BL21(DE3) are from Novagen. Reagents used in this project have been purchased from Sigma Aldrich and Aplichem Panreac. All aqueous solutions were prepared using water purified with Millipore BioCell system and also, their compositions are shown in Appendix 1. The thirty compounds that had been studied in this project were approved by FDA. However, for reasons of intellectual property protection, we cannot provide additional information of the same.

5.1.2. Instrumental

Instrument	Model	Software
Nickel Cartridge affinity column	His Trap™ HP 1mL	-
FPLC	AKATA FPLC W FRAC-950	Unicom 5.20
UV-Vis Spectrophotometer	Shimadzu UVmini-1240	UV Probe 2.42
Fluorimeter	Photon Technology International	Felix32™

5.2. SYNTHESIS OF THE FRET-BIOSENSOR

In order to synthesize the biosensor, three different steps have been performance: transformation, expression and purification.

5.2.1. Transformation

The DNA can be transfected into bacteria by transformation method (figure 8). Circular DNA fragments, which are called plasmids, contain the genes for the biosensor as well as the genes

that encode resistance to certain antibiotics, such as ampicillin (figure 7). Therefore, a selective medium containing an antibiotic was used so that only cells that contain the gene that encodes the resistance of a specific antibiotic can grow. In particular, the plasmid used was pET-19 (Novagen) and the bacterial cell line was BL21(DE3) (Novagen). This transformation was achieved by adding DNA plasmid to a cell stock of competent cells in an Eppendorf microtube of 1mL (normally 1µL from plasmid stocks between 80-100ng/µL). Then, cells were placed for 1minute 30seconds at 42 ℃ in a water bath. In these conditions, pores were formed in the membrane allowing DNA to enter into the cell. After that, cells were placed back in ice for 2minutes. Transformed cells can be stored as glycerol stocks at - 80 ℃.

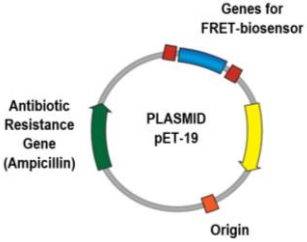


Figure 7. Structure of pET-19

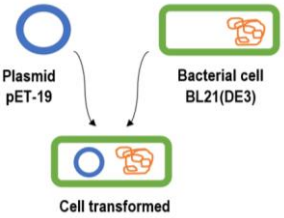


Figure 8. Protein transformation

5.2.2. Protein Expression

The protein expression starts from glycerol stocks already prepared in the previous step.

Firstly, a pre-culture step was done. It consists in amplifying a cell stock during an overnight period before starting a large scale growing (expression). In one falcon tube of 50mL, luria broth (LB) previously autoclaved was added (10mL*Number of expression flasks) with the corresponding antibiotic, in our case ampicillin (10µL*Number of expression flasks). Then, it was divided into the necessary falcon tubes to avoid a poor growth produced by a bad oxygenation. About 40µL of the aliquot of glycerol stocks was added to each falcon tube. Finally, these falcon tubes were left shaking at 37 ℃ overnight.

The next day, the content of the two falcon tubes was added to 2L flask of LB culture medium which had been previously autoclaved. Then, ampicillin was mixed in the flask (1µL of antibiotic for each mL of water). These cultures were incubated at 37 ℃ at 140rpm until the optical density (OD) at 600nm was 0.6 approximately. After, the protein expression was induced

by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1mM. When the induction started, cultures were incubated at 25 ℃ at 140rpm until were totally maturated (figure 9). In order to obtain the protein, cultures were centrifugated at 4500rpm for 20minutes at 4 ℃ and the supernatant was removed. In a sterile 50mL falcon tube, the remaining pellet was resuspended with lysis buffer, 500µL of protease inhibitor cocktail (PiC), 500µL of phenylmethylsulfonyl fluoride (PMSF) and stored at -80 ℃ till the start of purification.

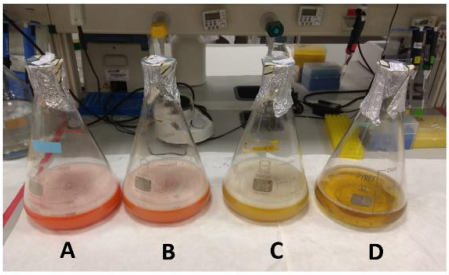


Figure 9. Guide of cell cultures: **A)** proper maturation of fluorescent proteins **B)** not enough maturation yet **C)** particles in suspension (NO maturation yet) **D)** LB after autoclave

5.2.3. Protein Purification

The resuspended pellet, obtained in the expression protocol, was thawed in a bucked with water in the cold room at 4 ℃. When it was thawed, dithiothreitol (DTT) was added at a concentration of 1mM. Then, the pellet was sonicated for 1minute 30seconds in intervals of 10 seconds repeating it three times. After that, a DNAase aliquot was added at a final concentration of 1µM and the sample was centrifuged at 20000rpm for 20minutes. This step was done to obtain the supernatant with the desired protein and discard cells organelles and membrane's debris from the pellet.

The supernatant was purified using the Nickel Cartridge affinity column which was connected to a peristaltic pump to produce a flux through the column and capture the protein of interest. Specifically, the desired protein has strong interaction with immobilized metal ion matrices due to its six consecutive histidine. After the protein was kept for Nickel column, it was eluted using a characteristic elution buffer that contains a high concentration of imidazole which competes for the active binding site and releases the protein from the column.

After the first purification, the sample was introduced into the fast protein liquid chromatography (FPLC) in order to separate the purest fractions of the desired protein. This is a

size exclusion chromatography. Before to start, it is important to condition the column with NaOH, water and buffer F which is going to be used to separate the protein for its biological characterises. When the chromatography was finished, only those fractions, that represent the relevant peak in the obtained chromatogram, were collected and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify the purest fractions. This part consists in denaturalising the protein by loading buffer before being introduced in the SDS-gel. In particular, one of the components of the loading buffer is SDS. Therefore, the presence of SDS in the sample allows to charge proteins with a negative charge in order to separate by their molecular weight with an electrophoretic method. By the SDS-PAGE gel, the purest fractions of the FRET-biosensor called Cloby were identified.

5.3. CONCENTRATION MEASURE

Analysis of the absorbance caused by chromophores was measured by UV-Vis spectrophotometer from 250nm to 650nm in order to calculate the concentration of the protein and each of its chromophores by Beer-Lambert's law:

$$\boldsymbol{A = l \times \varepsilon \times C}$$

In particular, **A** represents the amount of light absorbed by the sample at a certain wavelength, **ε** the molar absorptivity, **l** the distance of the cuvette which is 0.1cm and **C** the concentration of the absorbent species.

Aromatic residues such as tryptophan and tyrosine had an absorption maxim at 280nm. The chromophores of the fluorescent protein had maxim at 506nm for mClover3 and 558nm for mRuby3.

5.4. FRET ASSAY

Fluorescence's spectrums were measured by the fluorimeter of Photon Technology International (PTI) and processed with spectroscopy software called Felix 32™. The lamp of the fluorimeter was a mercury's lamp.

Screening analysis was performed with a ratio of 9:1 Buffer(FRET-Biosensor):DMSO(Drug) and the samples were prepared in Eppendorf microtubes. The concentration of FRET-Biosensor was 1μM and the final concentration of the solution was 100μM. As a control, a blank was done with a ratio of 9:1 Buffer:DMSO(Drug). So as to obtain data, samples were introduced into a quartz cuvette Hellma 8mm × 2mm.

In order to visualize the FRET phenomenon, the sample was excited at the wavelength of mClover3 (506nm) and then, emission spectrum was observed between 516nm-650nm. Therefore, the emission spectra of mClover3 and mRuby3 were obtained.

The excitation and emission wavelength for each chromophore is respectively is:

- **Excitation:**
  - mClover3: 506 nm
  - mRuby3: 558 nm
- **Emission:**
  - mClover3: 518nm
  - mRuby3: 592nm

5.5. BINDING ASSAY

Binding assay was also done with the fluorimeter of Photon Technology International (PTI) and processed with spectroscopy software called Felix 32™.

In this case, solutions from the range of 100μM to 0,1μM were prepared with a ratio of 9:1 Buffer(FRET-Biosensor):DMSO(Drug) with a constant concentration of FRET-Biosensor 1μM. To obtain data, samples were introduced into a quartz cuvette Hellma 8mm × 2mm. Also, the sample was excited at the wavelength of mClover3 (506nm) and then, the emission spectrum was observed from 516nm just 650nm.

6. RESULTS AND DISCUSSION

6.1. FRET-BIOSENSOR SYNTHESIS AND PURIFICATION

In order to confirm that the FRET-biosensor was correctly obtained, some tests had been realized such as mass spectrometry and SDS-PAGE gel into the different column fractions: flow-through fraction (FT), wash fraction (W), elution fraction (E) and several column factions (D9-E7).

6.1.1. SDS-PAGE gel

SDS-PAGE gel is a very common method for separating proteins of interest, due to the size protein, by electrophoresis. In this project, it was used to identify the purest fractions of protein purification and therefore, verify if the purification had been correctly done.

As already explained, firstly the FRET-biosensor was purified by a nickel affinity column and some fractions were collected (FT, W and E). Then, the elution fraction (E) which contained the desired protein was completely purified by the FPLC. According to the obtained chromatogram (figure 10), fractions of the main peak were chosen (D9-E7). Then, an SDS-PAGE gel was done to choose only the fractions in which not impurities were observed. The SDS-PAGE gel is shown in figure 11.

The flow-through fraction (FT) was obtained when the impure protein was loaded inside the nickel affinity column. In this fraction, we observed several brands that were the whole cell expression that had not interact with the column. In the wash fraction (W), there were four clearly brands. One band corresponded to the desired protein and the other three bands were proteins with non-specific interactions with the column. Finally, in the elution fraction (E) the desired protein was totally eluted because the elution buffer contained high concentration of imidazole (400mM) that released the desired protein from Ni-NTA column due to the presence of six consecutive histidine in the desired protein. In figure 11, the circle of the SDS-PAGE gel indicates the positive band for the desired protein.

According to SDS-PAGE gel, fractions of D10 to E1 were the purest because they had the positive band, the desired protein. The molecular marker (M) is used to identity approximately the zone where the protein has to be located depending on its molecular weight. Moreover, in the SDS-PAGE gel we observed a degradation zone. This was confirmed by FPLC (figure 10). In FPLC spectra, we could observe a big peak of the desired protein (FRET-biosensor, CLOBY) and another zone just closer that corresponded a degradation of the protein. This degradation was caused for the partial maturation of the chromophores.

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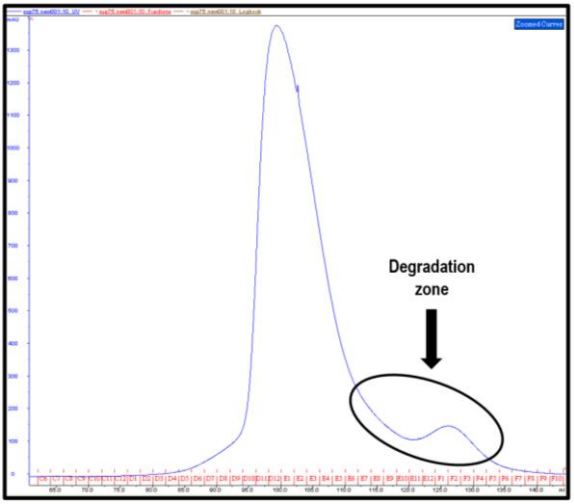


Figure 10. FPLC spectra of FRET-biosensor

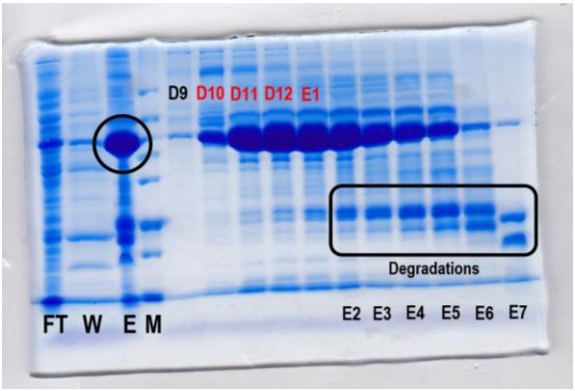
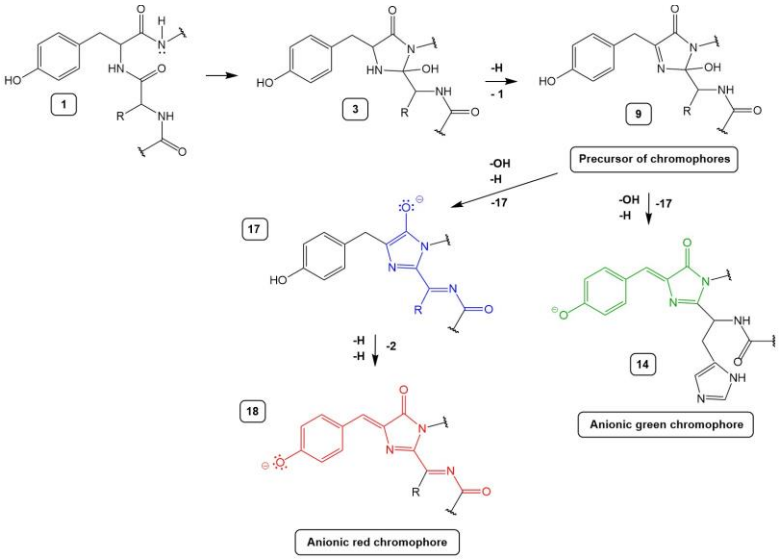


Figure 11. SDS-PAGE gel showing the purification of FRET-biosensor

6.1.2. Mass Spectrometry Analysis (LC-MS)

The purest fraction obtained by the FPLC was concentrated in order to send it to analyse by mass spectrometry.

Based on the mass spectrum obtained (figure 12), it was observed a significant peak of high abundance which coincided with the molecular weight of the biosensor taking into account the loss of some molecules by a post translational process (scheme 6) in order to convert the FRET-biosensor into a fluorescent substance.



Scheme 6. Maturation reactions of chromophores

Specifically, the molecular weight of the protein is 72.68 kDa. Considering this loss, the final molecular weight of the biosensor is: 72680-20-18= 72642 Da. That value matches with the significant peak of the mass spectrum. With this result, we can conclude that FRET-biosensor has been synthesize and purified correctly.

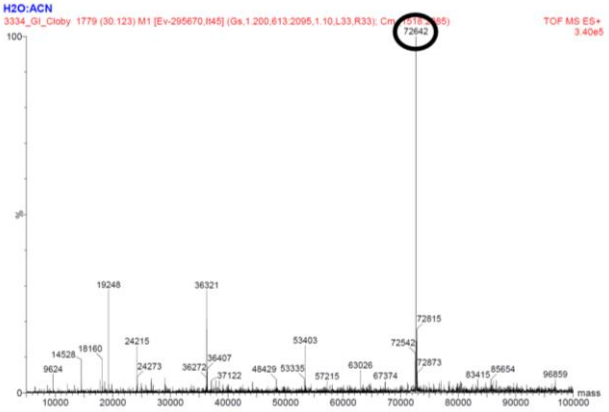


Figure 12. Mass spectrometry results of FRET-biosensor

6.2. MATURATION OF THE CHROMOPHORES

As already explained, in order to obtain the fluorescent protein a maturation's process takes place in some steps. The maturation mechanism is spontaneous and also time dependent, between two or three days are necessary to obtain the complete chromophores' maturation.

A quantitative evidence in order to determine the final concentration of each chromophore was done by an analysis UV-Vis. The results are shown in table 1.

	FRET-biosensor	**mClover3**	**mRuby3**
Wavelength [nm]	280	506	556
Molar absorptivity ε [$mM^{-1}cm^{-1}$]	66.24	109	128
Final concentration [%]	100	90-87	62-55

Table 1. Final concentration for FRET-biosensor and each chromophore at its wavelength

With these results, we can conclude that there was a fraction of mRuby3 that was not totally maturated. Therefore, we can conclude that maturation of mRuby3 is more complicated than mClover3 because more steps are necessary such as two oxidations. A correct oxygenation of the flasks could be an option to solve that issue.

6.3. FRET ASSAY

As already explained, the objective of this part was to screen a library of thirty compounds in order to detect FRET variation of mRuby3 and mClover3 because of changes produced between SH4, Unique and SH3 domains.

In particular, the screening of the thirty compounds consisted in measuring the fluorescence emission of FRET-biosensor from 516nm to 650nm. The sample was excited at the wavelength of mClover3 (506nm) so as to plot the intensity emission as a function of wavelength.

In addition, for each compound, a control of FRET-biosensor with Tobacco Etch Virus (TEV) was done in order to have a control with no FRET to confirm the absence of interactions between drugs and the fluorescent proteins. In particular, TEV is a protease which cuts the amino acid sequence of proteins. Also as another control, a screening of the blank of drugs was done with the aim to analyse if any of them present absorbance in the region to study. No one of the compounds that tested positive in the FRET's study had absorbance in that region.

Based on the results of the screening, compounds had been classified according to their FRET's effect (table 2). In particular, they were organized in two groups: the active compounds which presented a FRET's effect and the inactive compounds that no FRET's effect was observed. The group of active compounds was classified in two subgroups according to the difference of fluorescence's signal between the FRET-biosensor and the compound + FRET-biosensor at the wavelength of mRuby3.

ACTIVE COMPOUNDS		INACTIVE COMPOUNDS		
L13B-C2 L10B-C9	(236862 - 116404 counts/s) Pronounced FRET's effect	L04B-D2	L11B-H9	L16B-A10
		L06B-B2	L12B-B2	L17B-B9
L17B-C2 L06B-F9 L16B-H9 L17B-H9 L18B-H7	(81290 - 50311 counts/s)	L06B-F1	L13B-C2	L17B-G1
		L06B-F7	L13C-D2	L17B-G10
		L06B-F9	L14C-D2	L17B-H10
		L08B-H8	L14B-G1	L18B-B7
		L10B-A10	L15B-F1	L18B-C2
		L10B-E9	L15B-H8	

Table 2. Classification of the thirty compounds according to FRET's effect

To explain this classification, the most representative results are showed below.

The figure 13 shows an example of an inactive compound. No effects were observed, so the profile of the compound was the same as FRET-biosensor. Moreover, in figure 14 it was observed the same effect for the control with FRET-biosensor TEV. With these results we conclude that the presence of this type of compounds do not cause any effect to the protein. Therefore, there is not approximation between donor and acceptor chromophore.

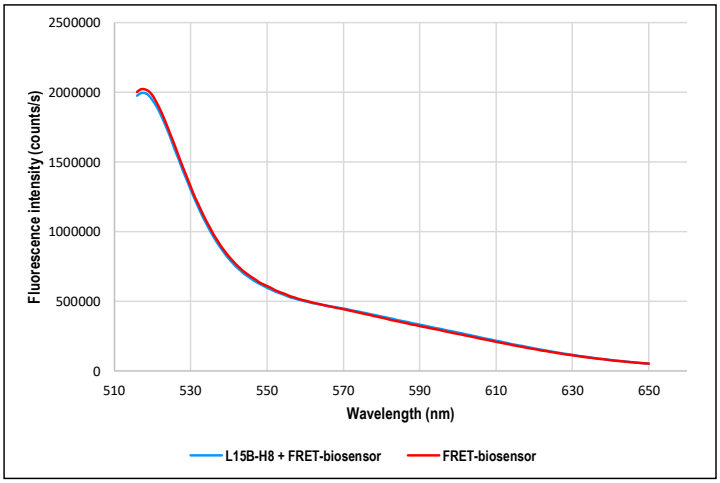


Figure 13. Excitation and emission spectrum of L15B-H8 + FRET-biosensor

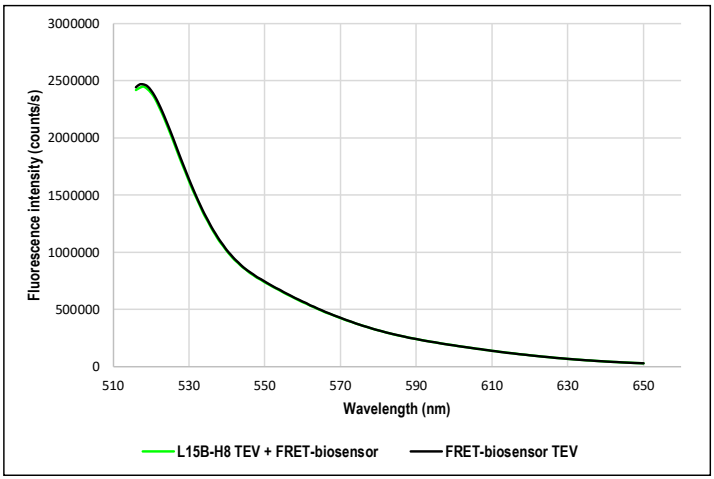


Figure 14. Excitation and emission spectrum of L15B-H8 TEV + FRET-biosensor

On the other hand, two compounds were found to present a pronounced FRET's effect with a wide range of fluorescence signal at the wavelength of mRuby3 (point 2) (236862 - 116404 counts/s). First, it is important to highlight that the first raised signal is from the chromophore mClover3 and the second signal is from mRuby3.

In figure 15 was observed that while fluorescence of donor was reduced (point 1), fluorescence of the acceptor was increased (point 2) when the drug was added in the FRET-biosensor (blue line). Therefore, a FRET's effect was observed because part of the energy of the excited chromophore (mClover3) was transferred to the second chromophore (mRuby3). However, the location of the drug in the FRET-biosensor was not known. With this result, we can only affirm that there is an approximation between the two chromophores.

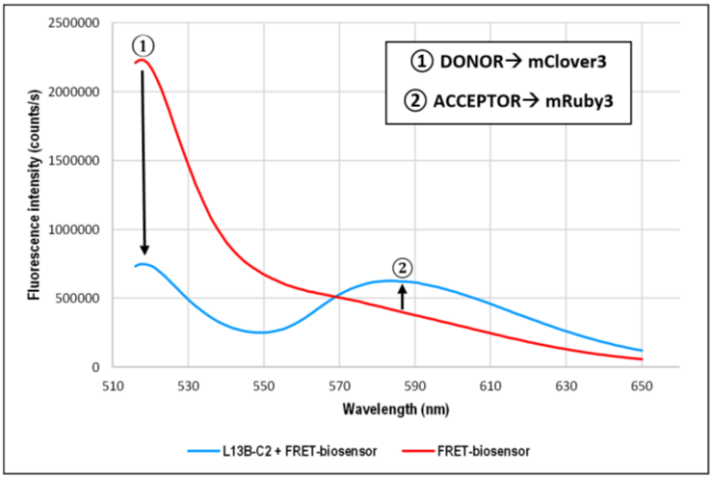


Figure 15. Excitation and emission spectrum of L13B-C2 + FRET-biosensor

Analysing the spectrum of the former compound with TEV (figure 16), there was an important decrease of the FRET signal. Specifically, there was a little change on the fluorescence of the donor which was reduced (point 1) and also the fluorescence of the acceptor changed (point 2) compared to figure 15.

With these results we can conclude that there is a less FRET's effect when the protein was cut by TEV. From this control we could suppose that the tendency to react between the drug and the FRET-biosensor was higher when it was fully connected.

This diminished FRET's effect could be caused by these two reasons:

- TEV could not be 100% efficient. Therefore, there is still complete protein in the medium which can suffer FRET.
- Drug could interact directly to donor or acceptor modifying the system. Therefore, chromophores may interact in spite of the FRET-biosensor being cut.

An example of an active compound in the range (81290 - 50311 counts/s) is shown in Appendix 2.

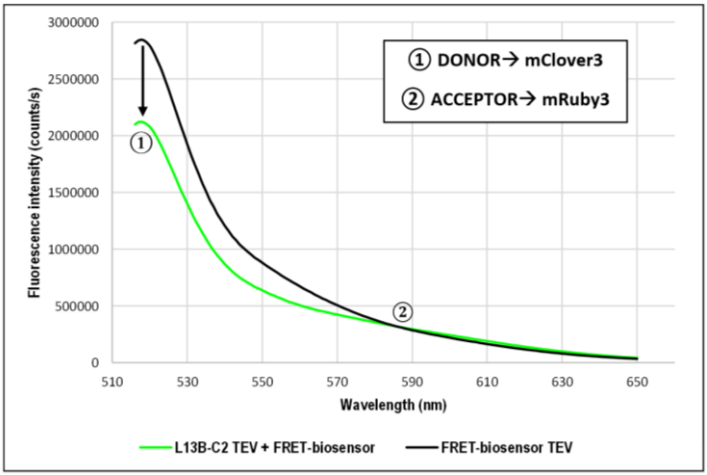


Figure 16. Excitation and emission spectrum of L13B-C2 TEV + FRET-biosensor

6.4. BINDING ASSAY

In this experimental part, a binding assay of the two compounds that presented a pronounced FRET's effect (L13B-C2 and L10B-C9) was done with two replicates for each case. The experiment was performed with a range of drug's solutions from 100μM to 0,1μM with a constant concentration of FRET-biosensor 1μM.

Based on the intensity obtained with the fluorimeter, we decided to focus the study at the emission wavelength of mClover3 (518nm) and mRuby3 (592nm). All samples had been represented by (F_∞-F/ F_∞-F_o) versus drug concentration [L] at a certain wavelength 518nm and 592nm.

The figure 17 shows the results of L10B-C9 at a wavelength of 518nm. Analysing the graph, two zones were clearly visible. Firstly, there was a first zone (from 0μM to 10μM) with a negligible effect of the drug on the observed fluorescence. From 10μM there was a strong

response that could be fitted to an apparent 1:1 model of binding. The abrupt change in response around 10μM was reminiscent of the response of compounds forming micelles and the critical concentration at which the changes were observed would correspond to the critical micelle concentration (CMC). L10B-C9 is a highly hydrophobic molecule with a single charge and a low aqueous solubility. Thus, the hypothesis that the active species was actually a micellar form of L10B-C9 is reasonable.

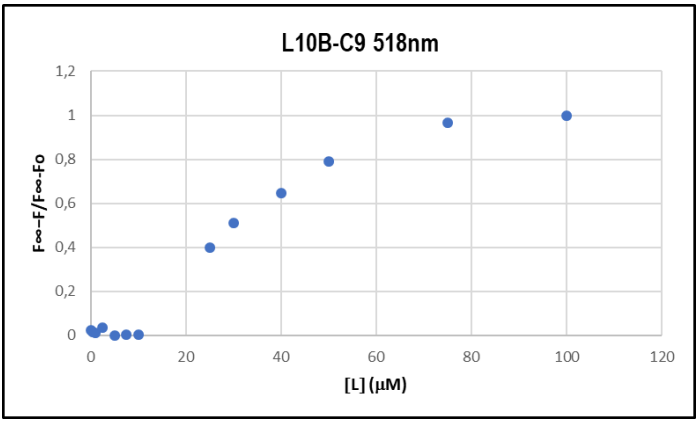


Figure 17. L10B-C9 curve form 0μM to 100μM at 518nm

The figure 18 shows the results of L10B-C9 at a wavelength of 592nm. Fluorescence at 592nm, when exciting at 506nm, was mainly the result of FRET and reflected the proximity of the FRET-biosensor. However, changes in the fluorescence of the donor would also affect the observed fluorescence at 592nm: bellow 10μM there was a negligible response. Above 10μM the fluorescence at 592nm was dominated by a strong FRET suggesting that interaction of the sensor with L10B-C9 (micelles) reverted in the approximation of the two fluorescent proteins.

The profile of the curve in the figure 18 did not adjust to a quadratic equation and therefore, the binding constant could not be determined.

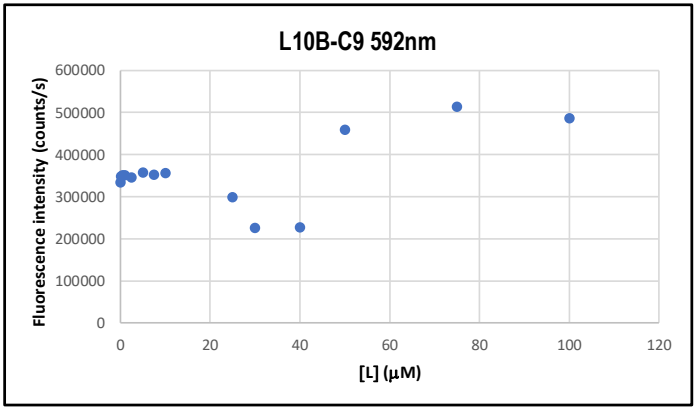


Figure 18. L10B-C9 curve form 0μM to 100μM at 592nm

The compound L13B-C2 showed the same profile as L10B-C9 in each wavelength. Its graphs are shown in Appendix 2.

Assuming that the second zone (10μM to 100μM) of the graph at 518nm followed a model's binding with a relation 1:1 protein:ligand, an approximate calculation of the apparent binding constant was done. First, the value of effective concentration was determined by subtracting the value of the actual concentration minus CMC. Then, the curve may be adjusted to a second-degree equation (figure 19). From this equation, the concentration of protein-ligand was determined and finally the binding association constant for each replicate was calculated. Then, the average of both replicates was calculated and its respective standard error of the mean (SEM).

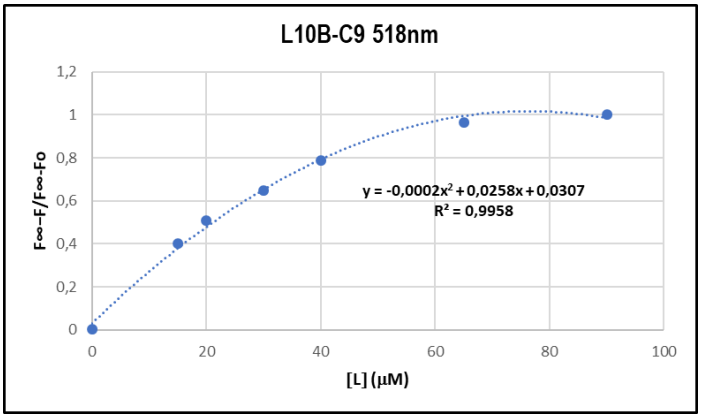


Figure 19. L10B-C9 curve form 0μM to 100μM at 518nm (effective concentration)

The results of all experiments which have been performed are shown in table 3.

DRUG	BINDING ASSOCIATION CONSTANT		
	Assay 1	Assay 2	Mean
L10B-C9	0,0077	0,0072	(0,0075±0,0003) μM^{-1} → **4% SEM**
L13B-C2	0,008	0,003	(0,006±0,002) μM^{-1} → **33% SEM**

Table 3. Results of the binding assay

According to the results of table 3, we may conclude that a part of our data fits to the binding model 1:1. In addition, after the analysis of the obtained values, we may conclude that L10B-C9 presents a higher affinity for the FRET-biosensor than L13B-C2 because the binding association constant is higher. The standard error of the mean for L13B-C2 was 33%, so the mean calculated is far from the real value of the real association constant. If we would be able to carry out more replicates, this error could be diminished.

7. CONCLUSIONS

The main conclusions that are obtained from this research are:

- The construct mClover3-linker-SH3-Unique-SH4-linker-mRuby3 (FRET-biosensor of c-Src) has been correctly expressed and purified, mass spectrum confirms it.
- The screening of compounds was successfully done. FRET has been studied with a wide range of drugs which have been classified according to that effect. Two compounds (L10B-C9 and L13B-C2) interact with the FRET-biosensor causing a spectacular FRET's effect.
- The interaction between these two former drugs and FRET-biosensor seems to take place when a minimum concentration of the drug is added. Probably, the active specie is actually a micellar form of the drug.
- Assuming a binding model protein-ligand 1:1, the compound L10B-C9 presents a higher affinity for the FRET-biosensor than L13B-C2.

8. REFERENCES AND NOTES

[1] E. Ingley, "Src family kinases: Regulation of their activities, levels and identification of new pathways," *Biochim. Biophys. Acta - Proteins Proteomics*, vol. 1784, no. 1, pp. 56–65, 2008.

[2] M. Maffei, M. Arbesú, A. L. Le Roux, I. Amata, S. Roche, and M. Pons, "The SH3 domain acts as a scaffold for the N-terminal intrinsically disordered regions of c-Src," *Structure*, vol. 23, no. 5, pp. 893–902, 2015.

[3] M. Arbesú *et al.*, "The Unique Domain Forms a Fuzzy Intramolecular Complex in Src Family Kinases," *Structure*, vol. 25, no. 4, pp. 630-640.e4, 2017.

[4] I. Amata, M. Maffei, and M. Pons, "Phosphorylation of unique domains of Src family kinases," *Front. Genet.*, vol. 5, 2014.

[5] Y. Pérez, M. Gairí, M. Pons, and P. Bernadó, "Structural Characterization of the Natively Unfolded N-Terminal Domain of Human c-Src Kinase: Insights into the Role of Phosphorylation of the Unique Domain," *J. Mol. Biol.*, vol. 391, no. 1, pp. 136–148, 2009.

[6] Y. Pérez *et al.*, "Lipid binding by the Unique and SH3 domains of c-Src suggests a new regulatory mechanism," *Sci. Rep.*, vol. 3, pp. 1–9, 2013.

[7] B. Hochreiter, A. P. Garcia, and J. A. Schmid, "Fluorescent proteins as genetically encoded FRET biosensors in life sciences," *Sensors (Switzerland)*, vol. 15, no. 10, pp. 26281–26314, 2015.

[8] C. L. Takanishi, E. A. Bykova, W. Cheng, and J. Zheng, "GFP-based FRET analysis in live cells," *Brain Res.*, vol. 1091, no. 1, pp. 132–139, 2006.

[9] F. Schaufele, I. Demarco, and R. N. Day, "FRET Imaging in the Wide-Field Microscope," *Mol. Imaging*, pp. 72–94, 2005.

[10] A. Miyawaki, "Development of Probes for Cellular Functions Using Fluorescent Proteins and Fluorescence Resonance Energy Transfer," *Annu. Rev. Biochem.*, vol. 80, no. 1, pp. 357–373, 2011.

[11] B. T. Bajar, E. S. Wang, S. Zhang, M. Z. Lin, and J. Chu, "A guide to fluorescent protein FRET pairs," *Sensors (Switzerland)*, vol. 16, no. 9, pp. 1–24, 2016.

[12] B. T. Bajar *et al.*, "Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting," *Sci. Rep.*, vol. 6, no. February, pp. 1–12, 2016.

[13] L. M. Costantini *et al.*, "A palette of fluorescent proteins optimized for diverse cellular

environments.," *Nat. Commun.*, vol. 6, no. May, p. 7670, 2015.

[14] S. J. Remington, "Fluorescent proteins: maturation, photochemistry and photophysics," *Curr. Opin. Struct. Biol.*, vol. 16, no. 6, pp. 714–721, 2006.

[15] E. Balleza, J. M. Kim, and P. Cluzel, "A systematic characterization of maturation kinetics of fluorescent proteins in live cells," *Nat. Methods*, vol. 6, no. November, pp. 1–10, 2017.

[16] P. Dedecker, F. C. De Schryver, and J. Hofkens, "Fluorescent proteins: Shine on, you crazy diamond," *J. Am. Chem. Soc.*, vol. 135, no. 7, pp. 2387–2402, 2013.

[17] O. Access, "GFP-Based Biosensors," *Long-Haul Travel Motiv. by Int. Tour. to Penang*, vol. i, no. tourism, p. 13, 2018.

[18] P. J. Tonge, "Quantifying the Interactions between Biomolecules: Guidelines for Assay Design and Data Analysis," *ACS Infect. Dis.*, 2019.

[19] P. Handcock and T. Cassidy, "Correction for Light Absorption in Fluorescence Studies of Protein-Ligand Interactions," in *Nsca Japan*, vol. 23, no. 10, 2016, pp. 52–56.

9. ACRONYMS

CLOBY	Clover Ruby
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography
FRET	Förster Resonance Energy Transfer
IDP	Intrinsically Disorder Region
IDR	Intrinsically Disorder Region
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Broth
LC-MS	Liquid Chromatography-Mass Spectrometry
OD	Optical Density
PiC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulfonyl Fluoride
PTI	Photon Technology International
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFK	Src Family Kinases
TEV	Tobacco Etch Virus
UD	Unique Domain
ULBR	Unique Lipid Binding Region

APPENDICES

APPENDIX 1: BUFFERS

– Lysis Buffer (pH 8) to 1L

Reagent	Concentration
TRIS-HCl	20mM
NaCl	300mM
Imidazole	10mM
NaN_3	0.01%

– Elution Buffer (pH 8) to 50 mL

The elution buffer is prepared by adding imidazole at a final concentration 400 mM to lysis buffer.

– Buffer F (pH 7.5) to 1L

Reagent	Concentration
NaH_2PO_4	0.2M
Na_2HPO_4	0.2M
NaCl	150mM
EDTA	0.2mM
NaN_3	0.01%

APPENDIX 2: GRAPHS' FRET - BINDING ASSAY

- **L17B-C2**

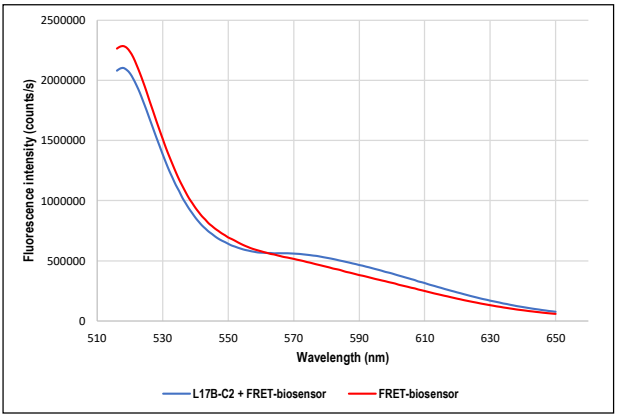


Figure 20. Excitation and emission spectrum of L17B-C2 + FRET-biosensor

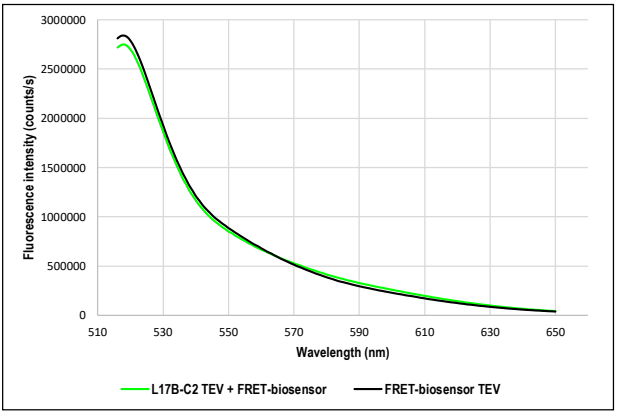


Figure 21. Excitation and emission spectrum of L17B-C2 TEV + FRET-biosensor

- **L13B-C2**

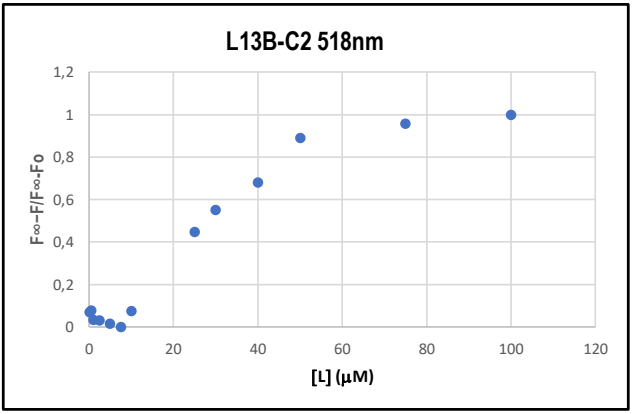


Figure 22. L13B-C2 curve from 0µM to 100µM at 518nm

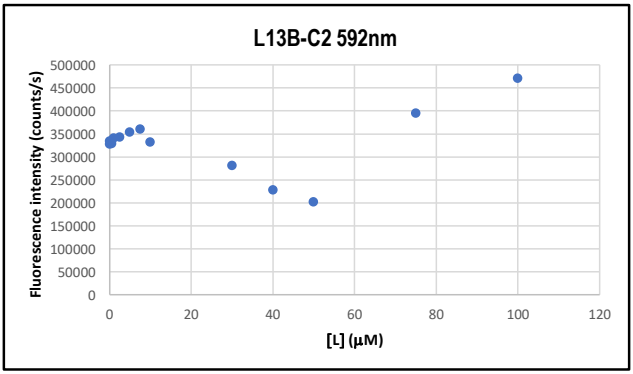


Figure 23. L13B-C2 curve from 0µM to 100µM at 592nm

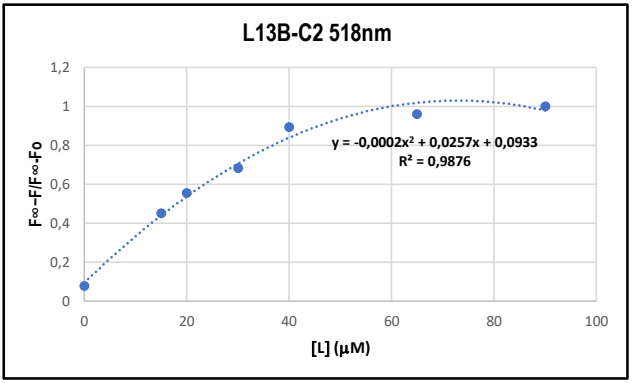


Figure 24. L13B-C2 curve from 0μM to 100μM at 518nm (effective concentration)