Aline Canani Viecinski

Licenciatura em Biomedicina

Development of biological and synthetic affinity ligands for human serum albumin

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientadora: Prof. Doutora Ana Cecília Afonso Roque

Júri

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"*Tudo de melhor nos acontece, quando agimos e pensamos positivamente."*

Abstract

This work aimed the development of biological and synthetic affinity ligands for human serum albumin (HSA). The first approach was to optimize the expression of a biological ligand, named WW Clone 3, which was previously selected from a phage display library using HSA as a target. The expression of WW Clone 3 in *Escherichia coli* strain BL21 (DE3) was attempted at three different temperatures (18ºC, 25ºC and 30ºC), with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG). The best temperature for cell growth was 30ºC, producing a larger amount of total protein with approximately 45 mg/ml in the soluble fraction and 10mg/ml in the insoluble fraction. However, WW Clone 3 was difficult to produce.

The second approach was to develop synthetic affinity ligands to HSA based on solid-phase synthesis of combinatorial libraries. The libraries were designed on the basis of amino acids from protein PAB (Peptostreptococcal albumin-binding) and drugs that bind naturally to the domain II of HSA. Two libraries were synthesized through the Ugi and Triazine reaction with 88 and 64 ligands, respectively.

The libraries were screened for binding to pure HSA and pure immunoglobulin G (IgG), to assess the selectivity towards HSA. The ligands that had the highest affinity for HSA and lowest affinity for IgG were re-screened to confirm the results. Two ligands, A3A2 and A6A5 from the Triazine library appeared as the most selective for HSA and therefore more promising for the capture of this protein.

Keywords: Human serum albumin; biological ligands; WW domain; synthetic ligand, combinatorial chemistry; affinity chromatography.

Resumo

Este trabalho teve como objetivo o desenvolvimento de ligandos de afinidade biológicos e sintéticos para albumina do soro humano (ASH). A primeira abordagem foi a de otimizar a expressão de um ligando biológico, intitulado WW Clone 3, que foi previamente selecionado a partir de uma biblioteca de *phage display* utilizando ASH como alvo. A expressão de WW Clone 3 em *Escherichia coli* estirpe BL21 (DE3) foi realizada a três temperaturas diferentes (18ºC, 25ºC e 30ºC), com 1mM isopropil-b-D-galactosídeo (IPTG). A temperatura mais adequada para o crescimento celular foi de 30ºC, produzindo uma maior quantidade de proteína total, com aproximadamente 45mg/ml na fracção solúvel e 10mg/ml na fracção insolúvel. No entanto, WW Clone 3 foi difícil de produzir.

A segunda abordagem foi desenvolver bibliotecas combinatórias de ligandos de afinidade sintéticos, em fase sólida, para ASH. As bibliotecas foram concebidas com base nos aminoácidos da proteína PAB (Peptostreptococcal albumin-binding) e drogas que se ligam naturalmente ao domínio II da ASH. Duas bibliotecas foram sintetizadas através da reação de Ugi e Triazina com 88 e 64 ligandos, respectivamente.

Foram realizados avaliações das interações das bibliotecas para ligação à ASH pura e posteriormente à imunoglobulina G (IgG) pura para aferir acerca da seletividade dos ligandos para ASH. Os ligandos que apresentaram a maior afinidade para a ASH e menor afinidade para IgG foram reavaliados para confirmar os resultados obtidos. Dois ligandos, A3A2 e A6A5 da biblioteca de Triazina, mostraram ser mais seletivos para ASH e, portanto mais promissores para a purificação desta proteína.

Palavras-chave: Albumina do soro humano; ligandos biológicos; domínio WW; ligando sintético, química combinatória; cromatografia de afinidade.

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Abbreviations

Aas- Amino acids residues

ABDs- Albumin Binding Domains

AI- After induction

APS- Ammonium Persulphate

Amp^R- Ampicillin resistance

B2M- β2-microglobulin

BCA- Bichinchoninic Acid

BI- Before induction

BSA- Bovine Serum Albumin

DNA- Deoxyribonucleic acid

DMF- Dimethylformamide

E.coli – Escherichia coli

EDTA- Ethylenediaminetetraacetic Acid

Eq- Equivalent

Fab- Fragment antigen- binding

FAs- Fatty acids

FcRn- Neonatal Fc receptor

GA- G-related Albumin binding

GFP- Green Fluorescent Protein

h- Helices

Hb- Hemoglobulin

His-tag- Histidine hexapeptide

HSA- Human serum albumin

IgG- Immunoglobulin G

IPTG- Isopropyl-β-D-thiogalactopyranoside

IS- Insoluble fraction

LB- Luria Broth

MHC- Major Histocompatibility Complex

N/A- Not Available

OD- Optical Density

OD_{600nm}- Optical Density at 600nm

PAB- Peptostreptococcal albumin-binding

PBS- Phosphate buffered saline suffer

PDB- Protein Data Bank

R1- Second nucleophilic substitution in the cyanuric chloride of Triazine

R2- The last nucleophilic substitution in the cyanuric chloride of Triazine

SA- Serum Albumin

SB- Soluble fraction

SDS- Page- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC- Super Optimal broth with Catabolite repression

TBS- Tris- buffered saline

TEMED- N,N,N,N-Tetramethylethylenediamine

Tris- Tris(hydroxymethyl) aminomethane

WW domain- Tryptophan tryptophan domain

I. Literature review

This first chapter describes the unique properties and main applications of human serum albumin (HSA). There is a particular focus on natural and synthetic HSA binders and its applications.

1.1 Human serum albumin

The human serum albumin (HSA) is a protein frequently found in plasma which is produced in the liver. The colloid osmotic pressure and the osmolality of the interstitial liquid around the hepatocytes are the major regulators of HSA biosynthesis (Fasano *et al*, 2005; Fanali *et al*, 2012).

When an individual reaches a human plasma albumin concentration of 34- 54 g/l and with an average plasma volume of 2.5- 3.0L for a 70kg person, the average intravascular albumin mass is approximately 120g. HSA circulates from the blood across the capillary wall into the interstitial compartments and also cerebrospinal fluid, and returns to the blood through the lymphatic system with a circulation half-life of 16 days (Fanali *et al*, 2012).

HSA is a component of many secretions from the human body including milk, sweat, tears and saliva, and is mainly degraded by intravascular space through larger organs, such as skin and muscles. The plasma HSA level decreases during periods of stress, trauma or sepsis despite its long half-life (Sleep *et al*, 2013).

1.2 Structural and functional features of HSA and their natural binders

1.2.1 Structural features

The primary sequence of HSA shows that the protein is a single polypeptide with 585 amino acids, having 17 pairs of disulfide bridges and one free cysteine at position 34 (Sugio *et al*, 1999). The disulphide bridges maintain the stability of HSA, explaining its relatively long biological half-life (Vusse, 2009). HSA has a single tryptophan residue at position 214. The Methionine, Glycine, and Isoleucine residues are in low levels, whereas Cysteine, Leucine, Glutamic acid, and Lysine are in high levels. The large number of ionized residues gives to HSA a high total charge (i.e., 215 ions per molecule at pH 7.0), facilitating its solubility (Fanali *et al*, 2012).

HSA is an α-helical, and a monomeric protein of 66kDa, composed by three structurally homologous domains (I, II, III) each being divided into two sub-domains (A, B) linked by random coil (Figure I.1) (Ghuman *et al*, 2005). The secondary structure of HSA is formed by α-helices (68%), without any β-sheet element. HSA has a globular heart-shaped form and each domain contains the following amino acids: I (1–195), II (196–383), and III (384–585). The three domains are comparable in the amino acid sequence as well as in the secondary and tertiary structure (Fanali *et al*, 2012).

Each domain has ten helices (h), and is divided into antiparallel six-helix for the subdomain A and four-helix for the subdomain B. It was constituted by six (h1-h6) and four (h7-

h10) α- helices, respectively, connected by an extended long loop (Varshney *et al*, 2010). To form the heart-shaped, the domain I and II are almost perpendicular to each other to constitute a T- shaped assembly in which the tail of subdomain IIA is attached to the interface region through the subdomains IA and IB by hydrophobic interactions and hydrogen bonds. In comparison, in an angle of 45°, the domain II overhang from subdomain IIB in a Y- shaped assembly for domains II and III. Domain III reacts only with subdomain IIB. The polypeptide chain folding and the disulfide bond topology are similar between the subdomains (Sugio *et al*, 1999). As a result of the large number of acidic and basic residues, the structure of HSA undergoes reversible conformational transitions at different pH values (Fanali *et al*, 2012).

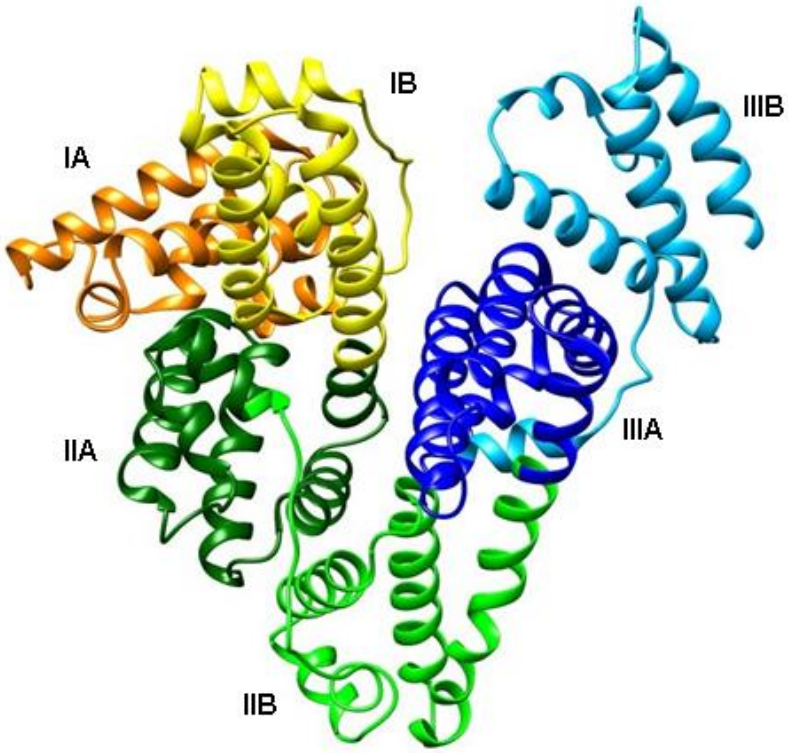


Figure I.1 : Three-dimensional structure of human serum albumin. Representation in ribbon models of HSA determined in 1999 by X-ray crystallography with a high resolution (2.5Å) Structure of HSA with the subdomains in different colors (subdomain IA, in orange; subdomain IB, in yellow; subdomain IIA, in dark green; subdomain IIB, in light green; subdomain IIIA, in dark blue; subdomain IIIB in light blue). Image built in UCSF Chimera software (PDB code: 1AO6) (Sugio *et al*, 1999).

Therefore, the structure of HSA changes when exposed to certain pH values: at pH lower than 2.7 it appears with an extended form; between pH 2.7 and 4.3 it appears in a fast-migration form, defined by an increase in viscosity, much lower solubility, and the loss of α-helix with respect to physiological conditions (the neutral form). On the other hand, when exposed between pH 4.3 and 8.0, it displays the neutral form, characterized by the heart-shaped structure. But when exposed at pH greater than 8.0, HSA changes its conformation to the basic

form, characterized by the loss of α-helix and an increased affinity for some ligands, with respect to the neutral form (Fanali *et al*, 2012).

1.2.2 Functional features

HSA has an extraordinary ligand binding capacity, as a large and wide variety of compounds bind to it. Moreover, HSA turns potential toxins harmless by transporting them to the target sites; it is responsible for most of the antioxidant capacity of human serum (Bolli *et al*, 2010). It is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia. Furthermore, HSA is largely used clinically to treat several diseases, like hypovolemia, shock, burns, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, acute liver failure, nutrition support and many others (Fanali *et al*, 2012).

Currently, the biotechnological and biomedical applications of HSA include implantable biomaterials, surgical adhesives and sealants; as a reagent in affinity chromatography, and as a fusion partner of therapeutic proteins to increase the circulation half-life (Fanali *et al*, 2012).

The main physiological functions of HSA, recognized for over 70 years, are the control of osmotic pressure and capillary membrane permeability and the ability of ligand binding and transport of many compounds (Fanali *et al*, 2012).

As an excellent carrier, HSA is involved in the transport of nutrients such as non-esterified fatty acids; heme group; lipophilic xenobiotics; hormones, such as thyroxine; amino acids; bile acids; steroids and it is also capable of transporting a wide range of therapeutic substances, through the bloodstream (Lexa and Jacobson, 2014; Curry, 2009; Lejon, 2004). In addition, it also binds a wide variety of drugs, being an important factor in the pharmacokinetic procedure, affecting their efficacy and rate of delivery. HSA is also related with the antioxidant capacity of human serum, by binding and transporting radical scavengers, or by sequestering transition metal ions with pro-oxidant activity (Fasano *et al*, 2005).

1.2.3 Ligand binding properties

HSA is well known for its excellent ligand binding capacity, due to its capacity to work as a reservoir for a great variety of compounds that may be available in quantities well beyond their solubility in plasma (Fasano *et al*, 2005). The tertiary structure of albumin, allows it to bind and transport a wide variety of molecules, such as metabolites (e.g., cholesterol, fatty acids, cations, and anions), gases (e.g., NO), exogenous substances (e.g.,Drugs) (Table I.1) (Fanali *et al*, 2012; Bolli, 2010). Although many ligands for HSA are hydrophobic or anionic, there are also

heavy metals and few cationic drugs also known to bind to HSA. This variability arises from the presence of multiple binding sites, which are dependent on many environmental factors like pH, temperature and ionic strength (Varshney *et al*, 2010).

It has the ability to bind many small molecules with micromolar affinity, and to control the outcome of certain chemical reactions. The broad binding action and chemical reactivity are related with the binding site located in the HSA subdomain IIA, also known as the Sudlow site I (Drug site I) (Luisi *et al*, 2013).

Other proteins can also interact with HSA, as part of its daily round through the circulation, to increase its half-life time. In addition, HSA has a size above the renal filtration cutoff and a pH-dependent binding to the neonatal Fc receptor (FcRn), which offers a rescue mechanism to avoid HSA and immunoglobulin G (IgG) degradation through the lysosomal degradation pathway (Nilvebrant and Hober, 2013).

Through a study of Ghuman and co-workers in 2005, it was possible to obtain several three-dimensional structures of HSA with many ligands and its specific binding sites (Figure I.2). In this study, the authors used drugs with acidic or electronegative features (e.g. warfarin, diazepam, ibuprofen and azapropazone) to test the binding to HSA. It was demonstrated that normally they bind at one of two primary sites (1 and 2), located in subdomains IIA and IIIA, respectively (Figure I.2) (Ghuman *et al*, 2005).

HSA can bind seven equivalents of long-chain fatty acids (FAs) at many binding sites with different affinities. In this case of FA1 to FA5 represented in the Figure I.2, the carboxylate part of fatty acids is anchored by electrostatic/polar interactions. On the other hand, sites FA6 and FA7 do not show a clear evidence of polar interactions that keep in place the carboxylate head of the fatty acid, thus suggesting that sites FA6 and FA7 have a lower affinity with the FA binding sites (Fasano *et al*, 2005).

When HSA is at physiological conditions, it binds not only endogenous and exogenous low molecular weight compounds but also peptides and proteins. There are thirty-five proteins that bind to HSA including high and low molecular weight proteins (e.g., angiotensinogen, apolipoproteins, ceruloplasmin, clusterin, hemoglobin (Hb), plasminogen, prothrombin, and transferrin). Any binding of proteins and peptides to HSA leads to proteomics and biomarker discovery studies, as the presence of both unbound and bound states of proteins in serum can change both the clearance and the detection of the free-state proteins and peptides (Fanali *et al*, 2012).

Table I.1: List of exogenous and endogenous molecules that bind to HSA and its respective binding site.

	Molecules	HSA binding site	References
Endogenous	Fatty acids (e.g., Myristic acid, Oleic acid and Decanoic acid)	FA1 (Subdomains IB and IIA), FA2 and FA3 (Subdomain IIIA), FA5 (Subdomains IIIB and IIIA), FA6 and FA7 (Subdomains IIA and IIIA).	Fasano *et al*, 2005; Petitpas *et al*, 2001; Bhattacharya *et al*, 2000
	Hormones (e.g., Thyroxine)	Subdomains IIA, IIIA and IIIB	Fanali *et al*, 2012
	Metal ions (e.g., Nickel (II) and Mercury (II))	N-terminal binding site (Aspargine 1, Alanine 2 and Histidine 3) and Free Cysteine 34 thiol	Fanali *et al*, 2012
	Bilirrubin	Subdomain IB	Fanali *et al*, 2012
	Vitamins (e.g., B12)	N/A	Hou *et al*, 2008
	Cholesterol	N/A	Fanali *et al*, 2012
Exogenous	Benzodiazepines drugs (e.g., Diazepam)	Subdomain IIIA (Drug site 2)	Ghuman *et al*, 2005
	Anti-inflammatory drugs (e.g., Ibuprofen)	Subdomain IIA, IIB and IIIA (Drug site 2)	Ghuman *et al*, 2005
	Anticoagulant drugs (e.g, Warfarin)	Subdomain IIA (Drug site 1)	Ghuman *et al*, 2005
	Anesthetic drug (e.g., Propofol)	Subdomain IIIA (Drug site 2) and IIIB	Ghuman *et al*, 2005
	Antibiotic drug (e.g., Daunomycin)	N/A	Tang *et al*, 2005

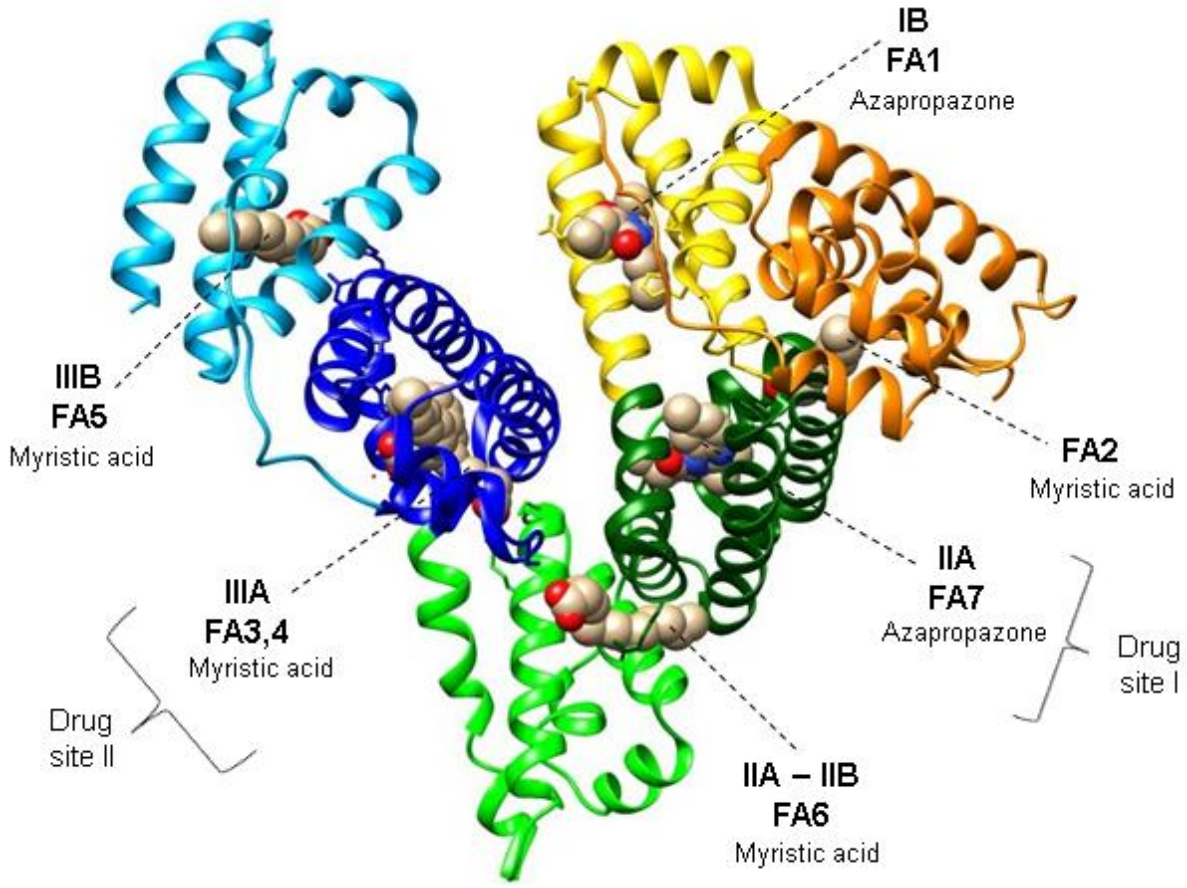


Figure I.2: Structure of HSA with ligand binding sites. Representation in ribbon models of HSA with myristic acid and azapropazone determined in 2005 by X-ray crystallography with a high resolution (2.5 Å). The six subdomains of HSA are colored as follows: subdomain IA: orange; subdomain IB: yellow; subdomain IIA: dark green; subdomain IIB: light green; subdomain IIIA: dark blue; subdomain IIIB: light blue. The drug site II is occupied by myristic acid (fatty acid) and the drug site II is occupied by azapropazone (drug), both white. Oxygen atoms are colored red and nitrogen atoms blue. Image built in UCSF Chimera software (PDB code: 2BXI) (Ghuman *et al*, 2005).

1.2.3.1 Human FcRn binding

The neonatal Fc receptor (FcRn) develops a central role in the regulation of IgG and serum albumin (SA) in mammals. FcRn is a 52kDa heterodimer, has a MHC (major histocompatibility complex) class I- related receptor consisting a heavy chain, which is non-covalently associated with β2-microglobulin (B2M) light chain (Figure I.3) (Mezo *et al*, 2010). FcRn may bind IgG and albumin simultaneously, and in a pH-dependent manner, with ligand binding at pH 6.0 and release at pH 7.4 (Oganesyan *et al*, 2014; Andersen *et al*, 2006).

The conjugation of proteins or peptides that have poor bioavailability or exhibit rapid clearance to the FcRn ligands (IgG, Fc molecule and albumin) can increase their pharmacokinetics owing to FcRn- mediated recycling and transport, and thereby obtaining greater therapeutic effects (Kontermann, 2012).

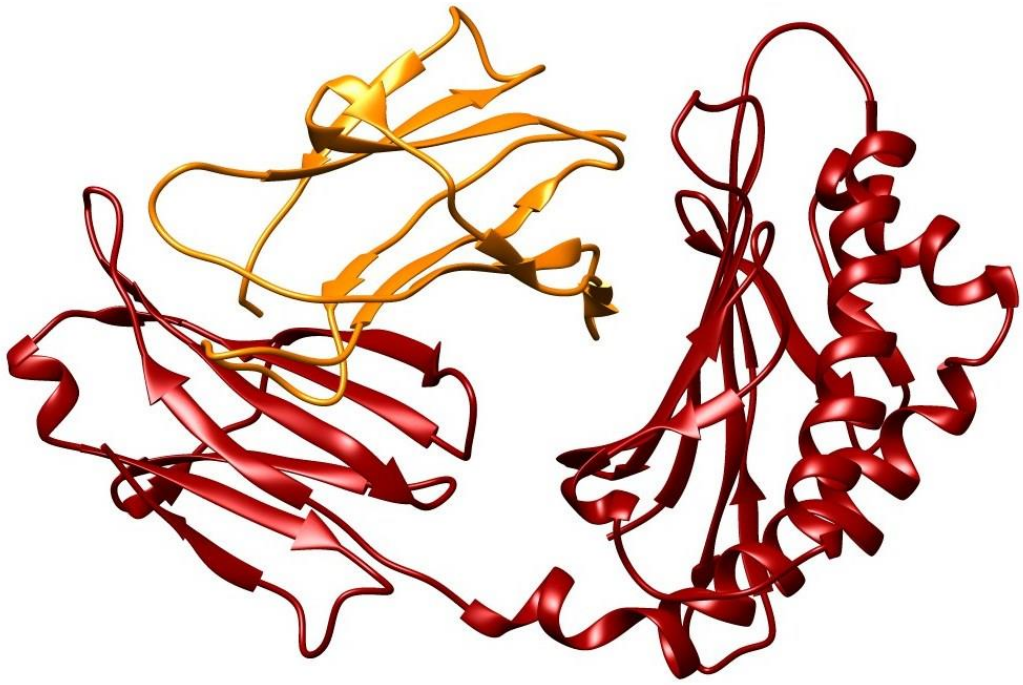


Figure I.3: Three-dimensional structure of the human neonatal Fc receptor. Representation in ribbon models of the human neonatal Fc receptor determined in 2010 by X-ray crystallography with a high resolution (2.6 Å). The red is the heavy chain called IgG receptor, and the light chain β2- microglobulin in orange. Image built in UCSF Chimera software (PDB code: 3M1B) (Mezo *et al*, 2010).

Binding to HSA has been shown to extend the in vivo half-life of proteins, and is known to have great potential as strategy for increasing the potency of biological drugs, through a mechanism which depends on the binding of HSA to FcRn at acidic pH in the endosomes (Nilvebrant *et al*, 2011). Endocytosis process allows the transport of substances through the extracellular space to the intracellular by endocytic vesicles, and many times, these substances are sorted to the lysosome and are degraded (Figure I.4) (Schmidt *et al*, 2013).

A study demonstrated that the concentration of albumin was approximately 40% lower in FcRn heavy chain deficient mice compared with normal mice. In addition, the data indicate that the capacity of FcRn is extraordinary, as albumin is degraded twice as fast in FcRn-deficient mice as in the wild type strain and almost four times more IgG is saved from degradation than is produced by the mouse (Andersen *et al*, 2006; Kim *et al*, 2006).

Oganesyan and colleagues, in 2014, indicate that the residue histidine 161 is the only potential direct contributor to the corresponding pH-dependent process (Figure I.5) (Oganesyan *et al*, 2014). However, a study by Sand and co-workers, in 2014, demonstrated that not only DIII but also DI of albumin is important for optimal pH-dependent binding to FcRn (Sand *et al, 2014*).

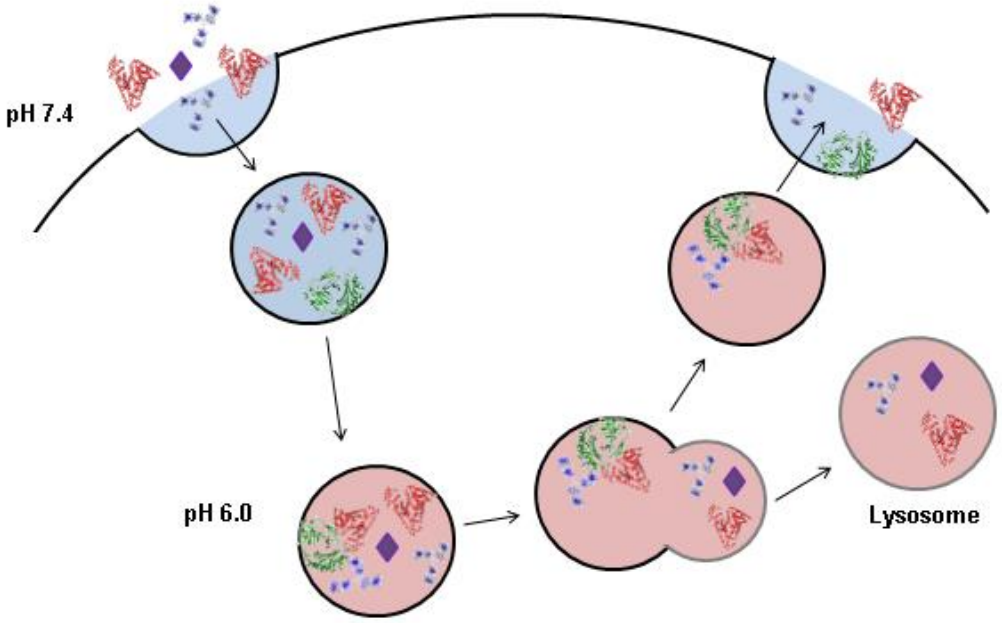


Figure I.4: FcRn mediated pH- dependent, recycling and transport of IgG and HSA. Plasma proteins are pinocytosed along with a variety of other constituents at neutral pH (pH 7.4) (light blue shading), then move to the acidic endosomal (pH 6.0) (pink shading) compartments where IgG (structure in dark blue) and albumin (structure in red) bind to FcRn receptor (structure in green) with high affinity at distinct binding sites. Unbound plasma proteins (purple lozenge shaped) or excess ligands, when FcRn is satured, are destined for degradation in lysosomes (circle in grey), while bound complex is sent to the cell membrane where IgG and albumin dissociate from FcRn at physiological pH, providing a longer survival for the two proteins. Image built in UCSF Chimera software (PDB code: 3M1B, 1AO6 and 1IGT) (Image adapted from Kontermann, 2012).

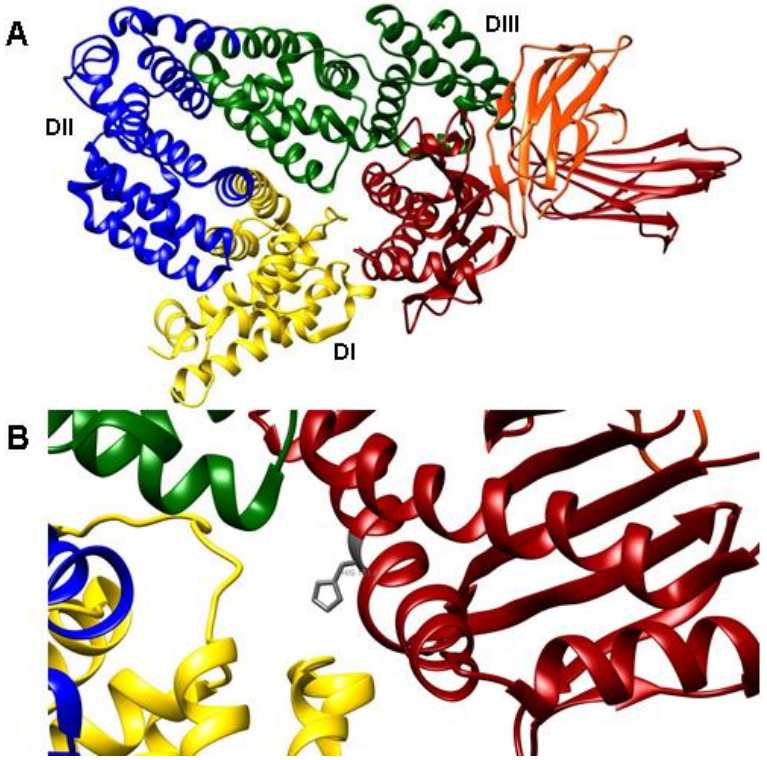


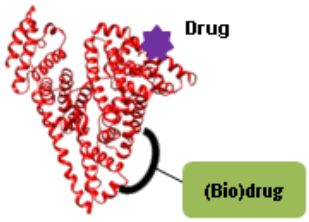
Figure I.5: Structure of human FcRn complexed with HSA. Representation in ribbon models of the human FcRn complexed with HSA determined in 2014 by X-ray crystallography with a high resolution (3.0 Å). (A) FcRn receptor with receptor IgG heavy chain bound mainly to the domain III of HSA. (B) Detail on the residue histidine 161 (grey) from FcRn, contributor to the pH-dependent process. Image built in UCSF Chimera software (PDB code: 4N0F) (Oganesyan *et al*, 2014).

1.3 Applications HSA

The average HSA consumption in the developed world is 200-400 kg/million inhabitants. Annually in the industry of therapeutic protein manufacture, more than 500 metric tons of HSA and more than 40 tons of intravenous immunoglobulins (IVIG) are produced from more than 22 million liters of source and recovered plasma, reaching up to 34 million liters. This $6.9 billion industry supplies products to more than one million inhabitants each year (Curling, 2004).

In the biomedical applications, HSA can be found in three different ways, such as HSA alone, HSA fused with biological proteins/peptides or HSA complexes, being of much interest in the therapeutic area (Table I.2).

Table I.2: List three different forms with HSA can be found in biomedical applications. HSA structure in red. Image built in UCSF Chimera software (PDB code: 1AO6).

	HSA Form	Application	Source	References
	**HSA/rHSA**	Treatment form and biomarker of many diseases	Blood fractionation/ Genetic Engineering	Burnouf, 2007; Wilkes and Navickis, 2001; Chuang and Otagiri, 2007
	**HSA fusion with biological proteins/ peptides**	Increase the half-life of biodrug	Protein engineering	Kontermann, 2009; Elsadek and Kratz, 2012
	**HSA complexes**	Improving the pharmacokinetic properties of the (bio)drugs	Protein engineering & development of HSA binders	Elsadek and Kratz, 2012

For a long time, HSA is emerging in the pharmaceutical industry due to its ability to bind a wide range of drug molecules and modify its pharmacokinetic parameters, contributing for the treatment of several chronic and acute diseases (Gundry *et al*, 2007; Grazzini *et al*,

2013; Varshney *et al*, 2010). Protein products fractionated from human plasma are widely used for therapeutic purposes, often as the only available option to the prevention, management, and treatment of severe diseases (Burnouf, 2007). Albumin administration is an alternative of treatment of many diseases as hypovolemia and shock; burns; hypoalbuminemia and hypoproteinemia; surgery; traumas; cardiopulmonary bypass; the acute nephrosis; hyperbilirubinemia; acute liver failure; ascites; and sequestration of protein-rich fluids in acute peritonitis, pancreatitis, mediastinitis, and extensive cellulitis (Wilkes and Navickis 2001).

HSA is emerging as a portable protein carrier for drug targeting and for improving the pharmacokinetic profile of peptide or protein-based drugs. Drugs, prodrugs or polypeptides can either be bound physically or covalently through a ligand or protein-binding group to HSA. More complex systems are known to have the attachment with numerous targeting ligands and prodrugs bound to the protein surface or nanobodies or bispecific antibodies that bind physically or are fused with albumin replacing the Fc fragment of immunoglobulin G (Elsadek and Kratz, 2012).

Many studies with direct fusion to whole albumin have been applied to a variety of proteins, such as interferons, IL-2 (Interleukin-2), insulin, human growth hormone, and antibody fragments. These proteins were fused to either the N- or C-terminus of HSA (Kontermann, 2009). A study *in vitro* used the thioredoxin (Trx), a redox-active protein, fused to HSA, resulting in about 40% reduction in the Trx biological activity. However the HSA–Trx fusion protein has proven to be therapeutically effective in the septic shock mouse model, because the HSA–Trx had higher distribution in the lungs than in other organs, as well as 10 times longer plasma half-life than Trx in normal mice (Furukawa *et al*, 2011).

1.4 HSA binders

Binders to HSA can find several applications, namely:

- The binder can capture HSA and be used for HSA depletion or purification
- The binder can be employed to detect HSA *in vivo* or *in vitro* for diagnostic purposes when HSA can be considered a biomarker;
- The binder can be covalently linked to a drug or biodrug and, once bound to HSA, be used to increase the half-life of the therapeutic agent.

1.4.1 Natural HSA binders

Many Gram-positive bacterial species, including human pathogens, express surface proteins that interact with host proteins like HSA and IgG with high specificity and affinity (Ascenzi *et al*, 2006). The surface proteins typically contain tandemly repeated serum protein-

binding domains with one or many specificities, which often include albumin binding (Nilvebrant and Hober, 2013).

The bacterial species that express albumin-binding domains (ABDs) are normally part of the normal human flora and they are opportunistic pathogens. There are several types of albumin-binding proteins with different sizes and functions (Nilvebrant and Hober, 2013; Jonsson *et al*, 2008). Examples of bacterial proteins that bind to HSA are protein G (Jonsson *et al*, 2008), protein PAB (Lejon *et al*, 2004), protein MAG (Jonsson *et al,* 1994), protein ZAG (Jonsson *et al*, 1995) and protein H (Frick *et al*, 1994) (Table I.3). The binding strength between different ABDs and serum albumin from different species can vary widely, which has been investigated in many studies (Jonsson *et al,* 2008). The fact that most of the ABDs recognize the domain II of HSA, having no overlap with the binding site of the FcRn receptor, is of greatest importance for the control of HSA homeostasis (Nilvebrant and Hober, 2013).

Table I.3: List of the most known protein with albumin binding domains (ABDs). Aas- Amino acid residues.

ABD	Organism	Size (Aas)	Reference
Protein G	Group C or G *Streptococcal*	46	Jonsson *et al,* 2008
Protein PAB	*Peptostreptococcus magnus*	45	Lejon *et al,* 2004
Protein MAG	*Streptococcus dysgalactiae*	60	Jonsson *et al,* 1994
Protein ZAG	*Streptococcus zooepidemicus*	52	Jonsson *et al*, 1995
Protein H	*Streptococcus pyogenes*	112	Frick *et al*, 1994

Proteins with ability to bind serum albumin, including ABDs, peptides or antiserum albumin antibodies, have been used in several important applications within biotechnology and biotherapy (Jonsson *et al,* 2008).

1.4.1.1 Protein G

Over the past 30 years, different streptococcal species, which bind to HSA with high affinity, have been identified (Egesten *et al*, 2011).

Protein G is expressed at the cell surface of certain group C and group G streptococcal strains, and it is known to be a bi-functional receptor, because it binds to IgG and HSA in different regions.

These regions have three independently folded domains (Figure I.6) (Sjöbring, 1992; Björck *et al*, 1987).

The ABD, also known as GA module, is at the N-terminal region, and the binding region to IgG is in the C domain of the C-terminal region (Egesten *et al*, 2011).

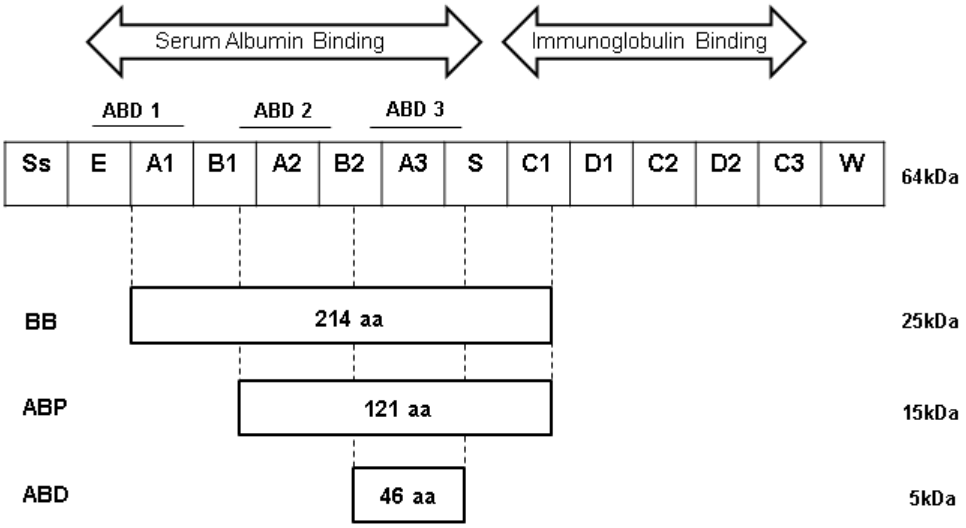


Figure I.6: An overview of the streptococcal protein G (SpG). The HSA and Imunoglobulin Binding sites are in different domainsof the protein. However, both have three homologous domains. Nomenclature according to Ståhl and Nygren (1999) (adapted from Linhult *et al*, 2002).

The three homologous HSA-binding domains from protein G have similar sequences and are called ABD1, ABD2, and ABD3. Through a study of Johansson and co-workers in 2002, it was possible to obtain the structure of ABD3 from protein G (strain G148) by Nuclear magnetic resonance (NMR) spectroscopy, (Figure I.7), which showed to have a left-handed three-helix bundle structure (Johansson *et al*, 2002). It consists of 46 amino acids and it is stable enough to be independent from disulfide bridges, does not require ligands, cross-linkers or metal ions for folding (Linhult *et al*, 2002). HSA contains one binding site for protein G, formed by loops 6–8 of HSA. This region also contains the binding sites for tryptophan, fatty acids, and thyroxine (Johansson et al, 2002).

The ABD has an excellent chemical and thermal stability, and therefore, is a suitable scaffold for protein engineering (Linhult *et al*, 2002). The amino acids from protein G responsible for most part of affinity with HSA were identified by alanine- scanning procedure, followed by binding studies using different methodologies- surface plasmon resonance (SPR), circular dichroism (CD) and affinity blotting. Most of these amino acids are located in the second helix. Moreover, it was possible to conclude that helix 1 and also most part of helix 3 are probably not involved in the binding to HSA (Linhult *et al*, 2002).

As an alternative to direct fusion to HSA, many molecules have been exploited as albumin-binding moieties, doing reversible, non-covalent interaction with HSA. Several *in vivo*

studies demonstrated the applicability of fusion to the ABD from protein G to increase the plasma half-lives of small antibody molecules (e.g. Fragment antigen-binding (Fab), biospecific antibody derivatives) and several antibody mimetics. Fusion of the ABD to the C-terminus of the light chain of an anti-HER2 Fab increased the terminal half-life in mice from approximately 2 hours to 20.9 hours. However, there are concerns due from the fact that this ABD is derived from a bacterial protein, which has been shown to be immunogenic. Impressively, it was shown that the ABD from protein G is capable of reducing the immunogenicity of a fusion partner, and that fusion proteins containing ABD may be administered repeatedly, without raising an immune response (Kontermann, 2009; Schlapschy *et al*, 2007).

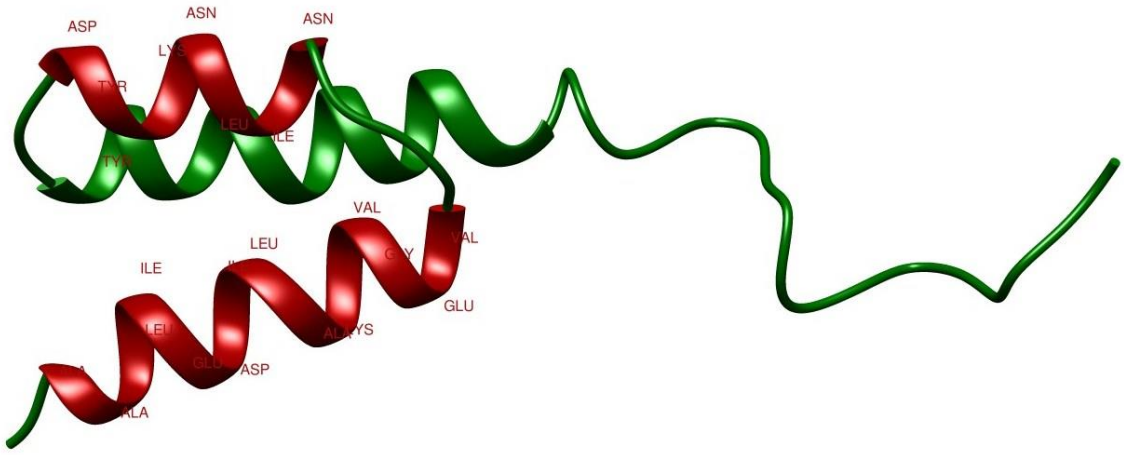


Figure I.7: Three-dimensional structure of albumin binding domain from Protein G. The structure was determined by NMR spectroscopy in 2002. ABD structure with three-helix bundle. The helices 2 and 3 are red with the code of the respective amino acids, site of highest affinity to HSA. Image built in UCSF Chimera software (PDB code: 1GJS) (Johansson *et al*, 2002).

Other studies have demonstrated that the non-covalent association with HSA has been useful to extend the half-life of short lived proteins. In an *in vivo* study, the recombinant fusion of the ABD from streptococcal protein G to human complement receptor type 1 was performed. Using this fusion protein, it was possible to increase its half-life 3-fold, for 5 hours in rats. Furthermore, fusion to this domain has served to enhance the immunological response directed to peptide antigens (Dennis *et al,* 2002; Makrides *et al*, 1996).

1.4.1.2 Protein PAB

The protein PAB (peptostreptococcal albumin-binding), is expressed at the surface of the Gram-positive anaerobic bacterium *Peptostreoptococcus magnus,* present in the indigenous flora of the skin, oral cavity, and the gastrointestinal and urogenital tracts (Lejon *et al*, 2004). The protein PAB contains a domain for binding to HSA named after the GA (protein G-related albumin-binding) module. This contains 45 residues, is composed by a left-handed three-helix

bundle, being therefore structurally and functionally similar to ABD from protein G (Figure I.8) (Johansson *et al*, 1997). It was postulated that the conserved region in the C-terminal part of the second helix and the flexible sequence between helices 2 and 3 could contribute to the albumin-binding activity (Lejon *et al*, 2008; Johansson et al, 2002). The GA module has a fold that is impressively similar to the immunoglobulin-binding domains of staphylococcal protein A, but it is not similar to the fold shared by the immunoglobulin-binding domains of streptococcal protein G and peptostreptococcal protein L (Johansson *et al* ,1997).

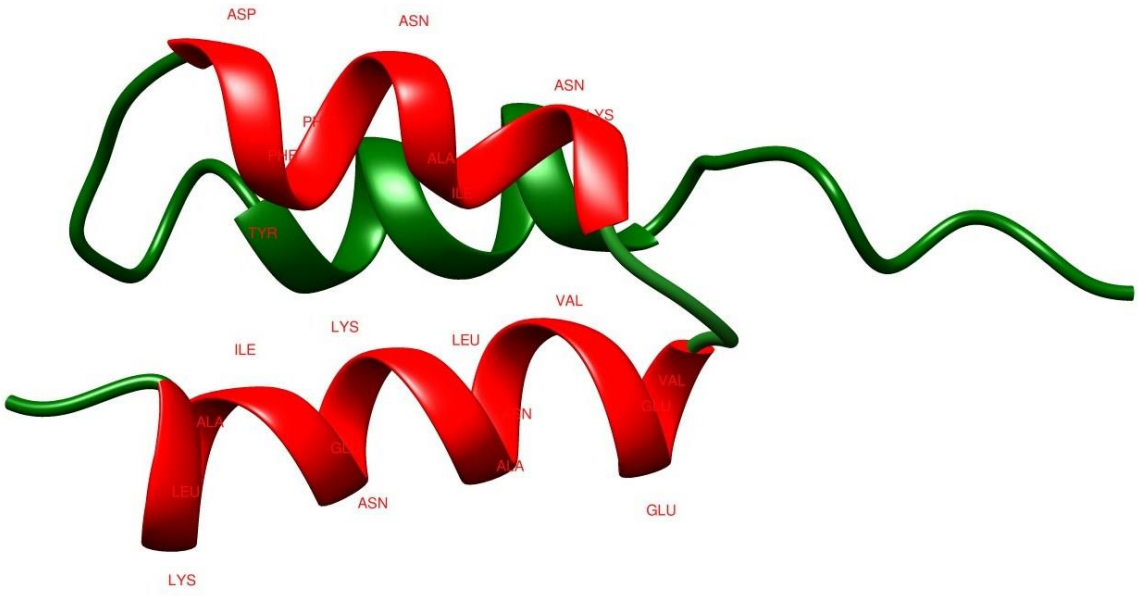


Figure I.8: Structure of the albumin-binding GA module. The structure was determined by NMR spectroscopy in 1997. Three- dimensional structure of GA module with the helix 1 in green and the helices 2 and 3 are red with the code of the respective amino acids, site of highest affinity to HSA. Image built in UCSF Chimera software (PDB code: 1GAB) (Johansson *et al*, 1997).

According to the study of Lejon and co-workers, in 2004, the GA module residues from the second helix and the two loops surrounding, bind to HSA in the domain II. More precisely, they bind in the helices 2 and 3 of domain IIA and in the helices 7 and 8 of domain IIB, close to a cleft bounded by helices 2 and 3 (Figure I.9). Residues phenylalanine 27, alanine 31, leucine 44, isoleucine 48, threonine 24, serine 25, tyrosine 28, threonine 37 from GA participates in the binding to HSA. The presence of fatty acid in the HSA-GA binding interface might influence the complex formation (Lejon *et al*, 2004). Based on previous observations of fatty acid-induced conformational changes in HSA, it is possible that the difference in global conformation of the albumin chain in the two structures of the HSA–GA complex is caused by the difference in fatty-acid content (Lejon *et al*, 2008).

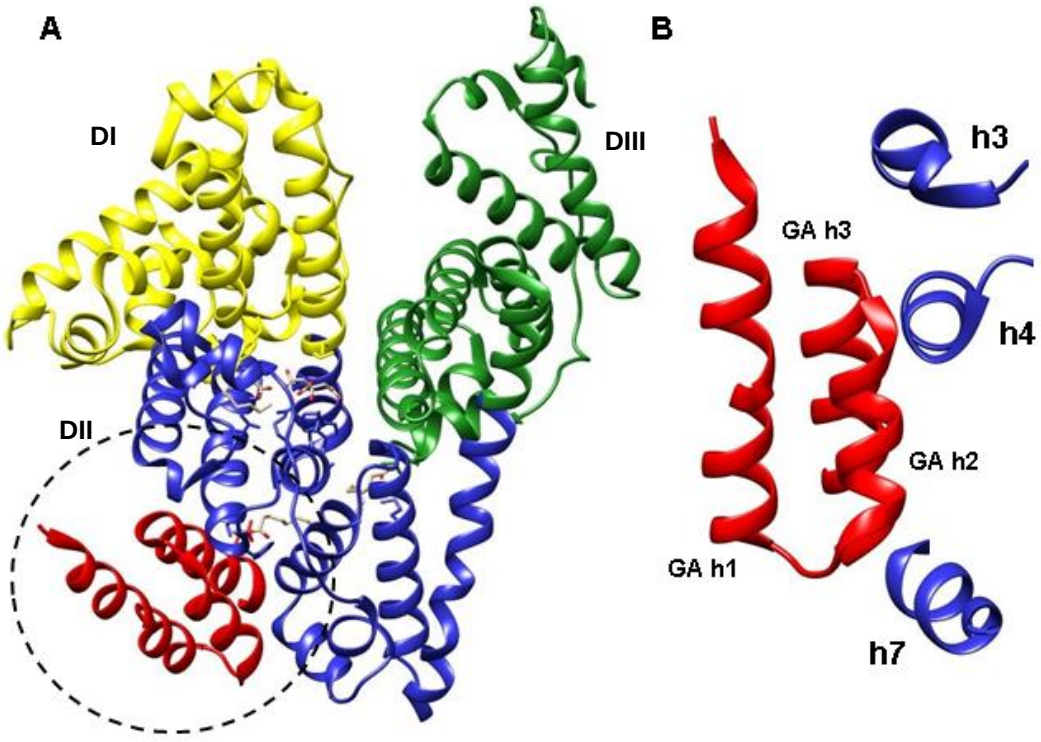


Figure I.9: Structure of the GA module complexed with HSA. Representation in ribbon models of HSA determined in 2004 by X-ray crystallography with a high resolution (2.7 Å). (A) The GA module binds in the domain II of HSA. The HSA molecule is shown in blue and the GA module in red. (B) Detail in the interactions between HSA side chains and the GA helices (red) pack at almost right angles to helices 3, 4, and 7 (h3, h4, and h7) in HSA domain II. Image built in UCSF Chimera software (PDB code: 1TF0) (Lejon *et al* ,2004).

A study by Johansson and co-workers, in 2002, through a comparison of the G148-GA3 protein (protein G) with ALB8-GA protein (protein PAB) demonstrate that both bind HSA. Both domains share 59% sequence identity and bind to the same site. However, the specificity of G148-ABD is much broader than for ALB8-GA, despite that the binding affinity of ALB8-GA for HSA is roughly two fold higher. In addition, streptococci expressing G148-ABD have much broader host specificity and this domain binds albumin from several non-primates better than ALB8-GA. As would be expected, competitive binding studies have shown that G148-ABD and ALB8-GA have the same binding site on HSA (Johansson *et al*, 2002).

1.4.2 Synthetic binders

Through of the progresses in molecular modeling, genetic engineering and both natural and synthetic combinatorial methodologies, have made possible the design and development of engineered of peptides and engineered protein domains and fully synthetic molecules (Roque *et al*, 2007).

The purification of HSA has attracted considerable attention in the pharmaceutical industry by its great binding potential. The biomimetic dye-ligands (e.g., triazine dyes) offer

many advantages as its low cost, ease of immobilization, stability and high adsorption capacity (Kassab *et al*, 2000).

Albumin-binding peptides (ABP) also represent an alternative to the use of bacterial ABDs, with the advantage of the smaller size. Such ABPs have been isolated from random peptide libraries applying phage and bacterial display, but were found to present a low affinity to HSA. The association, conjugation or fusion of therapeutic drugs to albumin is a well-accepted and established half-life extension methodology. The manipulation of the albumin–FcRn interaction will facilitate the modulation of the circulatory half-life of albumin-enabled drugs, leading to improve in the pharmacokinetics (Sleep *et al*, 2013).

Dennis and co-workers at Genentech Inc., in 2002, published a work on the attachment of albumin-binding single chain antibodies. They found a peptide with 18 amino acids (Ac-RLIEDICLPRWGCLWEDD-NH2) with high binding affinity for HSA (Kd~0.5μM). A related sequence QRLMEDICLPRWGCLWEDDF comprising 20 amino acids was then fused with a single chain antibody D3H44 Fab directed against the tissue factor and showed a similar affinity for HSA. This fusion protein was then injected in mice and rabbits and it was observed an increased in the plasma half-life 26-fold to 10.4 hours and 37-fold to 32.4 hours, respectively. These results demonstrated a 25–43% increase in the half-life in these animals. Thus, by attachment of a small peptide to a single chain antibody the rapid clearance of the native single chain antibody, that normally limits their therapeutic usefulness, could be overcome and can deeply affect its pharmacokinetic profile (Dennis *et al*, 2002).

1.5 Aims of the work

HSA besides being an important versatile carrier protein in plasma, can also is used clinically for the treatment of many diseases (e.g., hypovolemia, shock and burns) and as a biomarker for cancer, rheumatoid arthritis, and ischemia.

For this reason, the development of ligands with affinity for HSA may be an effective strategy for improving the pharmacokinetic properties of drugs or biodrugs and also for HSA purification, which posteriorly can be used for clinical purposes. Within this context, the aim of this research project is the development of biological and synthetic affinity ligands towards HSA (Figure I.10).

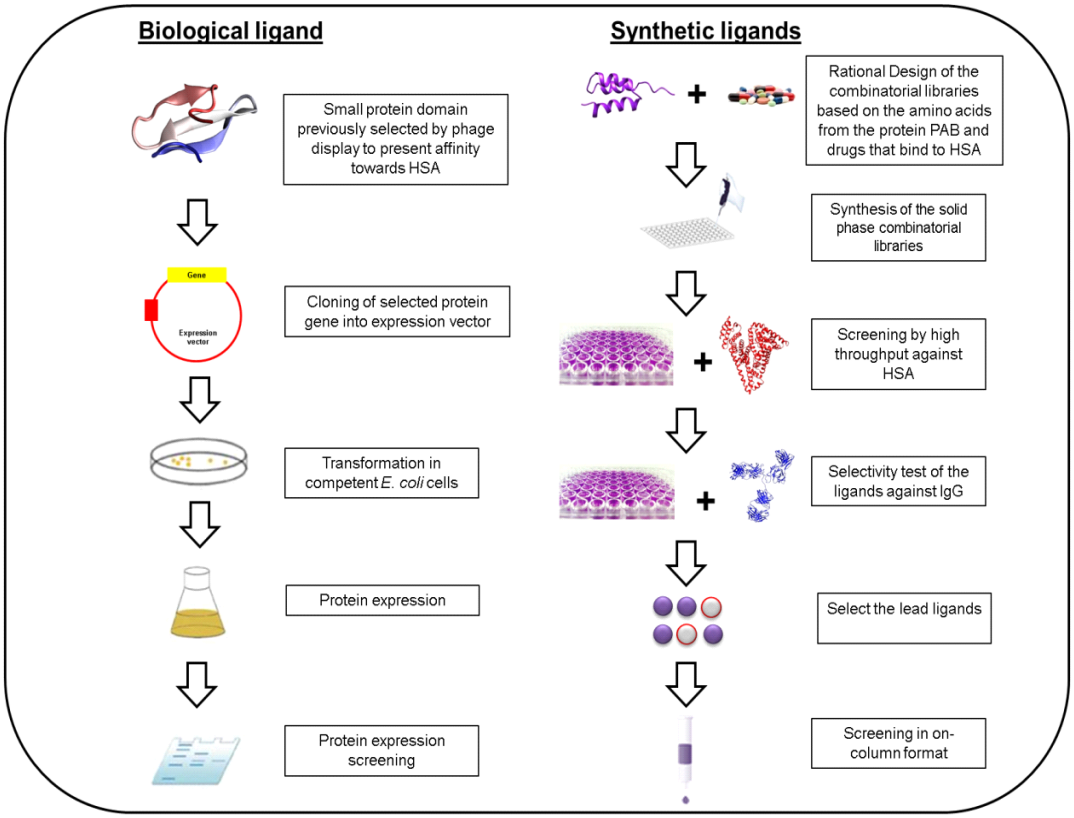


Figure I.10: Schematic representation of the procedures performed in this study. Image for the structures of the protein PAB and IgG were built in UCSF Chimera software (PDB code: 1AO6, 1GAB and 1IGT).

The first approach was to optimize the expression of a biological ligand, a small protein domain previously selected by phage display to present affinity towards HSA. This ligand is of biological origin, and therefore, has low toxicity and having a small size can be synthesized biologically and chemically.

In the second approach, the aim is to develop synthetic ligands with affinity to HSA, based on combinatorial chemistry to produce solid-phase libraries of ligands. These ligands are more resistant and are more easily produced. They can be used for the purification of HSA, or

to increase the half-life of drugs and therapeutic proteins when in complex with HSA. However, in this work was explored only the purification of HSA.

II. Optimization of the expression of and engineered biological ligand for human serum albumin

The aim of this chapter was to express the WW Clone 3 protein in *E. coli* strain BL21 (DE3) using pET expression vector system and test its affinity towards HSA to confirm the results obtained from the panning of the phase display library. From previous work in the laboratory, it was verified that the expression of this family of proteins without a partner protein (co- expression) was difficult.

2.1 Introduction

Biological ligands are biomolecules derived from natural sources and from *in vitro* selection techniques, as phage, ribosome or cell free display techniques. These are usually associated with high selectivity and affinity for the target (Pina *et al*, 2014b).

Protein domains are natural modulators of biological function through ligand binding. These domains can be modified in order to produce altered activities or binding specificities and create novel affinity reagents.

WW domains are a family of protein-protein interaction modules that are found in many eukaryotes and are present in approximately 200 human proteins. This small domain contains two conserved tryptophan residues, which justifies the name WW domain. The WW domains are sometimes present in multiple copies in a protein (for example, in Rsp5/Nedd4 or in the mouse YAP65) (Staub and Rotin, 1996; Ingham *et al*, 2005). While the WW domain was initially considered a 'cytoplasmic module', the proteins that have WW domains were also localized in the cell nucleus (Sudol *et al*, 2001).

WW domains are typically 35 to 40 amino acids in length and fold into a three-stranded antiparallel β- sheet with two ligand-binding grooves, while in the region between the N- terminus and C-terminus there are hydrophobic interactions (Ingham *et al*, 2005). This protein is one of the smallest protein module that folds as a monomer without disulfide bridges or cofactors. Moreover, this domain binds proteins containing short linear peptide motifs that are proline-rich or contain at least one proline. Through the recognition of proline-rich regions, the WW domain has a role in mediating protein-protein interactions (Sudol *et al*, 2001; Chen and Sudol, 1995).

Previously in our Research Group, a WW library has been used for *in vitro* selection, against HSA by phage display (Patel *et al*, 2013). The best binder selected was named WW Clone 3, with approximately 160bp (5kDa), and a predominately hydrophobic character.

2.2 Material and methods

2.2.1 Materials

Vector pEX WW Clone 3 was purchased from Eurofins (Germany) and the pET 21c expression vector kindly provided by Dr. Ana Pina (DQ, FCT, UNL).

The Top10 F' and BL21 (DE3) electro competent cells from *E. coli* were kindly provided by the group of Prof. Dr. João Gonçalves (Pharmacy Faculty, University of Lisbon, Portugal).

The restriction enzymes for modification of plasmid DNA were: *NheI* and *BamHI* FastDigest and the enzyme FastAP Thermosensitive Alkaline Phosphatase which were purchased from Thermo Scientific (Portugal). The DNase I was purchased from Roche (Germany).

Bovine Serum Albumin (BSA) and the Bicinchoninic Acid (BCA) Kit were acquired from Sigma- Aldrich (Portugal).

For DNA (Deoxyribonucleic acid) purification the NZY Gelpure kit was used, and for the preparation of pure plasmid DNA from recombinant *E. coli* strains the Miniprep kit was used, both acquired from NzyTech (Portugal).

The Luria broth (LB) medium, ampicillin antibiotic, T4 DNA ligase, Isopropyl-beta-D-thiogalactopyranoside (IPTG), Agarose (ultrapure grade), N,N,N,N,Tetramethylethylenediamine (TEMED) were purchased from NzyTech (Portugal). The Agar was purchased from HIMEDIA (Portugal).

For the DNA electrophoresis, the DNA markers used were NzyTech Ladder III and V, loading dye, Greensafe premium, purchased from NzyTech (Portugal). The Precision Plus Protein™ dual color standards was purchased from Bio- Rad (Portugal). For the Protein electrophoresis the ammonium persulphate (APS) was employed.

For the Western Blotting method, the Immobilon-P^{SQ} Membrane (0.2µm) was used and acquired from Merck Millipore (Germany). Tween 20, Coomassie Brilliant Blue R, β-mercaptoethanol were purchased from Sigma (Portugal). The Mini-PROTEAN Tris (Tris(hydroxymethyl)aminomethane)-Tricine precast gel and Acrylamide – Bis acrylamide 30:8 were purchased from Bio- Rad (Portugal). The Step™ TMB- Blotthing was acquired from ThermoScientific (Portugal). The Penta His HRP Conjugate Kit with Blocking Buffer were purchased from Qiagen (Portugal).

2.2.2 Equipment

Standard laboratory equipment were needed, including: Vortex mixer VX-200 Labnet International Inc., Micro centrifuge- ScanSpeed, Micro Centrifuge Frilabo, Incubator ZKA KS4000i control, Centrifuge Thermo (Bio-Rad T100™ Thermal Cycler), Spectrophotometer

Ultraspec 2100, Microplate reader TECAN Infinite F200, FrechPress and Nanodrop 1000 from Thermo Scientific (Portugal). Ultracentrifuge optima LE80K (Rotor type 45 Ti) from Beckman Coulter. To obtain the image of the gel, the GelDoc from Bio- Rad (Portugal) was employed. It was used software Chimera to obtain the image of the proteins structures.

2.2.3 Buffers

The following buffers and reagents were used: Tris (Tris-(hydroxymethyl) aminomethane) Acetate- EDTA (TAE) 50x (2M Tris- base, 1M Glacial acetic acid, 0,1M Ethylenediaminetetraacetic Acid (EDTA) at pH 8.5) from which was prepared TAE 1x; Tris-Tricine 10x (1M Tris, 1M Tricine, 1% Sodium dodecyl sulfate (SDS), pH 8.3) and was used in Tris- Tricine 1x; Phosphate buffered Saline buffer (PBS) (10mM Sodium phosphate, 150mM Sodium Chloride (NaCl), at pH 7.4) and was also used PBS with 0.05% Tween 20; Transfer Buffer (25mM Tris- Base, 195mM Glycine, 40% Methanol); Tris- Buffered Saline (TBS) 1x (50mM Tris- Base and 150mM NaCl, pH 7.5) and was also used TBS with 0.1% Tween 20; Coomassie Blue R-250 (0.1% Coomassie Blue R-250, 0.15% Glacial acetic acid, 0.9% Methanol); Tris- Glycine buffer (0.25M Tris-base, 1.92M Glicine, SDS, pH 8.3); Concentration of the gel Buffer II (0.5M Tris-base, 0.125M Tris-HCl (Hydrochloric acid), pH 6.8); Separating gel Buffer I (3M Tris-base, 0.375M Tris- HCl, pH 8.8); 10% APS, IPTG 1M stock 238.3g/mol; LB medium liquid (LB medium 25g/l in Milli-Q water) and LB medium solid (1.5% Agar in LB medium 25g/l in Milli-Q water), SOC (Super Optimal broth with Catabolite repression) medium (31.54g/l in Milli-Q water).

2.2.4 Methods

2.2.4.1 Cloning strategy

For expression of the protein WW Clone 3, it was necessary to first clone the gene codifying the protein of interest into the vector expression vector. As expression vector we selected a pET system (vector pET 21C, Ampicillin Resistance (Amp^R) for expression in *E. coli* BL21 (DE3). The gene codifying the protein was in a transporter vector pEX (Eurofins). These vectors have two sequences which are specifically recognized by the restriction enzymes *NheI* and *BamHI* surrounding the cloning site and have Amp^R. Figure II.1 represents a scheme of the cloning strategy of WW Clone 3 into pET 21c.

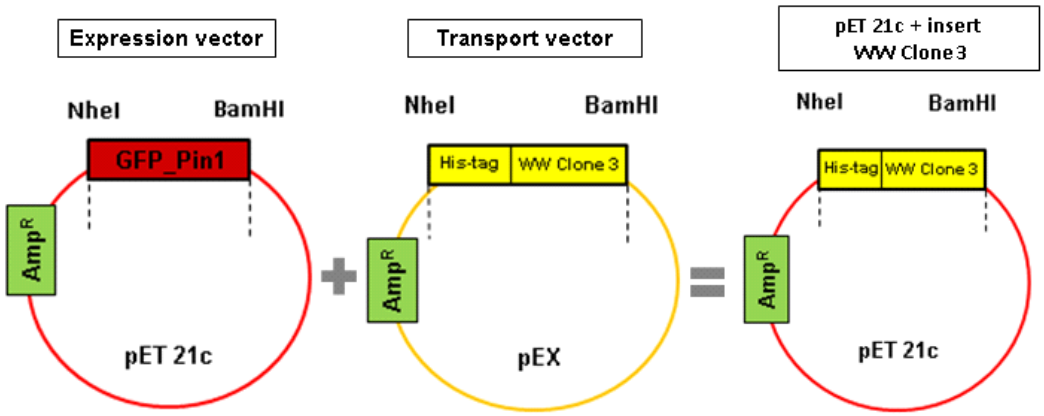


Figure II.1: Scheme of the cloning strategy of WW Clone 3 into pET 21c. Schematic representation of the cloning strategy of gene codifying WW Clone 3 into pET 21c vector.

2.2.4.2 Amplification of the vector pEX WW Clone 3

The pEX WW Clone 3 was synthesized by the company Eurofins (Germany) in a transport plasmid pEX A2. For the initiation of the cloning of the WW Clone 3, it was necessary to amplify the transport vector.

The vector was bought lyophilized and for this reason it was necessary to hydrate with 50µl of sterile Milli-Q water, as recommended by the manufacturer. After, *E. coli* Top10 F' bacterial cells were transformed by electroporation.

2.2.4.3 Bacterial transformation by electroporation

It was added 1µl of the vector pEX WW Clone 3 (2.5µg) in 50µl of TOP 10F' (10^9cfu/µg) cells *E. coli*, and the electroporation in mode EC1 with 1.8V of pulses as indicated by the manufacturer. Then, it was added in 1ml of SOC medium that was performed and incubated at 37°C with 220rpm for 1 hour.

After, it was added 50µl of the culture in the plates with LB solid and 10µl of ampicillin (100µg/ml). It was incubated at 37°C overnight to produce individual colonies.

2.2.4.4 Isolation and purification of plasmid DNA from *E. coli*

To proceed with the pre-inoculum, three colonies were picked in the plate and were added in 10ml of LB liquid and 10µl of ampicillin (100µg/ml) and incubated at 37°C overnight with 220rpm. The optical density (OD) of the growth media was measured at 600nm (OD_{600nm}) and selected the one presenting the highest OD values.

It was removed 1ml of the culture with the highest OD600nm to make a stock and it was centrifuged at 3826xg for 4 minutes and then, the supernatant discarded. Posteriorly, it was added 100µl of sterile glycerol 10% (v/v) and stored at -80ºC.

The remaining of the culture was centrifuged at 15303xg for 10 minutes and the supernatant was removed. Then, the purification of plasmid DNA from E.coli cells was performed with Miniprep kit, according with the manufacturer (NzyTech). The plasmid DNA was eluted in 40µl of sterile Milli-Q water.

2.2.4.5 DNA quantification

The plasmid DNA concentration of the pEX WW Clone 3 was determined with the Nanodrop system. The purity of plasmid DNA was determined based on absorbance ratios 260/280 and 260/230nm.

2.2.4.6 Cloning of the WW Clone 3 with the vector pET 21c

The enzymatic digestions of the vectors were performed with enzymes *NheI* and *BamHI* according to the supplier (Thermo Scientific). It was added 4.5µl of pEX WW Clone 3 DNA with concentration of 2000ng and 15µl of pET21c_GFP (Green Fluorescent Protein) DNA with concentration of 3000ng.

The samples were digested with the enzymes *NheI* and *BamHI* through the incubation in the Thermal Cycler at 37°C for 30 minutes, and for inactivation of the enzymes, 5 minutes at 80°C.

For the purification of the fragment that refers to the gene WW Clone 3 with a His-tag possessing a size of approximately 160bp (**Erro! A origem da referência não foi encontrada.**), was made an 2% agarose gel. For purification of the fragment of the gene pET 21c with size approximately 5400bp, was made in 1% agarose gel.

For a small gel, were added 35ml of gel, and 0.75µl of Greensafe premium. For a large gel, 80ml of gel and 1.5µl of Greensafe premium. After polymerization, to each gel added 5µl of the marker NzyDNA ladder V or 5µl of the marker NzyDNA ladder III (NzyTech) and to each DNA sample was added Loading Dye Buffer in 10% of the total sample volume. As a control was added 1µl of the undigested vector. The gel was run in 90V for 45 minutes.

The gel of each digestion reaction was visualized in GelDoc and registered. The bands correspondents to the fragment size of interest were extracted to WW Clone 3 and the digested pET 21c plasmid was digested.

For purification of the DNA from the agarose bands, the NzyGelpure kit was used. The procedure was followed as described by the supplier (NzyTech). The plasmid DNA and the insert were eluted in 30µl of sterile Milli-Q water. Then, the DNA concentration and purity were determined using Nanodrop described in 2.2.4.5.

To prevent re-ligation of the vector pET 21c, a dephosphorylation procedure was done. It was made with 50ng of vector to three ligation reactions.

The enzyme utilized was FastAP Thermosensitive Alkaline Phosphatase and was added 8.33µl of vector DNA, 2µl of buffer 1x from the FastAP Thermosensitive kit, 0.5µl of enzyme and up to 20µl with high quality water. The procedure was made of according with the protocol of the manufacturer (Thermo Scientific).

The sample was incubated in the Thermal Cycler for 10 minutes at 37ºC and enzyme inactivation for 5 minutes at 75ºC.

For the ligation of the WW Clone 3 with pET 21c, was made of two ratios (insert:vector) were studied: 3:1 and 5:1; and was used a negative control, NC (without insert). The amount of the insert was obtained through Equation 2.1:

$$\frac{50ng\ vector\ x\ 0{,}160kb\ insert}{5{,}4kb}\ x\ ratio \qquad \text{(Equation 2.1)}$$

For this procedure was added 4µl of vector for NC, 3:1 and 5:1; 0.7µl and 1.5µl of insert for 3:1 and 5:1 respectively; 2µl of Buffer 1x from T4 DNA ligase kit, T4 DNA ligase 1µl, and up to 20µl with Milli-Q water for NC, 3:1 and 5:1. The procedure was made according with the protocol of the manufacturer, and the samples were placed in the Thermal Cycler at 16°C, overnight.

The next day, were prepared the samples for the electroporation according to what has been described in 2.2.4.3 except that were added 2µl of each ligation sample to an aliquot of Top10F'.

To determine the success of the ligation, the plates of the different ratios (insert:vector) were analyzed. From each plate of the 3:1 and 5:1 three colonies of each plate were picked, and one from NC plate. Each clone was grown in 5ml of LB liquid with ampicillin at 37°C overnight at 220 rpm. In the next day, a stock of this culture was saved in 10% of glycerol (v/v) and stored at -80ºC.

After, it was made the purification of the clones according to what has been described in 2.2.4.4, followed by the quantification of DNA (2.2.4.5).

To observe the positive clones, it was made the digestion enzymes using 2µl of *NheI* and *BamHI,* 25µl of DNA, and 4µl of Buffer 1x Fast digest and incubated as described above, and visualized in 2% agarose gel.

One clone of the plate ratio 5:1 has shown to possess the WW Clone 3 gene with the expected size. So, it was made the expression in *E. coli* BL21 (DE3) competent cells.

It was added 1µl of the WW Clone 3 in 50µl of BL21 (DE3) *E. coli*, and the mixture proceeded for electroporation as described in 2.2.4.3.

In the next day, it was necessary to select one colony and add in 3ml of LB with 3µl of ampicillin. Then, the culture was incubated at 37°C for four hours. Posteriorly, were added 500µl

of the culture in 50ml of LB, with 50µl of ampicillin and incubated at 37°C, overnight with 220rpm. It was made a stock at -80ºC using 10% glycerol (v/v) of the positive clone WW Clone 3 in BL21 (DE3).

2.2.4.6.1 Protein expression at 18°C, 25°C and 30°C

For the expression of the WW Clone 3, 1 liter of LB, 1ml of ampicillin and 10ml of the culture were used and incubated at 37°C for three hours. Then, after obtaining an OD_{600nm} between 0.6 and 0.7, 1mM IPTG (stock 1M) was added, and protein expression was attempted at 18°C, 25°C and 30°C. To monitor cell growth the OD_{600nm} was measured in a cuvette (Spectrophotometer) and in a 96- well plate (Tecan) before and after induction, at intervals of 3, 4, 6 and 22 hours. In these intervals, samples were normalized the growth. After, it was stored at -20°C.

In the next day, the culture was centrifuged at 15303xg for 10 minutes at 4°C. The pellet was resuspended in 15ml of PBS buffer (10mM Sodium phosphate, 150mM NaCl, at pH 7.4).

2.2.4.6.2 Protein extraction by French Press from bacteria cellular extracts

Before starting the cell lysis with FrenchPress, it was necessary to weaken the cell membrane with three cycles of freeze and thaw. To thaw was heated in a bath at 37°C, and for the freeze cells were placed at -80°C, total of 3 repetitions.

After, cell suspension was processed in the FrenchPress to break the cell wall and the cell membrane of the bacteria. Then, it was added the DNase I, and was incubated in the ice for 15 minutes. It was centrifuged in 18517xg for 15 minutes at 4°C. After, it was added the soluble fraction in the flasks of the Ultra-centrifuge and was made the centrifuge for 1 hour at 204710xg. The soluble and insoluble fraction were collected and stored at -20°C.

2.2.4.7 Screening protein expression

2.2.4.7.1 Analysis by SDS-Page

A 17.5% Polyacrylamide gel was performed, according to the supplier (Bio-Rad). For this method, it was necessary add Tris-Glycine buffer (25mM Tris-Base, 195mM Glycine, 40% Methanol) that was the transfer buffer. In the insoluble fraction, it was necessary to add 25ml of PBS buffer (10mM Sodium phosphate, 150Mm NaCl), to dissolve the pellet, with the help of vortexing.

Posteriorly, it was added 10µl of the marker Precision Plus ProteinTM dual color standards with 5µl of sample buffer of β- mercaptoethanol in the gel. In each sample 5µl of β-mercaptoethanol were added in 30µl of sample at 18ºC, 25ºC and 30ºC. After the following samples were added in the gel: 10µl of after induction (AI) and before induction (BI), and 30µl of the soluble fraction (SF) and insoluble fraction (IF). As a control, it was used 10µl of WW Pin1 sample (a kind gift from Ms. Ana Dias), plus 5µl of sample buffer with β-mercaptoethanol. Before loading the gel, the samples were boiled for 5 minutes. The gel was run in 100 V for 60 minutes. After, it was made the coloring of the gel with Coomassie Blue R-250. (0.1% Coomassie Blue R-250, 0.15% Glacial acetic acid, 0.9% Methanol).

To analyze the samples of expression of the protein in soluble and insoluble fraction of all temperatures through the Western Blotting method, an SDS-Page with Mini-PROTEAN Tris-Tricine precast was made with Tris-Tricine 1x buffer, according to the supplier (Bio-Rad).

Then, it was added 10µl of the marker Precision Plus ProteinTM Dual Color Standards with 5µl of sample buffer with β- mercaptoethanol in the gel. In each sample 5µl of β-mercaptoethanol were added in 30µl of sample at 18ºC, 25ºC and 30ºC. After 30µl of the following samples were added in the gel: insoluble fraction (IF) and soluble fraction (SF). As a control, 10µl of GFP- Pin1, plus 5µl of sample buffer with β-mercaptoethanol were used. Before loading the gel, the samples were boiled for 5 minutes. The gel was run in 150 V for 80 minutes.

Through the gel SDS-Page with Mini-PROTEAN, it was possible to do the Western Blotting method, and it was necessary an Immobilon-PSQ Membrane (0.2µm) and Transfer Buffer with 40% of ethanol. The method was run in 200 mA for 35 minutes.

To wash the membrane, TBS buffer (50mM Tris-base in 150mM NaCl at pH 7.5) was added, and to block the membrane, it was added the Blocking Buffer from kit of Antibody used in this method. Then, for the detection, it was used the Penta Anti·His HRP Conjugate Kit of according with the protocol of the manufacturer. After, it was added 2ml of StepTM TMB-Blotting.

2.2.4.7.2 Total protein quantification by bicinchoninic acid assay (BCA)

The quantification of total protein in the soluble and insoluble fractions was made with the BCA (bicinchoninic acid assay) kit according with the supplier instructions. Firstly it was necessary to prepare a mixture with reagents A and B (A (bicinchoninic acid solution) and the reagent B (cooper (II) sulfate pentahydrate 4% Solution).

Then, were added 25µl of sample and 200µl of reagents in each well. After, it is necessary incubate at 37ºC for 30 minutes. Posteriorly, it was measured in a 96-well plate reader with OD_{560nm} (Tecan).

It was necessary to construct a calibration curve using solutions with known protein concentration, bovine serum albumin (BSA) from 0 to 1 mg/ml, resulting in the calibration curve of with a . Some samples were diluted 1:10 and 1:100.

2.3 Results and discussion

2.3.1 Amplification, construction and purification of plasmid DNA

The vector pEX WW Clone 3 contained the gene sequence that codified for the protein of interest. This gene sequence was optimized to contain non rare codons. This vector was ready for bacteria transformation and amplification. After amplification, the vector DNA was quantified, and presented the following values: Concentration = 455.1ng/µl; the purity of Abs 260/280 = 1.87 and Abs 260/230 = 2.26. After analyzing of the sample, the DNA extracted showed a high degree of purity.

To clone the gene that encodes to the protein WW Clone 3 directly into the expression vector pET 21c, the sites of restriction enzymes *NheI* and *BamHI* were used. The pET 21c used in this assay, had an insert that encodes for the protein GFP-Pin1 (800bp), which had to be removed first. This gene was removed by the restriction enzymes *NheI* and *BamHI.* Subsequently to verify if the vector and insert were well cleaved, and proceed with the extraction, it was made an agarose gel 1% for the pET 21c with a fragment of size of 5400bp, and an agarose gel 2% for the fragment of WW Clone 3 with an expected size of 160bp (Figure II.2).

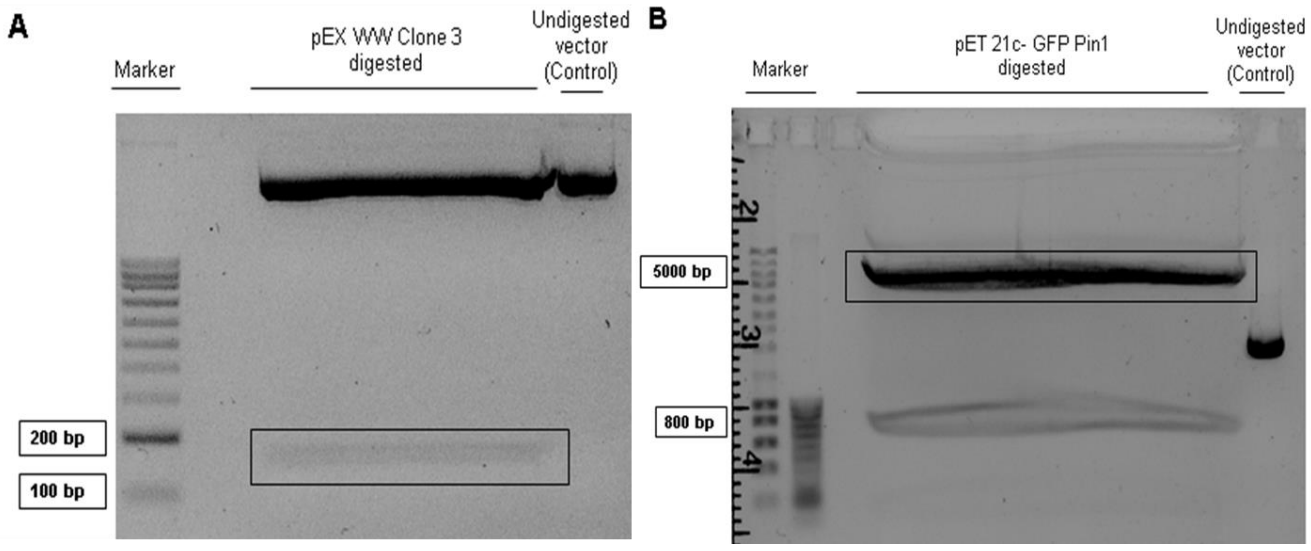


Figure II.2: Agarose gel purification of the DNA fragment. (A) 2% agarose gel of the insert WW Clone 3 with a fragment of 160bp. (B) 1% agarose gel of the pET 21c with a fragment of 5400bp. It was used the marker NzyDNA ladder V to the 2% agarose gel and the marker Nzy DNA ladder III to the 1% agarose gel.

In order to obtain the desired fragments, we proceeded with the extraction, purification and quantification of the DNA. The concentration of pET 21c was 33.2ng/µl and the ratios of purity were Abs 260/280= 1.87 and Abs260/230= 0.52; the concentration of the insert WW

Clone 3 was 7.9ng/µl. Then, it was made the dephosphorylation of the vector pET 21c to prevent its re-ligation.

Based on the concentration of the DNA, the ratios for ligation were determined between the pET 21c and WW clone 3. Two ratios (insert:vector) were attempted: 3:1 and 5:1; and also a negative control, NC (without insert). After the incubation of the plates, clones were found in all plates, but the plate 5:1 contained more.

The concentration and purity were determined for one colony from NC and 3:1, and three colonies from the 5:1 ratio assay. The average concentrations employed of NC, 3:1 and 5:1 was 90ng/µl and the average of purity Abs 260/280 was 1.90 and Abs 260/230 was 2.2.

These concentrations were utilized to proceed with the screening of the clones by digestion. And after the enzyme digestion, the screening of the clones with 2% agarose gel was performed, where one positive clone was verified, with a fragment of 160bp correspondent to the WW Clone 3 (Figure II.3).

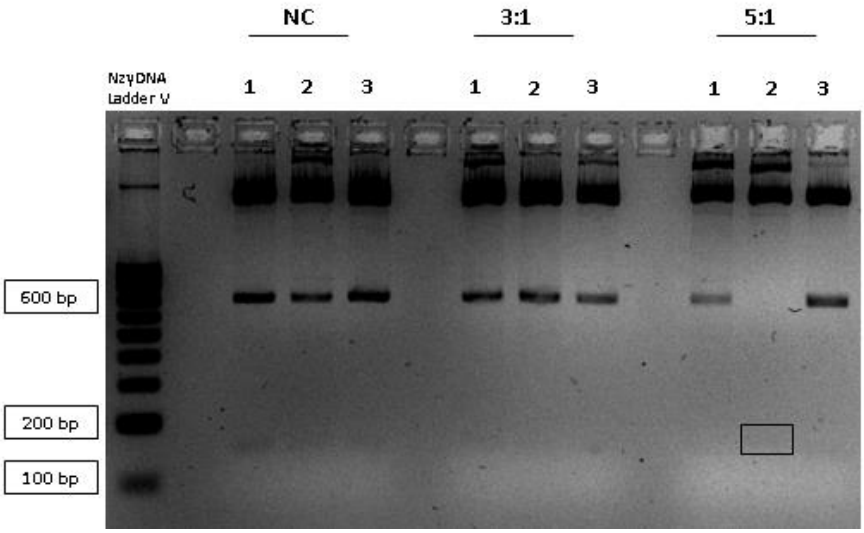


Figure II.3: Screening of the Clones NC, 3:1 and 5:1. It was possible verify one clone positive, 5:1-2, that has a fragment of 160bp that corresponding to the WW Clone 3. It was used the marker NzyDNA ladder V.

In order to make sure that was a positive clone, with correct ligation, a new screening was performed with a higher amount of sample (Figure II.4).

In other clones it was possible to observe a band with 5400bp and two bands related to GFP- Pin1 protein which was previously in the expression vector. This must have occurred in the enzyme digestion process which might have been inefficient, and for that reason the vector may have re-ligated. It is also possible that the vector undigested was also cut during band extraction, due to the low resolution of the gel and the fact that the sizes are very close. Thus, in clone 5:1-2, the gene encoding the protein WW Clone 3 was successfully cloned into the vector pET 21c.

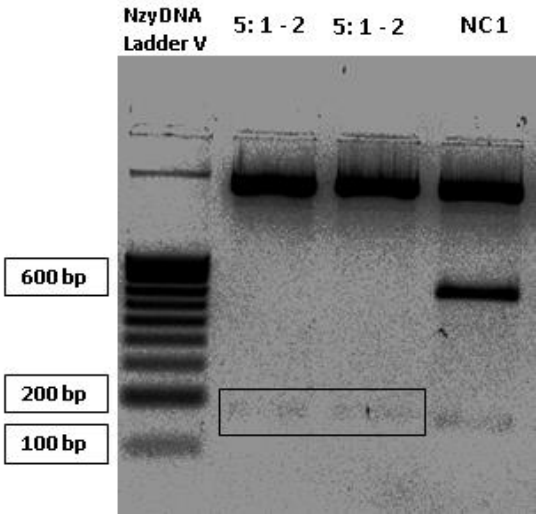


Figure II.4: Screening of the clone 5:1-2. A greater amount of sample was added, showing that the clone 5:1-2 is a positive clone. It was used the marker NzyDNA ladder V.

2.3.2 Protein expression of the clone WW Clone 3

For the expression of the WW Clone 3 protein, the transformation in *E. coli* BL21 (DE3) was performed. In order to assess the best temperature for expression, three different temperatures were studied, 18°C, 25°C and 30°C. Cell growth was monitored over time by measuring the absorbance at 600nm (Figure II.5).

Before induction, the first 3 hours, the growth was at maintained 37°C, and only then, IPTG was added to induction. After this, growth was carried out at different temperature. During the first 3 hours it is possible to observe the lag phase, and after induction with IPTG a significant increase in the culture growing at 18°C and 25°C and a smaller increase in the culture growing at 30°C. After 6 hours, there was a significant increase in the cell growth for all temperatures, mainly at 30ºC. The last measurement at 22 hours was made, to obtain the maximum cell growth. The optical density was monitored simultaneously using a spectrophotometer and a multiplate reader, showing high similarity (Figure II.5).

To verify the expression of the clone WW Clone 3, an SDS- Page with polyacrylamide gel 17.5% was performed as shown in Figure II.6.

It was not possible to observe the band corresponding to the protein in study, due to presence of large amounts of other proteins or because the protein was not expressed. A solution of WW Pin1 (kindly provided by Ms. Ana Dias, FCT-UNL) was also added as control because they have the same size.

After, a SDS- Page Tris- tricine gel was performed, which is more appropriate for small proteins, with the sample of soluble fraction (SF), insoluble fraction (IF) of all temperatures, and for the control, a sample of the protein GFP-Pin1. After, it was made a Western blotting method, through colorimetric detection (Figure II.7).

Through Western blotting method, it was not possible to detect the protein in study, but positive control was detected and the lower molecular weight marker was also detected. This might be due to some limitations of this method, if the protein of interest was produced in a very small amount, the detection does not occur.

Assuming a very small amount of protein was expressed, and also to verify the optimum temperature of expression, it was performed total protein quantification, through the BCA test.

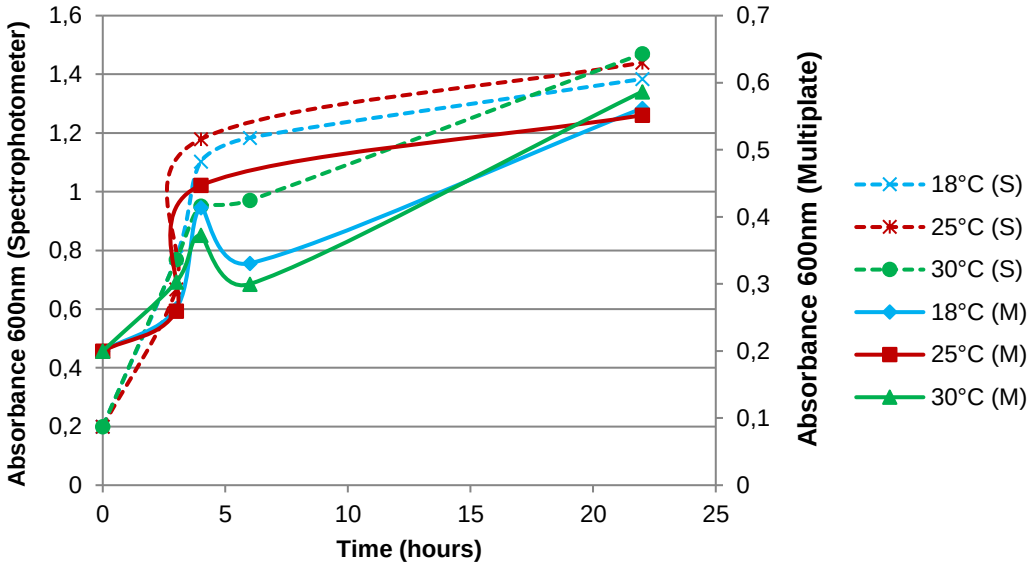


Figure II.5: Graphic of cell growth by measuring the absorbance at 600nm. (S) Optical density values obtained during the growth of the WW Clone 3, by Spectrophotometer. (M) Optical density values obtained during the growth of the WW Clone 3, by a multiplate reader.

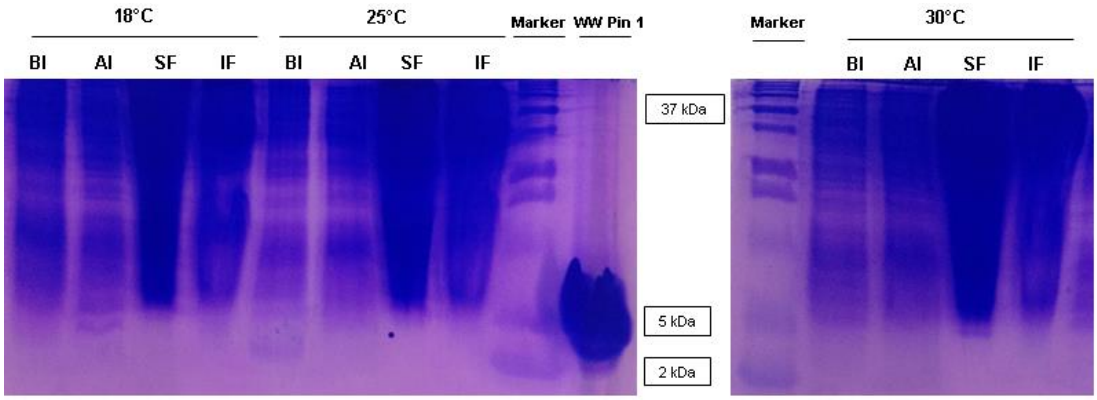


Figure II.6: SDS-Page with polyacrylamide gel 17.5% to verify the expression of the clone WW Clone 3. Polyacrylamide gel (17, 5%) from electrophoresis, stained with Coomassie blue with the samples of 18ºC, 25ºC and 30ºC. The control used was the protein WW Pin1. It was used the marker Precision Plus Protein Dual Color Standards.

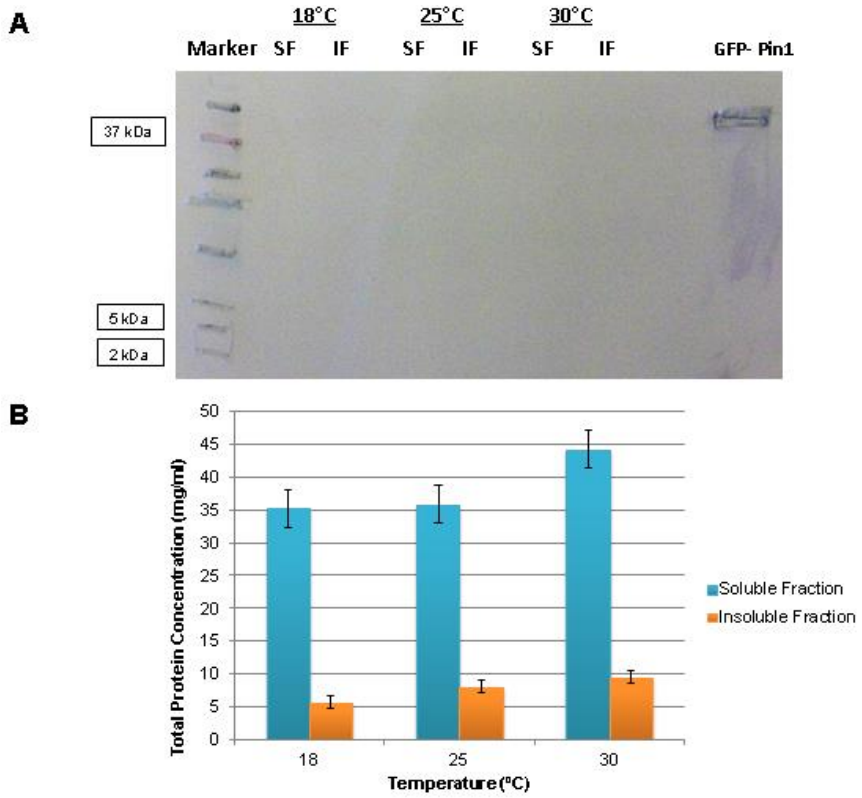


Figure II.7: Western Blotting method and Total protein quantification. (A) Western blotting membrane from the tris-tricine gel for all samples in study, with a band of a positive control, GFP-Pin1. It was used the marker Precision Plus Protein dual color standards. SF (soluble fraction); IF (insoluble fraction). (B) Column chart for total protein concentration obtained by BCA test. The concentration for soluble fraction is in blue and for insoluble fraction in orange.

The BCA test allows us to obtain the total protein concentration in the soluble and insoluble fractions of the samples. Using the calibration curve previously shown, it was possible to calculate the concentrations of the soluble and insoluble fractions for all temperatures above mentioned (18ºC, 25ºC and 30ºC). The results obtained for soluble fraction at 18ºC and 25ºC was approximately 35mg/ml and approximately 45mg/ml for 30ºC. In the case of insoluble fraction it was obtained approximately 5mg/ml for 18ºC, approximately 8mg/ml for 25ºC and approximately 10mg/ml for 30ºC, as is shown in the Figure II.7.

According to the columns chart (Figure II.7), it was possible to observe that the best temperature is 30ºC, because the concentration of total protein is higher than at the other two temperatures. Although it was possible to determine the protein concentration, it was not possible to observe in the Western blotting, the WW Clone 3 protein.

The protein gene from WW Clone 3, was sent for sequencing and the result was positive, which means that the gene codifying for the protein WW Clone 3 is present in the sample.

According to the results obtained, it was not possible to continue this work. This may be due to the size of the protein of interest. The WW Clone 3 is a very small protein, of approximately 5kDa, and with a hydrophobic character, being considered difficult to express in *E. coli*.

2.4 Conclusions

Several studies have been conducted with the WW domain protein, due to its excellent ability to establish protein-protein interactions. Protein domains are natural modulators of biological function through ligand binding. These domains may be engineered in order to generate altered activities and novel affinity reagents and provide a versatile platform to link individual proteins into physiologically important networks (Patel *et al*, 2013; Ignham *et al*, 2005).

This chapter aimed at optimizing the expression of WW Clone 3 protein, since another WW domain (WW Pin-1) was already previously co-expressed in our group with GFP. In this work we used molecular biological techniques to construct a protein expression vector using the expression vector pET 21c, adding to insert the gene encoding the protein WW Clone 3. This step was successful, in the positive clone 5:1-2 it was possible to obtain the gene encoding the protein WW Clone 3.

Three different temperatures were tested for expression, 18ºC, 25ºC and 30ºC, and through the result of the optical density absorbance, it was possible to conclude that the best temperature for cell growth was 30ºC. Also, we obtained the total protein concentration in the soluble and insoluble fractions at each temperature, and the temperature that produced a larger amount of total protein was at 30ºC, with approximately 45mg/ml in the soluble fraction and 10mg/ml in the insoluble fraction.

In conclusion, in this study it was possible to obtain the gene encoding the protein of interest through a sequencing confirmation, but the quantity of protein expressed was too small to be detected by Western blotting method, or in fact protein was not expressed. With these results, it was not possible to continue this work because the result was not sufficient to achieve the goal initially proposed; therefore, a better strategy to optimize the expression of this protein in a detectable amount is required.

As a future strategy, we could try to optimize the expression using another type of competent cells of *E. coli*, and the pET System, e.g. pET 28. In addition, the concentration of IPTG can be adjusted; the time and the temperature of induction changed; and the gene the sequence can be optimized again. Other strategies could be to express the protein in insoluble form by decreasing the temperature of expression and subsequently try to solve the inclusion bodies. It can also change the position of the protein tag and its type, to a more hydrophilic, to provide more solubility to the protein, e.g. Ubiquitin, SUMO and GST (Glutathione- S-transferase); or an affinity tag to further purification.

III. Design, synthesis and screening of solid phase combinatorial libraries of affinity ligands towards HSA

In this work two solid phase combinatorial libraries were design based on amino acids of the protein PAB and drugs that bind naturally to HSA, and synthesized using the Ugi and Triazine based reactions. Subsequently, the libraries were screened against to HSA in two different pH values by microscale affinity chromatography. Different pH conditions were tested for binding of albumin to the neonatal Fc receptor in order to try increase the half-life of drugs or even from the therapeutic proteins as this binding is pH-dependent. In addition, these synthetic ligands can serve as a new alternatives for the purification of the HSA in a lower cost, more durability and stability manner.

3.1 Introduction

Currently there is an increase in the popularity of low molecular weight ligands that mimic the action of biologics, not only for drug discovery and inhibition of enzymes, but also in many protein purification applications (Gupta and Lowe, 2000).

Affinity ligands are classified into two types: biospecific and pseudobiospecific. Biospecific ligands are molecules derived from natural sources that have affinity for the target protein-binding site (e.g. natural antibody-binding molecules include the antigen, anti-antibodies, lectins or bacterial immunoglobulin-binding proteins). Pseudobiospecific ligands can be biological or non-biological molecules that have affinity to the target protein, although do not occur naturally in the biological systems (e.g. synthetic dyes, peptides or oligonucleotide aptamers) (Roque and Lowe, 2006; Roque *et al,* 2007).

Employing combinatorial chemistry approaches, a new class of pseudobiospecific ligands, termed biomimetic has been developed, as a more advanced version of the natural affinity ligands (Roque *et al*, 2005). This class of ligands, mimic the structure and binding of natural biological ligands and result from the integration of rational design, combinatorial chemistry and screening in parallel for binding to the target protein (Sousa and Taipa, 2014; Roque *et al*, 2004).

There are four main strategies on ligand design that can be followed: (i) use of the natural binding site of the biological ligands as a template; (ii) development of a novel molecule with the same binding of the complementary ligand for the target protein; (iii) exploitation of the natural biological recognition interactions between the pairs ligand and protein; and (iv) analysis of the structure of the target protein and selection of the best binding sites (Roque *et al*, 2004).

Lowe and colleagues were pioneers in the synthesis of synthetic ligands through the Triazine based reaction, a type of combinatorial chemistry reaction employed on a solid support, for affinity purification of different target biomolecules (e.g. immobilized artificial Protein A to purify IgG from human plasma, ascites fluid and fetal calf serum) (Li and Lowe, 1998; Lowe *et al*, 1992; Roque *et al*, 2004).

The Triazine reaction (2,4,6-trichloro-1,3,5-triazine) is based on the reactivation of the cyanuric chloride under relatively mild conditions. Normally, the three chloride atoms from cyanuric chloride can be replaced in a sequential and controlled fashion by amines in aqueous solution through nucleophilic substitutions; the first substitution occurs between the solid matrix activated with amine groups and the cyanuric chloride molecule at approximately 0ºC, the second (R1) at 30ºC and the last substitution (R2) occurs at 80ºC (Figure III.1) (Filippusson *et al*, 2000; Sproule *et al*, 2000; Teng *et al*, 1999; Roque *et al*, 2004).

The Ugi multicomponent reaction has also been employed for the synthesis of combinatorial libraries of affinity ligands, and is a reaction with several advantages, as its simplicity, low cost and time saving (Pina *et al*, 2014a). Furthermore, the Ugi reaction is considered more flexible than the Triazine scaffold due to its structure that is less planar (Qian *et al*, 2012). Haigh and co-workers synthesized a ligand that mimics the protein L through the

Ugi reaction on Sepharose™ solid-support. This ligand binds preferentially to the Fab domain over the Fc domain (Haigh *et al*, 2009). Therefore, this study and many others demonstrated that Ugi reaction can be a great alternative to synthesize synthetic ligands for different protein purification applications (Haigh *et al*, 2009; Chen *et al*, 2014; Pina *et al*, 2014a).

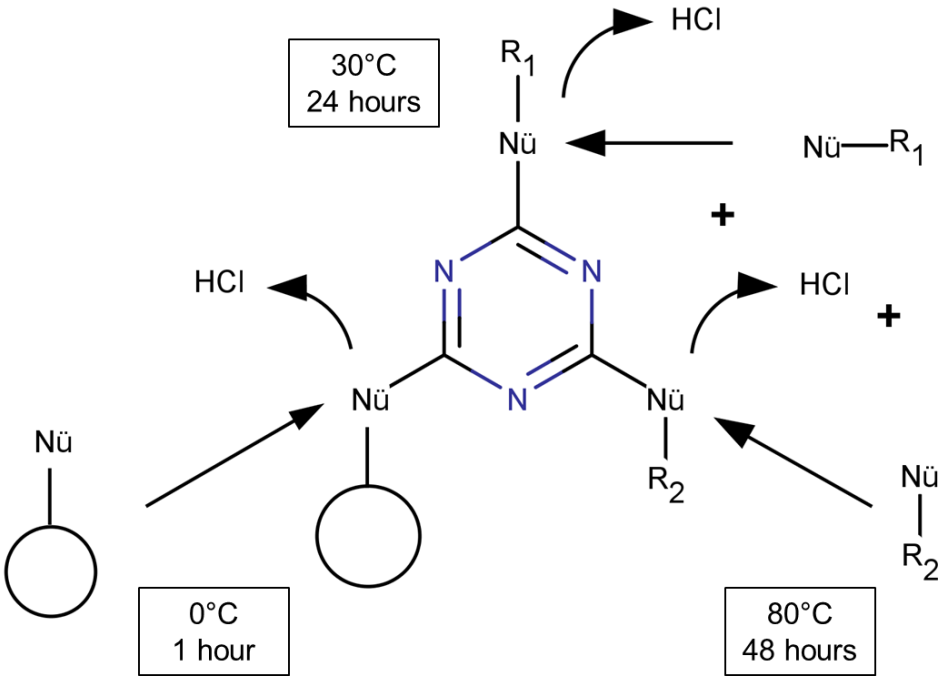


Figure III.1: The solid phase synthesis of Triazine reaction. For occur the combinatorial synthesis of a Triazine-based ligand library, the cyanuric chloride compound, will be undergoes three nucleophilic substitution (denoted by Nü), and these nucleophiles can be amines, hydroxyls, thiols and others. For the first substitution, a solid matrix (sphere) with a nucleophile will react with cyanuric chloride at 0°C for 1 hour. Subsequently two other nucleophilic substitutions will occur: R1 at 30 ° C for 24 hours and R2 at 80ºC for 48 hours. R1 and R2 can contain a varied functional group (Lowe *et al*, 2000).

This multicomponent reaction consists in a four-component reaction which involves a the strictly governed interaction primary amine, an aldehyde or ketone, an isocyanide, and a carboxylic acid to form a bis-amide, losing just one molecule of water during the whole process (Figure III.2) (Tron, 2013; Qiana *et al*, 2012).

The most commonly adsorbents used on protein purification protocols are biological ligands which presents several limitations as higher costs associated, greater fragility and they are not readily amenable to scale-up. Moreover, the adsorbents based on monoclonal antibodies or phage display peptides are more prone to chemical and biological degradation. On other hand, entirely synthetic ligands presents more advantages over the biological ligands such as higher stability, defined chemical structure, resistance to chemical and biological degradation, sterilization *in situ* without loss of performance, durable and reusable over multiple cycles and can be produced with lower costs (Haigh *et al*, 2009; Gupta and Lowe, 2000; Teng *et al*, 1999; Lowe, 2001; Pina *et al* 2014b).

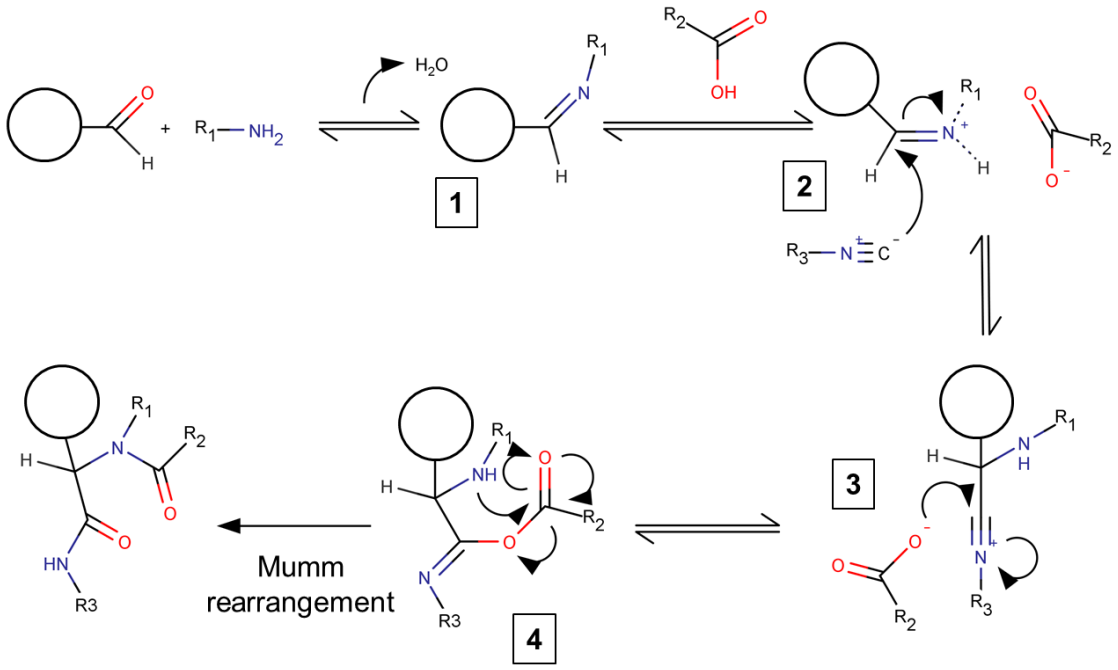


Figure III.2: Mechanism for the Ugi reaction. The reaction begins with an interaction between the amine and the aldehyde or ketone in a solid matrix, to give the corresponding imine (1). The imine will react in an acid-base reaction with the carboxylic acid to form an iminium ion (2), which is posteriorly arrested by the isocyanide to obtain a nitrilium ion (3). This reacts with carboxylate ion produced in the imine activation step to afford an imino-anhydride unstable (4), and lastly occurs the Mumm rearrangement (Image adapted from Tron, 2013).

The most common adsorbents used on protein purification protocols are biological ligands which present several limitations high cost, great fragility and difficult amenable to scale-up. Moreover, the adsorbents based on monoclonal antibodies or phage display derived peptides are more prone to chemical and biological degradation. On other hand, entirely synthetic ligands, as those based on the Triazine and Ugi scaffold, present more advantages over the biological ligands such as higher stability, defined chemical structure, resistance to chemical and biological degradation, sterilization *in situ* without loss of performance, durable and reusable over multiple cycles and can be produced at lower costs (Haigh *et al*, 2009; Gupta and Lowe, 2000; Teng *et al*, 1999; Lowe, 2001; Pina *et al* 2014b).

3.2 Material and methods

3.2.1 Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents Tyramine (A1), Isopentylamine (A2), 3,4-Dimethylaniline (A3), 1,4 Diaminobutane (A4), Phenethylamine (A5), Sulfisoxazole (A6), Amino-2-propanol (A7), 2-aminopentane (A8), 3,4-dihydroxyhydrocinnamic acid (C1), 9- Anthracenecarboxylic acid (C2), 4-Methylvaleric acid (C3), 3,5-Diiodosalicylic acid (C4), 4-Imidazolecarboxylic acid (C5), Indomethacin (C6), Arabic Acid (C7), Aspirin (C8), Nicotinic acid (C9), 3-indole acetic acid (C10), DL-Proline (C11), human serum albumin and bovine serum albumin (BSA) were purchased from Sigma- Aldrich (Portugal).

Acetone, Epichlorohydrin, Cyanuric chloride, Isopropyl isocyanide, Phenol, Sodium bicarbonate ($NaHCO_3$), Sodium periodate (NaIO4), Sodium thiosulfate ($Na_2S_2O_3$), Silver nitrate (AgNO3), Glutaraldehyde, Glycine, Sodium Chloride (NaCl) and QuantiPro™ BCA Assay Kit were acquired from Sigma-Aldrich (Portugal). Methanol (MeOH) and Ammonium hydroxide solution (NH4OH) were obtained from Roth. Dimethylformamide (DMF), Methanol (MeOH), Di-Sodium-hydrogen Phosphate 2-hydrate and Sodium hydroxide (NaOH) were purchased from VWR. Ethanol absolute PA, Sodium-di-hydrogen Phosphate 1-hydrate, Hydrochloric acid 37% (HCl), were acquired from Panreac (Spain).

3.2.2 Chromatographic materials

Cross linked agarose (SepharoseTM CL-6B) was acquired from GE Healthcare (Portugal); Captiva 96-well filtration block was purchased from Agilent Technologies (EUA); Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Germany), respectively.

3.2.3 Buffers

The following buffers and reagents were used: Phosphate Buffer Saline (PBS) (10mM Sodium phosphate, 150mM NaCl, pH 7.4 and pH 6.0); Regeneration Buffer (0.1M NaOH in 30% isopropanol); 0.1M NaOH in 50% isopropanol; 0.1M glycine- NaOH at pH 9.0 in 50% Ethileno glycol; 0.1M Glycine – NaOH at pH 11; 0.1M HCl; 0.1M Glycine at pH 2.5; 0.2M NaOH in 50% isopropanol; 1M NaCl in Phosphate buffer saline.

3.2.4 Equipment

The synthesis of the synthetic ligands on solid-phase based on Triazine and Ugi reaction were carried out in Incubator ZKA KS4000i (VWR); Microplate reader TECAN Infinite F200 from Thermo Scientific (Portugal); Big SHOT III from Boekel Scientific. The screening was performed using a LMC 3000 laboratory centrifuge from Biosan.

3.2.5 Methods

3.2.5.1 Epoxyactivation of agarose

Firstly the SepharoseTM CL-6B (83g) was washed with distilled water (10x volume of resin"s weight) and filtered with the aid of a filter under vacuum. Posteriorly, the resin was resuspended in 83ml of distilled water and 3.3ml of 10M NaOH (40ml per 1kg of moist gel). After, the suspension was incubated for 30 minutes at 30ºC with orbital shaking at 200rpm. Then 5.97ml Epichlorohydrin (72ml per 1kg of moist gel) was added to the moist gel and incubated for 3 hours at 36ºC at 200rpm. In the end, the agarose was extensively washed with distilled water (10x resin volume).

Afterwards, the extension of epoxyactivation was determined through the quantification of OH- groups released from the epoxy ring opening. Firstly 3ml of 1.3M Sodium Thiosulfate was added to 1g of epoxy-activated resin and incubated for 20 minutes at room temperature. After, the released OH- groups were titrated with 0.1M HCl until pH reached 7. The volume added corresponded to the number of OH- released (10µmoles per 100µl added). Normally, the epoxyactivation of SepharoseTM CL-6B yielded 20µmol of epoxy/g of gel (Roque and Lowe, 2008). In this work, the epoxy-activation obtained was 20µmol/g of agarose.

3.2.5.2 Functionalization of epoxyactivated agarose with amine groups

40g of epoxyactivated agarose was resuspended in 5M Ammonium hydroxide (1.5ml per gram of moist resin) and then incubated overnight at 40ºC with agitation at 200rpm. The aminated agarose was then washed with distilled water (10x resin volume)

In order to confirm the presence of the amines in agarose, the Kaiser test was performed. The test allows to quantify free amines (-NH2) present in a given sample and is based on the reaction of ninhydrin with primary amines, which gives a characteristic dark blue color. Thus, 50µl of 5% ninhydrin in ethanol (w/v), 50µl of 80% phenol in ethanol (w/v) and 50µl of 2% 0.001M potassium cyanide in pyridine (v/v) were added to 100mg of aminated agarose. Then 0.9ml distilled water was added to the samples and heated in a water-bath at 100°C for 5 minutes. A calibration curve with glycine solutions (0-5µmol/ml) was performed in triplicates of

$y = 3.4949x - 0.051$ with a $R^2 = 0.9939$. Typically, the amount of amine groups was estimated as 10µmol/g support.

3.2.5.3 Functionalization of epoxyactivated agarose with aldehyde groups

40g of epoxyactivated agarose was resuspended in 40ml 5M NaOH (1ml/g moist resin) and incubated overnight at 30°C with agitation at 200rpm. After, the resin was extensively washed with distilled water using vacuum filtration, resuspend in 40ml 0.1M sodium periodate (1ml/g moist resin) and incubated at 45ºC, 200rpm for 6 hours. At the end of the functionalization, the resin was washed with distilled water (10x resin volume).

After functionalization, the amount of aldehyde groups was analyzed through the Tollens Test. The presence of a silver mirror or a black precipitate after the test reveals the presence of aldehyde groups (Figure III.3). Firstly 1ml of freshly prepared Tollens reagent was added to 1g of aldehyde-activated agarose. For the positive control it was used 1ml of glutaraldehyde and for the negative control was used 1g of SepharoseTM CL-6B unmodified. The Tollens reagent was prepared by adding 2ml of 0.2M Silver nitrate and a drop of 3M NaOH in a test tube, which was previously cleaned with 3M NaOH. Subsequently, 2.8% Ammonium hydroxide was added dropwise with constant agitation until almost all precipitate of silver oxides has dissolved. In order to guarantee the removal all the precipitate, 8.8% Ammonium hydroxide was added dropwise. Afterwards, the Tollens reagent was ready to use.

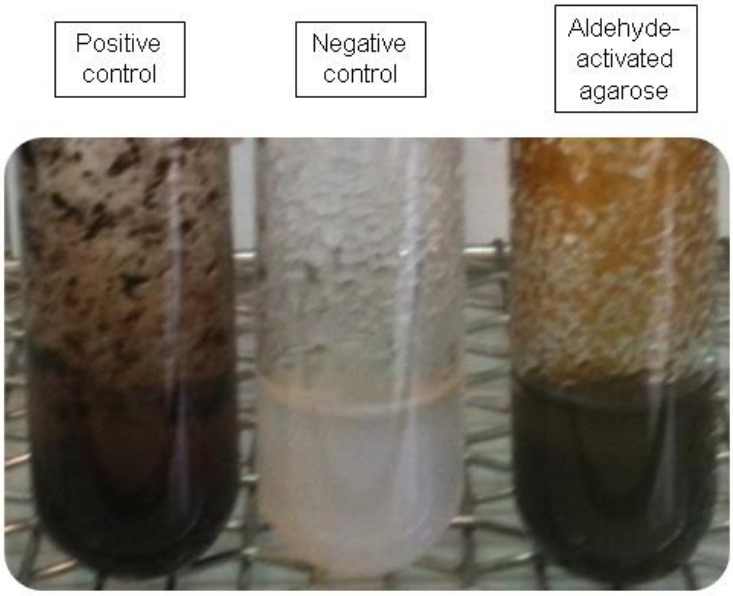


Figure III.3: Tollens test of the agarose with aldehyde groups. The presence of a silver mirror or a black precipitate in the aldehyde- activated agarose revealed the presence of aldehyde groups.

3.2.5.4 Synthesis of the solid phase combinatorial library based on the Triazine reaction

In order to perform the Triazine reaction, the agarose gel functionalized with amine groups was washed with distilled water. Then, two solutions were prepared: first a cold solution of 50% (v/v) acetone/distilled water with 1 molar eq. of Sodium bicarbonate relative to the content epoxy (1ml/g moist agarose), and a second with a solution containing Cyanuric chloride (5 eq. molar excess relative to epoxy content) dissolved in acetone (8.6ml/g Cyanuric chloride). These solutions were added to the aminated resin, followed by incubation for 1hour in ice at 200rpm.

Posteriorly, the agarose was washed with acetone (2x resin volume), 50% (v/v) acetone/ distilled water (3x resin volume) and distilled water (5x resin volume). The agarose functionalized with Cyanuric chloride was resuspended in 18ml 50% DMF/distilled water, and then with a 1-ml pipette tip that was cut 4mm, was distributed in 64 wells of a 96-well filter plate (0.25g/ well). After that, the end cap of the reaction block was removed to occur the solvent drain by gravity. Then, the end cap of the reaction block was again placed, and the amines A1 to A8 to each column of the filter plate (2 molar eq. of each relative to epoxy content; 0.5ml/ well) were added (R1) (Figure III.4). Then, an upper cover was placed, and the filter plate was incubated for 24h at 30°C with agitation (150rpm).

Subsequently, the ligands were washed with 50% DMF/distilled water (1x 0.75ml) and distilled water (4x 0.75ml). In the last wash, the solvent was left to drain by gravity. Afterwards, the end cap was again placed and the amines A1 to A8 were added to each well of the filter plate (5 molar eq. of each relative to epoxy content; 0.5ml/ well) (R2) (Figure III.4). After, the block was sealed and incubated for 48 hours at 80°C in the orbital shaker (150rpm).

At the end of the reaction, the ligands were washed with 50% DMF/distilled water (5x 0.75ml), 0.1M HCl (1x 0.75ml), distilled water (1x 0.75ml), 0.1 NaOH in 30% isopropanol (v/v) (regeneration buffer; 0.75ml), and finally distillated water (5x 0.75ml), and 20% ethanol (v/v) for store at 4ºC.

In this reaction, all the amines (A1 to A8) were firstly dissolved in 50%DMF/distilled water, before add to the wells. Moreover, it was added 1M Sodium bicarbonate (1 molar eq. relative to epoxy) in each amine.

Two types of negative controls were added in two different wells of the multiwell plate i) 0.25g SepharoseTM CL-6B ii) 0.25g Aminated agarose.

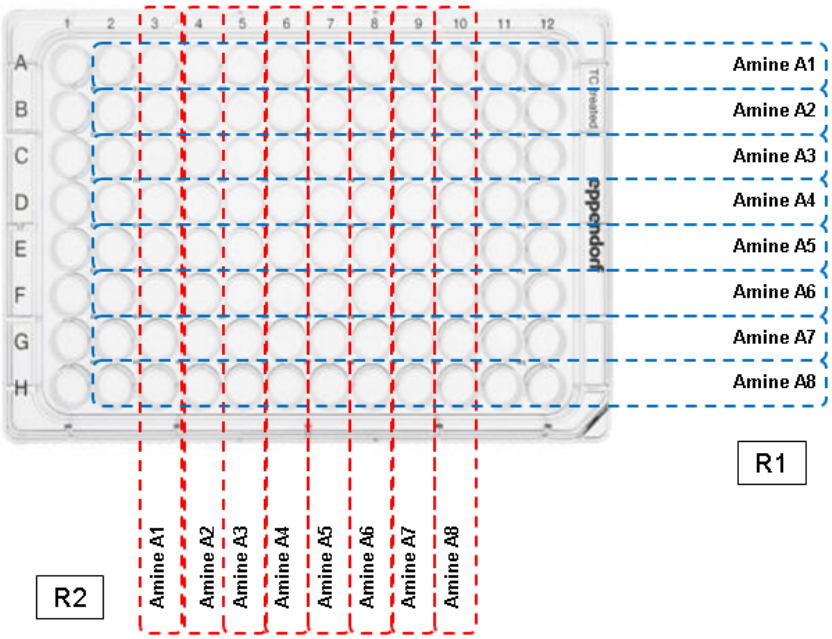


Figure III.4: Illustrative image of the 96-well filtration block used for the synthesis of the ligands based on Triazine reaction. From the top, with the order of addition of the compounds. R1 indicates the amines that were added to the second nucleophilic substitution and the R2 indicates the amines that were added to the last nucleophilic substitution used on the Triazine library.

3.2.5.5 Synthesis of the solid phase combinatorial library based on Ugi reaction

In order to perform the Ugi reaction, the agarose functionalized with aldehydes groups was washed with 20% of methanol, from 0% (v/v) to 100% (v/v) methanol. After, the agarose was resuspended in 24ml of 100% methanol (v/v). Thus, with a 1-ml pipette tip that was cut 4mm, agarose was distributed in 88 wells of a 96-well filter plate (0.25g/ well), and the slurry was left to drain by gravity in order to remove the solvent. Then, the end cap of the reaction block was again placed.

First, the amines A1 to A8 (5 molar eq. of each relative to epoxy content; 0.25ml/well) were added in the block (Figure III.5), to form the imine compound that is required in the Ugi reaction. Then, an upper cover was placed, and the filter plate was incubated for 2 hours at 60°C with agitation (180rpm). After, the carboxylic acid compounds C1 to C11 (5 molar eq. of each relative to epoxy content; 0.25ml/ well) were added in the block (Figure III.5), with the isopropyl isocyanide (5 molar eq. relative to epoxy). Then, the block was sealed and incubated for 48 hours at 60°C in the orbital shaker (150rpm).

In the end of the reaction, the ligands were washed with 1ml of the following reagents: 100% methanol (v/v), 50% methanol (v/v), 50% DMF/distilled water (v/v), distilled water, 0.1M HCl, distilled water, 0.2M NaOH in 50% isopropanol (v/v), 2x distilled water and 20% ethanol (v/v) for store at 4ºC.

In this reaction, all the amines and carboxylic acid compounds were dissolved in 100% methanol, with the exception of C7 compound, that was dissolved in 50% methanol/distilled water (v/v).

Two types of negative controls were added in two different wells of the i) 0.25g Sepharose™ CL-6B ii) 0.25g Aldehyde-functionalized agarose.

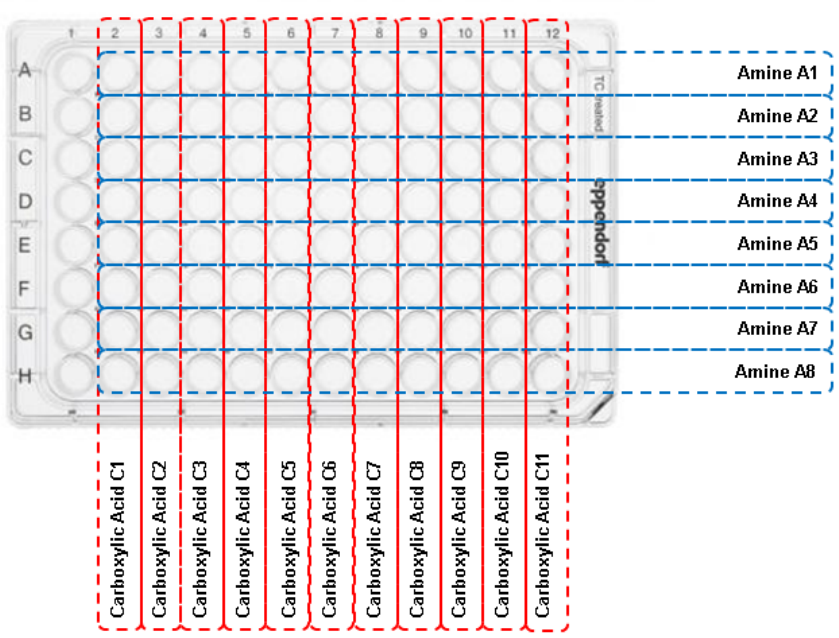


Figure III.5: Illustrative image of the 96-well filtration block used for the synthesis of the ligands based on Ugi reaction. From the top, with the order of addition of the compounds amine and acid carboxylic used in the Ugi library.

3.2.5.6 Regeneration and equilibration of the combinatorial libraries

The regeneration and equilibration of the libraries were necessary prior to screening. The libraries of Ugi and Triazine containing the affinity ligands and the controls were washed for binding with regeneration buffer (0.1M NaOH in 30% isopropanol) followed by distilled water (3 cycles of washes, 0.75ml/well). After, the equilibration of the libraries was conducted with the addition of the binding buffer (15x 0.75ml/well on average). The 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 and 6.0 was used as binding buffer. In the last wash, 100µl were collected in a half-area UV-Star 96-Well microplate, and read at 280nm. The last wash was performed when the absorbance values were lower than 0.005. Then, it was added 20% ethanol (v/v) (0.75ml/well) in the libraries for store at 4ºC.

3.2.5.7 Screening of the synthetic ligands

For the screening procedure, it was a solution of pure HSA in Phosphate Buffer at pH 7.4 (0.25mg/ml) or Phosphate Buffer at pH 6.0 (0.25mg/ml) were prepared. After, 0.25ml of the solutions were loaded to each well of the combinatorial libraries. The blocks were incubated with the loading for 1 hour at 25ºC with agitation (200rpm). Posteriorly, the flow-through and the washes were collected with its respective binding buffer (8x 0.25ml). Both were collected in 96-well transparent microplates by centrifugation at 500rpm during 1 minute.

After, the total protein present in the samples was quantified by using the QuantiPro™ BCA assay kit. Thus, 150µl of the QuantiPro™ BCA reagent was added to 150µl of each sample added and incubated at 60ºC for 1 hour according to supplier instructions. The absorbance of the samples was then determined by measured at OD in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 and pH 6.0 from 0 to 200µg/ml with the values of $y = 0.0184 + 0.0362$ with a $R^2 = 0.0995$ and $y = 0.0193x + 0.0104$ with a $R^2 = 0.989$, respectively.

After the screening, the libraries were washed with 1ml of the following solvents: 0.1M glycine- NaOH at pH 9.0 in 50% Ethileno glycol (v/v) (1x); 0.1M Glycine – NaOH at pH 11 (1x); distilled water (1x); 0.1M HCl (2x); distilled water (1x); 0.1M Glycine at pH 2.5 (1x); 0.2M NaOH in 50% isopropanol (v/v) (2x); Phosphate buffer saline in 1M NaCl (1x). Then, it was made the regeneration described in 3.2.5.6.

3.3 Results and discussion

3.3.1 Rational design of the combinatorial libraries

In order to select the amine and carboxylic compounds to incorporate in the combinatorial libraries, several HSA ligands were considered. The study focused initially on proteins that bind to the domain II of HSA. The aim was to leave domain III, responsible for FcRn interaction, free to bind to the natural receptor.

The protein PAB naturally binds in the domain II of HSA, and through of the study of Lejon and coworkers, it was possible to determine the amino acids involved in this interaction (Figure III.6) (Table III.1) (Lejon *et al*, 2004). To ensure greater variability of the libraries, the drugs that bind to Domain II of HSA were also included in the design (Table III.2).

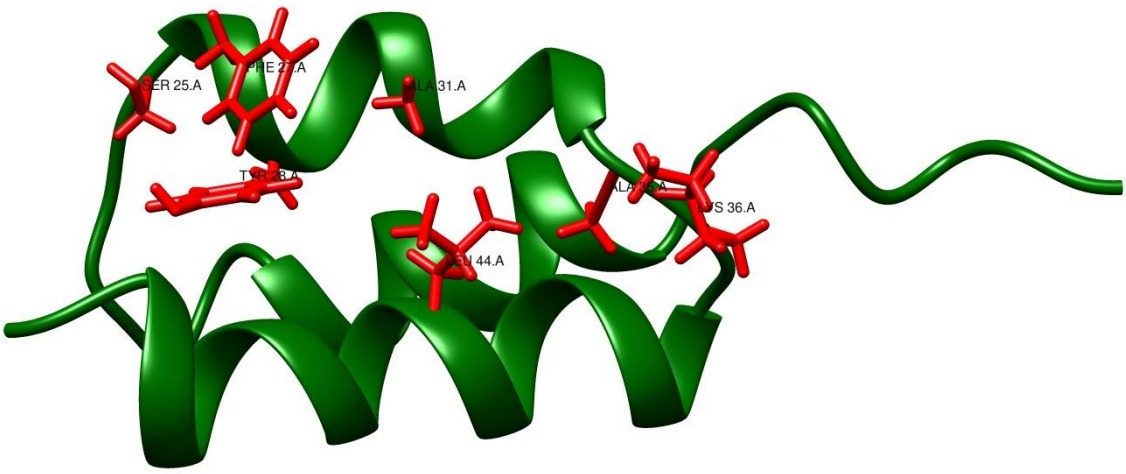


Figure III.6: Structure from the protein PAB with the amino acids that bind to the domain II of HSA. Representation in ribbon model of the protein PAB (green), and the selected amino acids in stick (red) with the code, that were introduced for the synthesis of libraries. Image built in UCSF Chimera software (PDB code: 1GAB) (Lejon *et al*, 2004; Johansson *et al*, 1997).

Table III.1: List of the amino acids from the protein PAB that bind to the domain II of HSA (Lejon *et al*, 2004).

Amino acids from protein PAB
Phenylalanine 27
Leucine 44
Lysine 36
Serine 25
Tyrosine 28
Alanine 31, 35

Table III.2: List of the drugs that bind to the domain II of the HSA used in the libraries.

Drug	Reference
3,5-Diiodosalicylic acid	Ghuman *et al*, 2005
Indomethacin	Ghuman *et al*, 2005
Sulfisoxazole	Ascenzi *et al*, 2006
Aspirin	Fanali *et al*, 2012

From these amino acids and drugs, different amines (Table III.3) and carboxylic acid (Table III.4) were selected for the synthesis of these libraries. Aromatic moieties were also introduced to mimic hydrophobic interactions, to improve the variability within the libraries.

Considering that the Ugi reaction is a multicomponent reaction, it is possible to incorporate three, four or even more reagents, and then have a wide variety in the final structure of the ligand. In this study, only two components, an amine (Table III.3) and other carboxylic acids were varied binding a library with 88 different affinity ligands (Table III.4). The isopropyl isocyanide component was kept constant (Haigh *et al*, 2009).

For the Triazine reaction, eight amines were used to construct the library leading to 64 different affinity ligands (Table III.3). In the triazine reaction, is indifferent use a type of amine in the first or second nucleophilic substitution, being theoretically symmetric. In the first substitution (R1), a lower temperature (30°C) is used to occur only a nucleophilic substitution between the first amine compound and the support; however, in the second substitution (R2), the temperature is increased (80°C), to the second compound make the remaining substitutions.

Table III.3: Amine compounds (A1- A8) used in the libraries of Ugi and Triazine with structure and name. The reagent who has the small rectangle indicates the amino acid which it mimics.

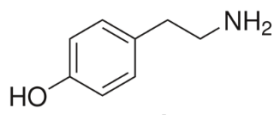
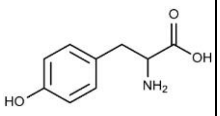
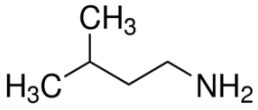
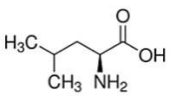
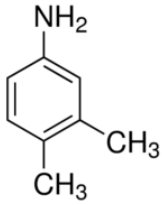
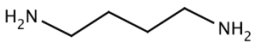
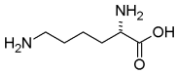
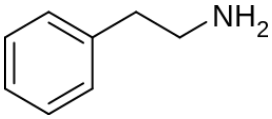
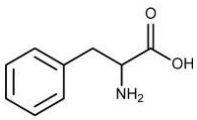
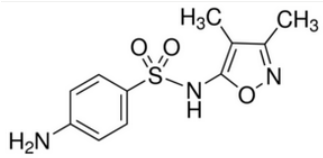
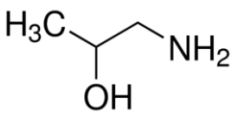
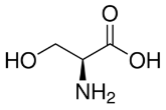
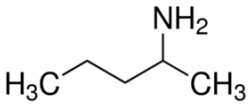
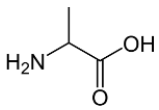
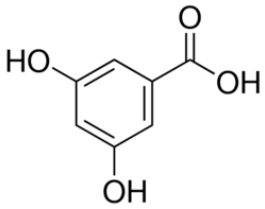
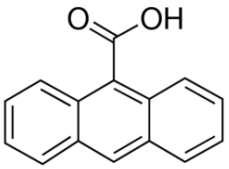
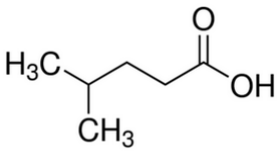
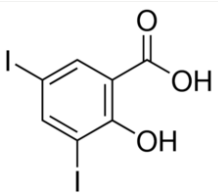
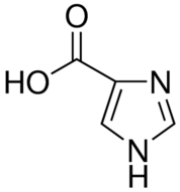
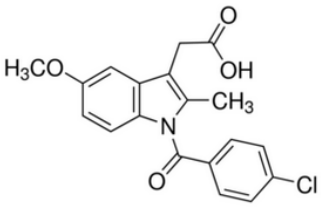
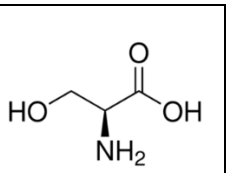
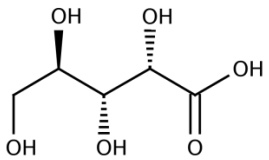
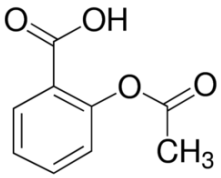
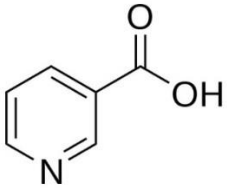
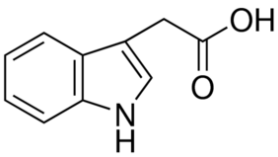
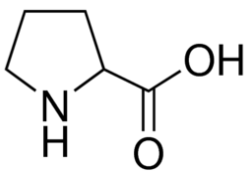
	Reagent/ Amine		Reagent/ Amine
A1	 **Tyramine**  Tyrosine	**A2**	 **Isopentylamine**  Leucine
A3	 **3,4-Dimethylaniline**	**A4**	 **1,4 Diaminobutane**  Lysine
A5	 **Phenethylamine**  Phenylalanine	**A6**	 **Sulfisoxazole (Drug)**
A7	 **Amino-2-propanol**  Serine	**A8**	 **2-aminopentane**  Alanine

Table III.4: Carboxylic acid compounds (C1- C11) used in the libraries of Ugi reaction with structure and name. The reagent who has the small rectangle indicates the amino acid which it mimics.

	Reagent/ Carboxylic acid		Reagent/ Carboxylic acid
C1	 **3,4-dihydroxyhydrocinnamic acid, 98%**	**C2**	 **9- Anthracenecarboxylic acid**
C3	 **4-Methylvaleric acid**	**C4**	 **3,5-Diiodosalicylic acid – (Drug)**
C5	 **4-Imidazolecarboxylic acid**	**C6**	 **Indomethacin (Drug)**
C7	 Serine  **Arabic Acid**	**C8**	 **Aspirin (Drug)**
C9	 **Nicotinic acid**	**C10**	 **3-indole acetic acid**
C11	 **DL-Proline**		

3.3.2 Initial screening of combinatorial library with pure HSA

All libraries were screened with pure HSA (250µg/ml) in PBS at pH 7.4 and pH 6.0. The screening was performed at different pH, because the binding between FcRn receptor and HSA is pH- dependent, causing an increase in the half-life of HSA. The receptor binds to HSA, after HSA enters in the endosome (pH 6.0 through pinocytosis), and then the dissociation occurs in the blood stream (pH 7.4), avoiding the degradation of the HSA by the lysosome. As one of the possible applications for HSA ligands is to increase the half-life of drugs or therapeutic proteins by binding to HSA, it is important that these synthetic ligands bind HSA at both pH.

The unbound HSA was quantified by the QuantiPro™ BCA assay and is based on the same principals of the bicinchoninic acid assay (BCA), however, the QuantiPro BCA gives a linear response from 0.5 to 30µg/ml of protein, and the BCA gives a linear response at higher concentrations of protein (200-1000µg/ml). Both techniques rely on the formation of a Cu^{2+} - protein complex under alkaline conditions. Cysteine, tryptophan, tyrosine and peptide bonds are able to reduce Cu^{2+} to Cu^{1+}, producing a purple-blue complex which absorbs at 562nm. The amount of reduction is proportional to protein.

The determination of the unbound protein was determined by Equation 3.1 and Equation 3.2.

$$Amount\ of\ protein\ bound\ (\mu g) = Amount\ of\ protein\ loaded\ (\mu g)\ - \sum amount\ of\ protein\ washed\ (\mu g)$$

(Equation 3.1)

$$\%\ Bound = \frac{Amount\ of\ protein\ bound\ (\mu g)}{Amount\ of protein\ loaded\ (\mu g)} \times 100$$

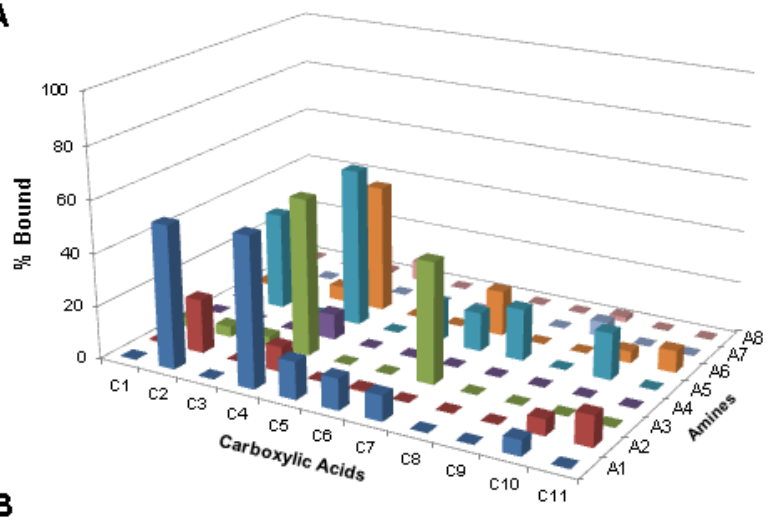
(Equation 3.2)

The results obtained with the Ugi library showed that the pH may be a strong influence on the binding of synthetic ligands for HSA. In the screening at pH 6.0 (Figure III.7), some of the ligands as C2A1, C4A1, C4A3 and C4A5 revealed an approximately HSA binding of 60%. In the screening at pH 7.4 (Figure III.8), the results were more promising with up to 100%, namely for C2A1, C2A5, C4A2, C4A3, C4A5 and C7A3 ligands. All ligands which obtained good percentage of HSA bound at pH 6.0, also performed well at pH 7.4 as C2A1, C4A3 and C4A5.

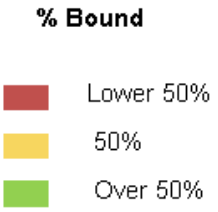
In the Triazine library, when comparing with the Ugi library, more ligands with affinity to HSA were obtained. The pH continues to affect the binding potential of the ligands to HSA. In the screening at pH 6.0 (Figure III.9), a maximum of 66% binding to HSA was observed in some ligands as A1A3, A2A3, A2A6, A3A3, A3A5, A3A6, A5A3, A5A6, A6A3, A6A2 and A6A5. In the screening at pH 7.4 (Figure III.10), many ligands present 100% of HSA bound, namely ligands

A1A3, A2A3,A2A5, A3A1, A3A2, A3A3, A3A4, A3A5, A3A6, A3A8, A5A3, A5A5, A5A6, A6A1, A6A2, A6A3, A6A5 and A8A3. All ligands with good percentage of HSA bound at pH 6.0, also performed well at pH 7.4 as the ligands A1A3, A3A3, A6A3, A6A5, A3A6 and A5A6. The negative controls showed than 2% of HSA bound, meaning that unspecific binding effects with the solid matrix are low.

A



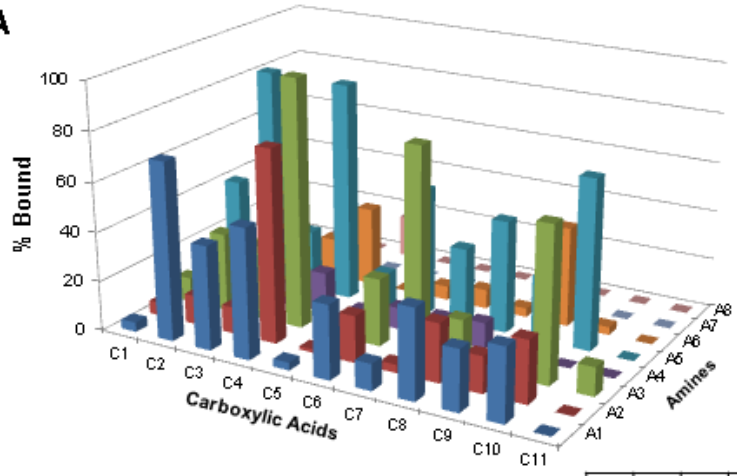
B



	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
A1	0	55	0	57	14	12	10	0	0	6	0
A2	1	21	0	10	0	1	0	0	0	6	12
A3	4	4	4	60	0	0	46	0	0	0	0
A4	0	0	0	10	0	0	0	0	0	0	0
A5	0	37	0	60	0	15	15	20	0	18	0
A6	1	0	6	49	0	0	17	0	0	4	8
A7	7	0	0	0	0	0	0	0	6	0	0
A8	0	0	1	8	0	0	0	0	2	0	0

Figure III.7: Screening results of Ugi reaction at pH 6.0. Screening was performed a loading pure HSA solution (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 6.0, to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 6.0. (B) Schematic diagram representing the amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 6.0.

A



B

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
A1	4	72	42	52	3	30	11	36	24	29	0
A2	5	12	11	78	1	19	3	24	15	25	0
A3	10	32	30	100	3	27	82	18	0	62	12
A4	0	6	9	16	4	9	9	10	4	0	0
A5	40	89	25	89	14	52	31	45	26	68	0
A6	0	5	16	32	0	6	8	4	40	3	0
A7	36	0	5	0	0	0	0	0	0	0	0
A8	0	3	0	17	0	0	0	0	0	0	0

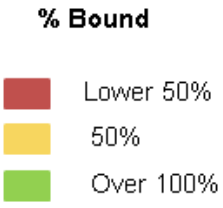
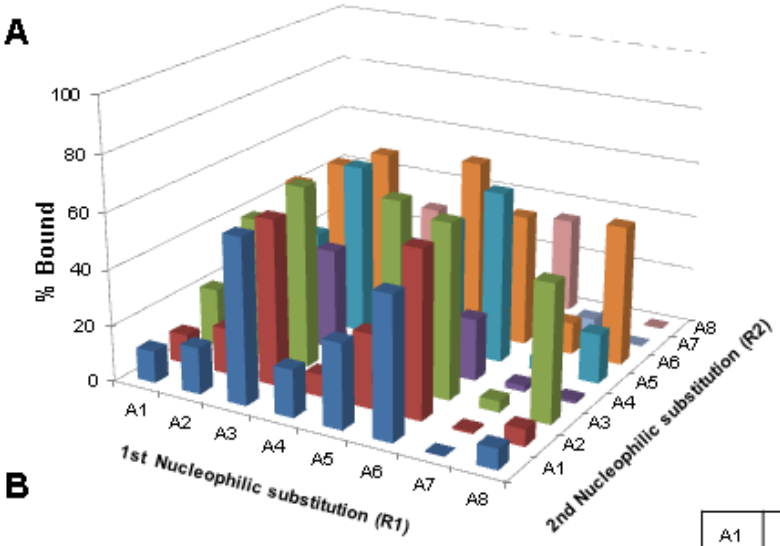


Figure III.8: Screening results of Ugi reaction at pH 7.4. Screening was performed a loading pure HSA solution (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 7.4, to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 7.4. (B) Schematic diagram representing the amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 7.4.



B

	A1	A2	A3	A4	A5	A6	A7	A8
A1	11	17	59	17	30	50	0	7
A2	11	16	59	8	26	59	1	6
A3	21	50	65	3	66	62	4	49
A4	7	5	36	2	9	22	2	1
A5	20	33	61	10	47	61	5	18
A6	44	54	61	16	63	47	11	50
A7	6	1	21	0	11	14	6	0
A8	0	0	29	0	10	34	0	0

R2 ↓

R1 →

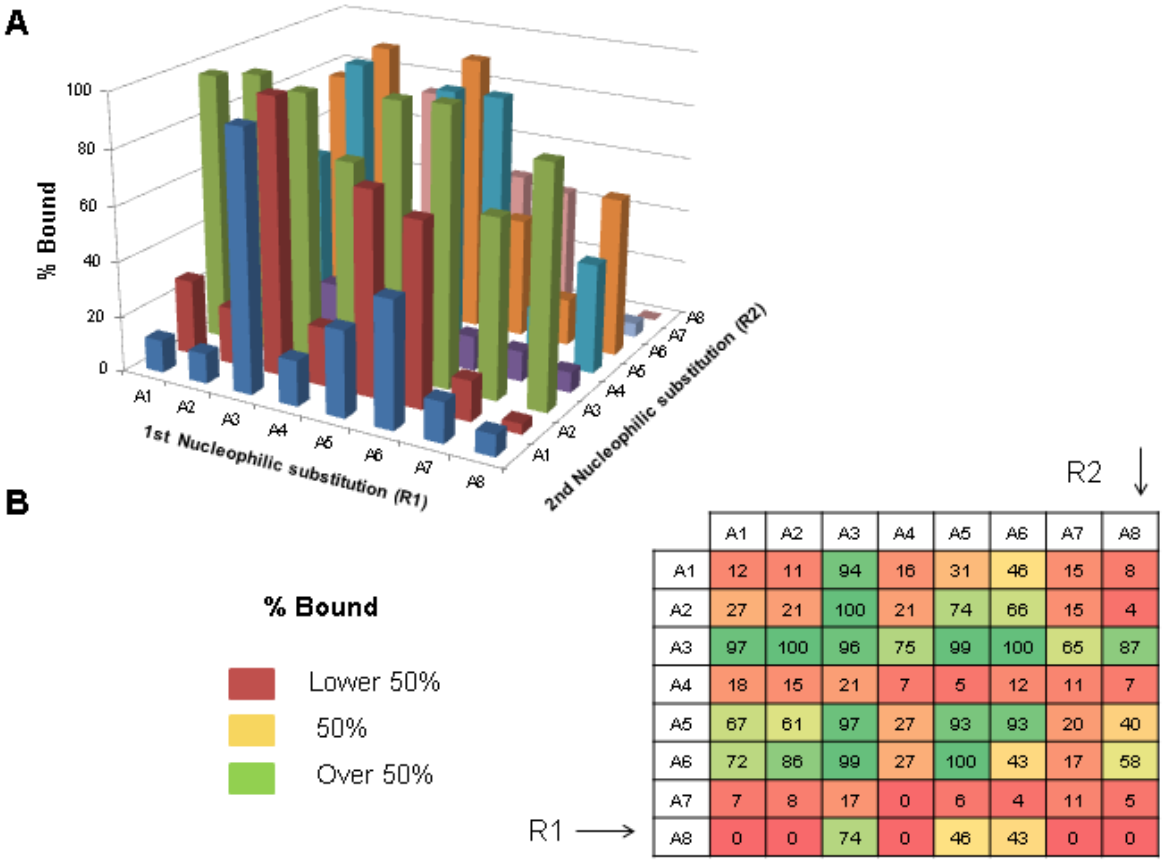
% Bound

Lower 50%

50%

Over 50%

Figure III.9: Screening results of Triazine reaction at pH 6.0. Screening was performed a loading pure HSA solution (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 6.0, to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 6.0. (B) Schematic diagram representing the amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 6.0.



	A1	A2	A3	A4	A5	A6	A7	A8
A1	12	11	94	16	31	46	15	8
A2	27	21	100	21	74	66	15	4
A3	97	100	96	75	99	100	65	87
A4	18	15	21	7	5	12	11	7
A5	67	61	97	27	93	93	20	40
A6	72	86	99	27	100	43	17	58
A7	7	8	17	0	6	4	11	5
A8	0	0	74	0	46	43	0	0

Figure III.10: Screening results of Triazine reaction at pH 7.4. Screening was performed a loading pure HSA solution (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 7.4, to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 7.4. (B) Schematic diagram representing the amount in percentage of HSA bound in each ligand-functionalized support, using PBS at pH 7.4.

3.4 Conclusions

In this chapter, two combinatorial libraries of affinity ligands based on the triazine and Ugi reaction were designed, synthesized and screened for binding HSA.

The two libraries were focused on the scaffold molecules derived from Ugi reaction (the multicomponent reaction) and Triazine reaction (mixed and split synthesis). In order to create diversity in the libraries, the amine and carboxylic acid compounds were designed based on the i) interactions between HSA and protein PAB; ii) drugs binding to domain II of HSA. We focused on targeting domain II, to increase the potential application of the ligands not only the purification of HSA as to increasing the half-life of therapeutic proteins or increasing the pharmacokinetics of drugs or biodrugs. In particular, by targeting domain II, it is possible to have the domain III of HSA available to bind to FcRn and to survive to lysosome conditions (Figure I.5). Therefore, during the screening process, the tests for HSA binding were performed at pH 6 and 7.4.

In total, 88 and 64 synthetic ligands were produced in the Ugi and Triazine libraries, respectively. Subsequently, the libraries were tested against pure HSA at pH 6.0 and 7.4. All libraries have a series of ligands that bind well to HSA, between 50 to 100% of binding, and presented higher percentages of binding at pH 7.4 and pH 6.0, but principally at pH 7.4.

In the Ugi library, C2A1, C4A1, C4A3 and C4A5 were the ligands that presented the higher proportion of HSA bound to both pH's (above 50%). In addition, compounds that stood out were the A1 (Tyramine) and C4 (3,5 Diiodosalicylic acid). The A1 compound must bind to HSA through hydrogen bonds or hydrophobic interactions, but the compound C4 is more to exert links covalent, such as hydrogen bonds, or even by dispersal forces, for present iodine in its structure.

In the Triazine library, the ligands that have a percentage of HSA bound higher than 50% in both pH values were: A1A3, A2A3, A2A6, A3A2, A3A3, A3A5, A3A6, A5A3, A5A6, A6A2, A6A3, A6A5 and A6A8. The compounds that stood out were the A3 (3,4 Dimethylaniline), A5 (Phenethylaniline) and A6 (Sulfisoxadole). Through the structure of the compounds A3, A5 and A6, the interaction with HSA may be by hydrogen bonds or intermolecular forces and hydrophobic interactions.

The synthetic ligands showed a percent bound to HSA at higher pH 7.4, in this way, this pH was chosen to continue the studies to test the selectivity of the ligands, in order to re-synthesize and re-screen the lead ligands to HSA and confirm the reproducibility of the results.

IV. Purification of HSA with synthetic ligands

The aim of this chapter is to evaluate the selectivity of the synthetic ligands based on Ugi and Triazine reactions for HSA binding. The combinatorial libraries were screened against pure IgG. Subsequently the lead ligands were selected, re-synthesized and re-screened against HSA and IgG to confirm the reproducibility of the results. Afterwards, a single elution condition was attempted to recover HSA and IgG in on-column format employing a single elution condition at low pH.

4.1 Introduction

HSA is a protein frequently found in plasma and, together with immunoglobulins, constitutes 80% of all plasma proteins. Moreover, HSA is also found in tissues and bodily secretions throughout the body, being an excellent biomarker and also a therapeutic agent in several conditions (Denizli, 2011; Rajak *et al,* 2013).

The plasma proteins have a wide variety of concentrations (from less than 1mg/ml to 40g/l), becoming its fractionation a major challenge (Denizli, 2011). In addition, the removal of abundant serum proteins can help in the discovery and detection of less abundant proteins which may serve as disease markers (Steel *et al*, 2003). Many therapeutic products can be obtained from human plasma. Purification of albumin is very important for therapeutic applications and to study other plasma proteins, which may be masked by the high amount of HSA found in plasma. Therefore, it is necessary to develop purification methods selective for HSA, with the potential of reuse and protein recovery, at lower costs. Three industrial techniques are well established for the purification of albumin from serum or plasma: (i) ethyl alcohol fractionation (Cohn method), (ii) heat shock and (iii) chromatography (Denizli, 2011).

The ethyl alcohol fractionation was developed by Cohn and colleagues, through a process that consist in the difference of solubility of albumin and other plasma proteins based on pH, ethanol concentration, protein concentration, temperature and ionic strength. This method can produce a large volume of fractionated plasma and may be regarded as safe for therapeutic use. However, the Cohn method requires demanding refrigeration systems (e.g. cold areas or refrigerated tankers), there is the risk of protein denaturation, and needs high quality starting material (Tanaka *et al*, 1998; Denizli, 2011).

The thermal shock method is also very used to purify HSA due to its higher thermal stability. Plasma proteins are exposed to a very high temperature (60ºC), and HSA does not denature readily as opposed to the other plasma proteins which rapidly denature becoming insoluble in such conditions. Normally, the purity of a heat shock HSA is greater than 98% (Denizli, 2011).

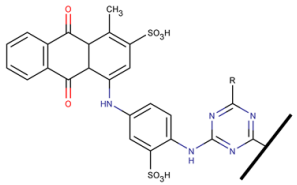
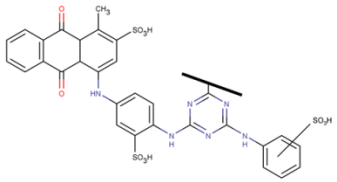
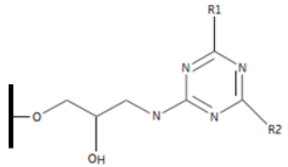
A wide variety of chromatographic techniques may be used for the albumin purification (Matejtschuk, *et al*, 2000). Berglöf, in 1983, described a chromatographic procedure to purify albumin from plasma fractionation. Plasma is transferred into the process buffer by gel filtration. After removal of the euglobulin fraction by precipitation, the plasma is added to a column packed with an anion exchanger adsorbent. This separation medium binds the albumin under starting conditions but allows the IgG fraction to pass through. The bound albumin fraction is eluted for further purification on the cation exchanger before final polishing on the gel filtration medium. Pure albumin is bottled and pasteurized (60°C for 10 hours) in accordance with the European Pharmacopoeia (Berglöf,*et al*, 1983).

Affinity chromatography is a powerful technique for the purification of many proteins being based on highly specific molecular recognition (Denizli, 2011). Currently there are on the

market a few HSA purification kits (Table IV.1) based on affinity chromatography. The immobilized Cibacron Blue F3G-A, a sulfonated triazine dye, is one of the earliest examples of the use of synthetic ligands in affinity chromatography for isolation and purification of HSA (Williams *et al*, 2014). This Dye-ligand adsorbent offers many advantages such as the resistance to chemical and biological degradation and low cost (Zhixin and Kongchang, 1993). However, the heterogeneity and low purity of the Cibacron dye can affect its use in the introduction of the purification of HSA at production scale for clinical use (Burnouf and Radosevich, 2001). The Albupure kit by triazine method, is a high-performance affinity capture adsorbent, however, it was designed for the purification of albumin fusion proteins.

The process of using immunoaffinity resins for the purpose of selectively purify or remove HSA from solution is also well established. In the market, there are several immuno-adsorbents, such as Vivapure® kit from Vivascience, and the Anti-HSA IgY Coupled Beads kit from GenWay. Nevertheless the use of biological ligands is very expensive and the scale-up is limited (Curling, 2004).

Table IV.1: HSA purification kits currently available on the market based on affinity chromatography. The structures were designed in MarvinSketch, ChemAxon.

Product	Structure	Advantage	Disadvantage	Cost (USD)	Company
AlbuPure®	N/A	• Robust and long-life adsorbent; • Colorless ligands; • High dynamic binding capacities.	• Low selectivity for plasma.	15.40/ml	ProMetic
Mimetic Blue® P6XL		• HSA and albumin related proteins from variety of sources; • Robust and long life adsorbents; • High purity; • High sensitive ELISA assay.	• Low selectivity.	12.60/ml	ProMetic
Cibacron Blue 3GA		• Purification of HSA and others proteins and enzymes; • Resistance to chemical and biological degradation.	• Low selectivity; • Toxicity of the dyes; • Low purity; • Usage limit.	228.99/g	Sigma Aldrich
Capto Blue		• Resistance and long life adsorbents; • Chemically stable; • Low cost.	• Low selectivity; • Toxicity.	7.63/ml	GE Healthcare

4.2 Material and methods

4.2.1 Chemicals

All reagents were used with a high purity and the solvents were pro-analysis.

The reagents Isopentylamine (A2), 3,4-Dimethylaniline (A3), 1,4 Diaminobutane (A4), Phenethylamine (A5), Sulfisoxazole (A6), 2-aminopentane (A8), 9- Anthracenecarboxylic acid (C2), 3,5-Diiodosalicylic acid (C4), Arabic Acid (C7), 3-indole acetic acid (C10) , human serum albumin, IgG from human serum and bovine serum albumin were purchased from Sigma-Aldrich (Portugal).

Acetone, Epichlorohydrin, Cyanuric chloride, Isopropyl isocyanide, Phenol, Sodium bicarbonate ($NaHCO_3$), Sodium periodate (NaIO4), Sodium thiosulfate ($Na_2S_2O_3$), Silver nitrate (AgNO3), Glutaraldehyde, Glycine, Sodium Chloride (NaCl) and QuantiPro™ BCA Assay Kit were acquired from Sigma-Aldrich (Portugal). Methanol (MeOH) and Ammonium hydroxide solution (NH4OH) were obtained from Roth. Dimethylformamide (DMF), Methanol (MeOH), Di-Sodium-hydrogen Phosphate 2-hydrate and Sodium hydroxide (NaOH) were purchased from VWR (Portugal). Ethanol absolute PA, Sodium-di-hydrogen Phosphate 1-hydrate, hydrochloric acid 37% (HCl) were acquired from Panreac (Spain).

4.2.2 Chromatographic materials

Cross linked agarose (Sepharose™ CL-6B) was acquired from GE Healthcare; Captiva 96-well filtration block was purchased from Agilent Technologies (USA); Half-area UV-Star® 96-well microplates and 96-well transparent microplates were obtained from Greiner Bio-One (Germany) and Sarstedt (Portugal), respectively. Agilent bond Elut 3ml and Frits from Agilent Technologies (USA).

4.2.3 Buffers

The following buffers and reagents were used: Phosphate Buffer Saline (PBS) (Sodium phosphate 10mM, 150mM NaCl, pH 7.4 and pH 6.0); Regeneration Buffer (NaOH 0.1M in 30% isopropanol). The buffers used in the elution step were 0.1M Glycine- HCl at pH 2.5 and Tris (hydroxylmethyl) aminomethane- base at pH 9.0 for neutralization of eluted samples.

4.2.4 Equipment

The synthesis of the synthetic ligands on solid-phase based on Triazine and Ugi reaction were carried out in Incubator ZKA KS4000i from VWR (Portugal). Microplate reader TECAN Infinite F200 from Thermo Scientific (Portugal); Big SHOT III from Boekel Scientific and LMC 3000 laboratory centrifuge from Biosan.

4.2.5 Methods

4.2.5.1 Screening against pure IgG

The two combinatorial libraries produced in Chapter 3 were screened for binding to pure human IgG at pH 7.4. The procedure was similar to 3.2.5.7. After, proceeded with the selection of the lead ligands, its synthesis, and re-screened against HSA and IgG to confirm the previous results.

4.2.5.2 Re-synthesis of the lead ligands based on Triazine reaction

Cyanuric chloride-modified agarose was prepared according to 3.2.5.4. After, 6g of agarose was weight to different flask. Subsequently, the amines (2 molar eq. of each relative to epoxy content; 1ml/g of resin/flask) were added in its corresponding flask, for the first nucleophilic substitution (R1) for 24 hours at 30°C with agitation (150rpm).

Posteriorly, the ligands were washed with 50% DMF/distilled water (5x resin volume) and distilled water (5x resin volume). Then, the second amines (R2) (5 molar eq. of each relative to epoxy; 1ml/g of resin/volume) were added and incubated at 80ºC for 48 hours, in the orbital shaker (150rpm).

All the amines used in these reactions (Table IV.2) were dissolved in 50% DMF/distilled water (v/v). Moreover, Sodium bicarbonate 1M (1 molar eq. relative to epoxy; 1ml/g of resin/volume) was added in each amine.

At the end of the reaction, the ligands were washed with 50% DMF/distilled water (5x resin volume), 0.1M HCl (1x resin volume), distilled water (resin volume), Sodium hydroxide 0.1M in 30% isopropanol (v/v) (regeneration buffer; 1x resin volume) distillated water (5x resin volume), 20% ethanol and stored at 4ºC.

Two types of negative controls were added in two different wells i) 0.25g Sepharose™ CL-6B ii) 0.25g Aminated agarose.

Table IV.2: List of amines used in the Triazine synthesis.

Nomenclature	Reagents/ Amine
A1	Tyramine
A2	Isopentylamine
A3	3,4-Dimethylaniline
A4	1,4 Diaminobutane
A5	Phenethylamine
A6	Sulfisoxazole
A7	Amino-2-propanol
A8	2-aminopentane

4.2.5.3 Re-synthesis of lead ligands based on the Ugi reaction

The Aldehyde-functionalized agarose was prepared according to 3.2.5.3. After, the agarose functionalized with aldehydes was washed with methanol, from 0% (v/v) to 100% (v/v) methanol with increments of 20%. Then, 6g of agarose was weighted to each flask. Thus, the amines (5 molar eq. of each relative to epoxy content; 1ml/g resin/volume) were added in its corresponding flask, and incubated for 2 hours at 60ºC in the orbital shaker (180rpm). After, the carboxylic acid compounds (5 molar eq. of each relative to epoxy content; 1ml/g resin/volume) were added together with isopropyl isocyanide (5 molar eq. relative to epoxy content, 1ml/g resin volume). In this reaction, all the amines and carboxylic acid compounds used (Table IV.3) were dissolved in 100% methanol, with the exception of C7 compound, that was dissolved in 50% methanol/distilled water (v/v). Then, the flasks were incubated for 48 hours at 60°C in the orbital shaker (150rpm).

At the end of the reaction, the ligands were washed with 1x resin volume of the following reagents: 100% methanol (v/v), 50% methanol (v/v), 50% DMF/distilled water (v/v), distilled water, 0.1M HCl, distilled water, 0.2 M Sodium hydroxide in 50% isopropanol, 2x distilled water and 20% ethanol for store at 4ºC.

Two types of negative controls were added in two different wells i) 0.25g Sepharose™ CL-6B ii) 0.25g Aldehyde-functionalized agarose.

Table IV.3: List of Carboxylic acid and amine used in the Ugi synthesis.

Nomenclature	Reagent/Carboxylic acid	Nomenclature	Reagent/Amine
C2	9-Anthracenecarboxylic acid	**A1**	Tyramine
C4	3,5-Diiodosalicylic acid	**A2**	Isopentylamine
C7	Arabic Acid	**A3**	3,4-Dimethylaniline
C10	3-indole acetic acid	**A5**	Phenethylamine

4.2.5.4 Re-screening of the lead ligands against pure HSA and pure IgG

Firstly 0.25g of ligand-functionalized agarose and negative controls (SepharoseTM CL-6B; Aminated agarose; Aldehyde-functionalized agarose) were added in each well in a 96-well filter microplate for the regeneration and equilibration of the ligands according to 3.2.5.6.

The ligands were then re-screened for binding pure HSA and pure IgG to confirm the previous results. The lead ligands were added in triplicate in two blocks, being that in the first block was tested against pure HSA in 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 (0.25mg/ml) and in the other block was tested against pure IgG in 10mM Phosphate Buffer, 150mM NaCl at pH 7.4 (0.25mg/ml). The procedure was similar to 3.2.5.7.

4.2.5.5 Screening of the lead ligands in on-column format

For the ligands A3A2 and A6A5 that were selected for binding HSA selectively, 0.5g of ligand-functionalized agarose was packed in a small column, in duplicate. For each ligand, two columns were made, one to test against HSA and other to test against IgG. Then, the columns were washed with regeneration buffer (0.1M NaOH in 30% isopropanol) followed by distilled water (3 cycles of washes, until the maximum amount of column). After, the resins were equilibrated with the addition of the binding buffer with 10mM Phosphate Buffer 150mM NaCl at pH 7.4 (5 times until the maximum amount of column). In the last wash, 100µl of each sample was collected in a half-area UV-Star 96-Well microplate, and the absorbance was read at 280nm. The washes came to an end when they reached a lower absorption than 0.005.

Then 0.5ml of pure HSA (0.25mg/ml) 10mM Phosphate Buffer 150mM NaCl at pH 7.4 was loaded in the column and 0.5ml of pure IgG (0.25mg/ml) in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 was added in other column. Posteriorly, several washes with 10mM

Phosphate Buffer 150mM NaCl at pH 7.4 (8x 1ml) were performed. After, the total protein was quantified on the samples using the QuantiPro™ BCA assay kit. For this 150µl of the each sample was added in a transparent microplate , and then, 150µl of the QuantiPro™ BCA reagent was added to each sample and incubated at 60ºC for 1 hour according to supplier instructions. The absorbance of the samples was then determined in the microplate reader at 560nm, with a calibration curve using solutions with known protein concentration BSA in 10mM Phosphate Buffer 150mM NaCl at pH 7.4 from 0 to 200µg/ml with the values of $y = 0.0222x + 0.0501$ with a $R^2 = 0.9974$.

4.2.5.6 Elution of the pure HSA and pure IgG in on-column format

After the screening on-column, 0.5ml 0.1M Glycine- HCl at pH 2.5 was added in each column with the lead ligands (A3A2 and A6A5) and collected in 1.5 ml microcentrifuge tubes. This procedure was performed 5 times. To avoid denaturation of the eluted proteins,1M Tris-base pH 9.0 was added to collect the elution fractions, to adjust the pH value to 7. The analysis of the samples was made using the QuantiPro™ BCA assay kit. Thus, in a transparent microplate 150µl of the each sample was added followed by 150µl of the QuantiPro™ BCA reagent and then incubated at 60ºC for 1 hour according to supplier instructions. The absorbance of the samples was then determined in the microplate reader at 560nm. It was necessary to construct a calibration curve using solutions with known protein concentration (BSA) in 0.1M Glycine- HCl at pH 2.5 from 0 to 190µg/ml with the values of $y = 0.0012x - 0.0252$ with a $R^2 = 0.969$. In each samples of the calibration line was added 0.05ml 1M Tris (hydroxylmethyl) aminomethane at pH 9.0.

4.3 Results and discussion

The two combinatorial libraries based on the Ugi and Triazine reaction previously tested against HSA (Chapter 3) were assessed for binding human IgG.

The binding assays were performed at pH 7.4, and the results were compared with the screenings performed against HSA at pH 7.4 (Chapter 3). The quantification of the bound IgG and the percentage bound were determined by Equations 3.1 and 3.2, respectively.

The enrichment factor was determined through Equation 4.1. In the enrichment factor, all values higher than 1 mean that a ligand preferentially binds to HSA over IgG.

$$Enrichment\ Factor = \frac{\%\ HSA\ bound}{\%\ IgG\ bound} \qquad \text{(Equation 4.1)}$$

In the Ugi library, from the 28 ligands that showed more than 20% of binding to HSA and that were compared with the results obtained with the screening against IgG (Figure IV.1), 14 ligands had an enrichment factor of equal or more than 1. The ligands C4A3 and C8A5 presented the same percentage of binding between the HSA and IgG. Furthermore, ligands C4A3 and C8A5 are the ones that had an enrichment factor of 1.

In the Triazine library, 23 ligands that showed more than 50% of binding to HSA and that were compared with the results obtained with the screening against IgG (Figure IV.2). All chosen triazine ligands have a greater percentage of binding to HSA when compared with the binding to IgG, except the A8A6, which consequently has an enrichment factor less than 1.

As the ligands have only been screened once against HSA and IgG, it is fundamental re-synthesize and re-screen the best ligands in order to determine the reproducibility of results. The criteria used to select the best ligands were different for each library and are presented in Table IV.4. The structure of the ligands was also taken into consideration to ensure higher diversity.

From the selectivity criteria, the 14 Ugi ligands initially selected were narrowed to 5, since some of the ligands did not display relevant structural differences. For the 22 Triazine ligands, 8 were selected based on selectivity criteria (Table IV.4). The Triazine ligand A5A5 was eliminated by presenting a very hydrophobic structure and the ligands A2A3 and A5A6 were eliminated due structure similarity with ligands A3A2 and A6A5, respectively. The negative controls had less than 10% of IgG bound, meaning that the solid matrix is not involved in the interaction with IgG as already observed for HSA. Therefore, a total of 5 Ugi ligands and 8 Triazine ligands were re-synthesized and re-screened at pH 7.4.

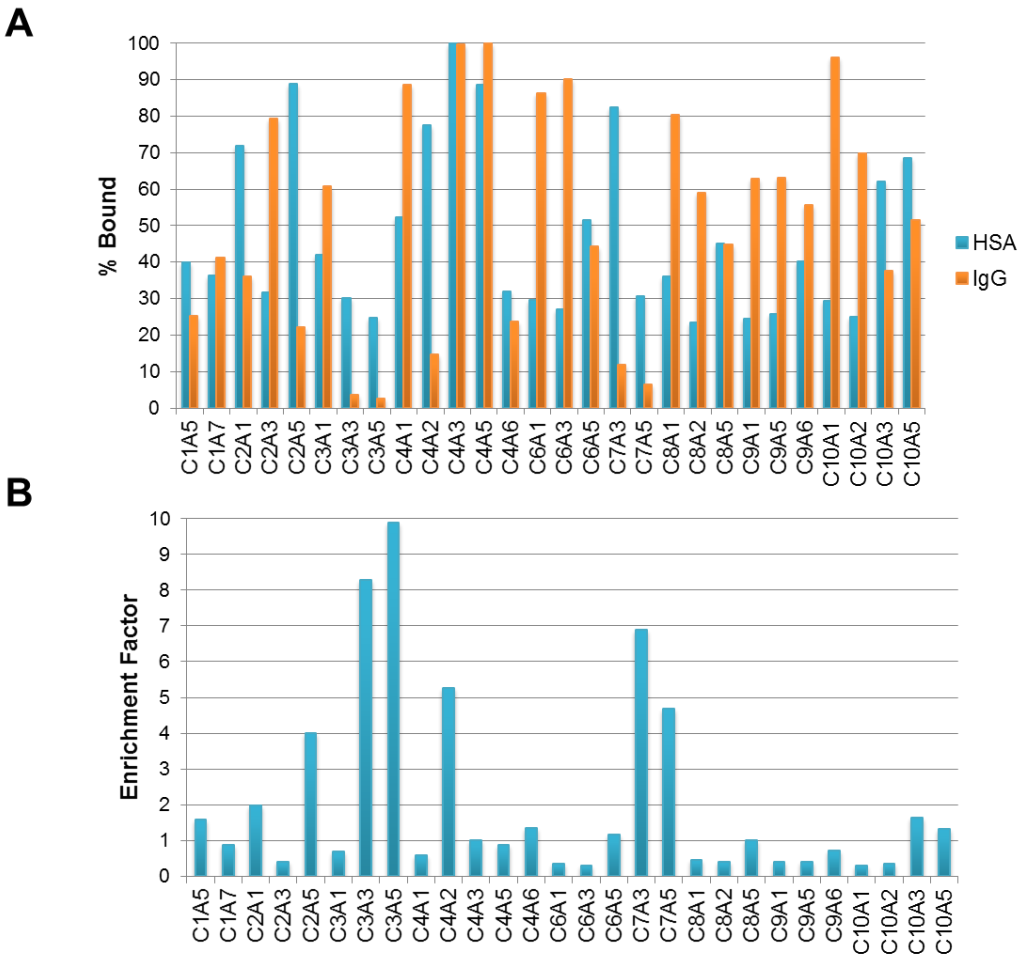


Figure IV.1: Screening results of putative Ugi ligands against IgG at pH 7.4. Screening was performed by adding a loading with pure IgG (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl at pH 7.4), to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) The 28 selected ligands that have more than 20% binding to HSA at pH 7.4, compared with the results obtained from the screening against IgG. (B) Enrichment factor of each selected ligand.

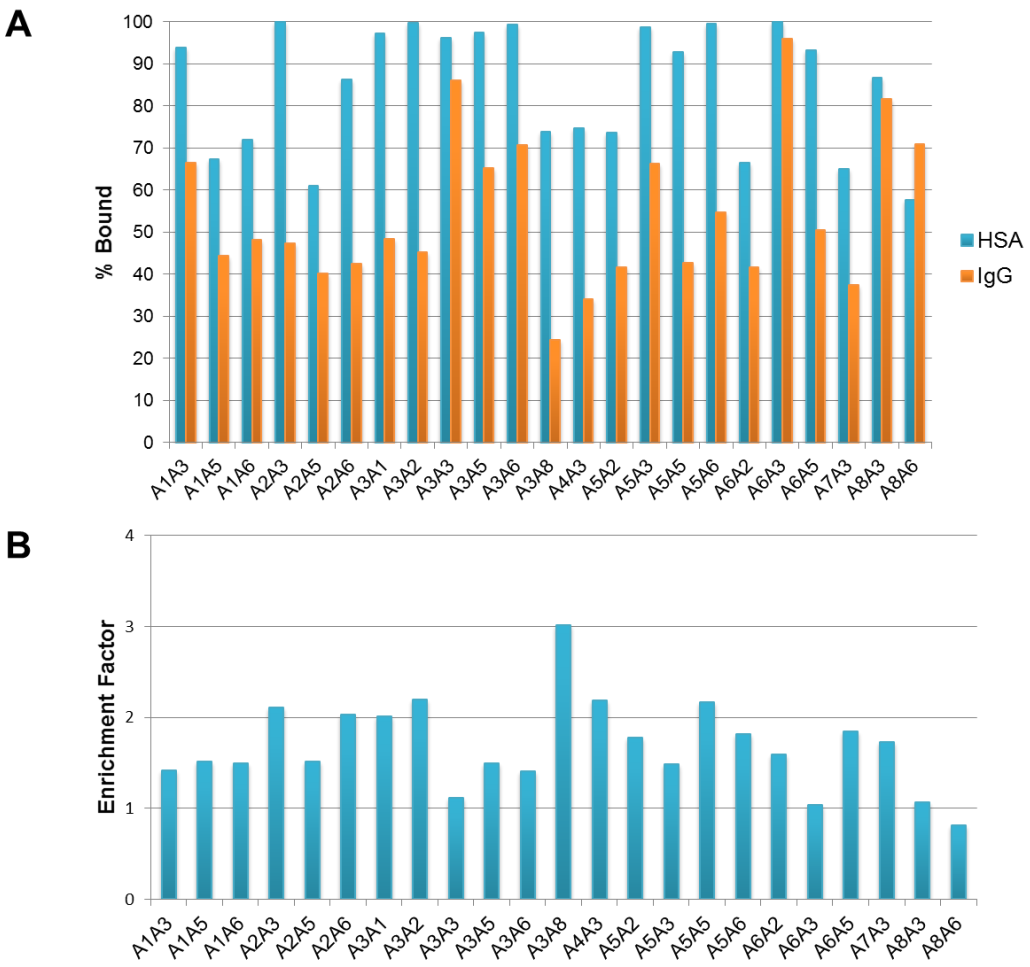


Figure IV.2: Screening results of putative Triazine ligands against IgG at pH 7.4. Screening was performed by adding a loading with pure IgG (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl at pH 7.4), to each well of a 96-well filtration block containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) The 23 selected ligands that have more than 50% binding to HSA at pH 7.4, compared with the results obtained from the screening against IgG. (B) Enrichment factor of each selected ligand.

Table IV.4: Criteria used for the selection of best ligands.

Ligand	% Bound HSA	Enrichment Factor
Ugi	≥ 50%	≥ 1.5
Triazine	≥ 50%	≥ 1.7

The lead ligands were re-synthesized and re-screened against pure HSA and pure IgG in triplicate, to confirm the previous results (Figure IV.3 and Figure IV.4). Although The Ugi lead ligands presented promising percentage of binding to HSA they also showed a strong binding to IgG. Ligand C7A3 has the better result with a 1.3 of enrichment factor, but the difference in binding percentage between HSA and IgG is only 22%. Except for C2A1 binder, all the other ligands showed a stronger binding to HSA rather than for IgG, but with a small difference. The results obtained with the Triazine ligands revealed to be more promising than

the results obtained with Ugi ligands. The triazine ligands showed a higher percentage of binding to HSA than for IgG, having an enrichment value of at least 1.5, with the exception of ligands A4A3 and A7A3. The ligands highlighted are the A3A2 and A6A5, with a difference of at least 50% higher for HSA than for IgG.

The fact that some ligands did not provide the same results as the previous screening for IgG, might be due to an inefficient regeneration. For this reason, some contaminants might be present thus preventing the binding to IgG. This shows the importance of re-synthesize and re-screen new ligands, to confirm the reproducibility of the results. Nonetheless most of the ligands of both syntheses showed an enrichment factor higher than 1 which indicates that they bind more to HSA than to IgG. Ligands A3A2 and A6A5 showed the most consistent values of enrichment factor 3.0 and 2.1, respectively. Therefore, these ligands were chosen to proceed with the work.

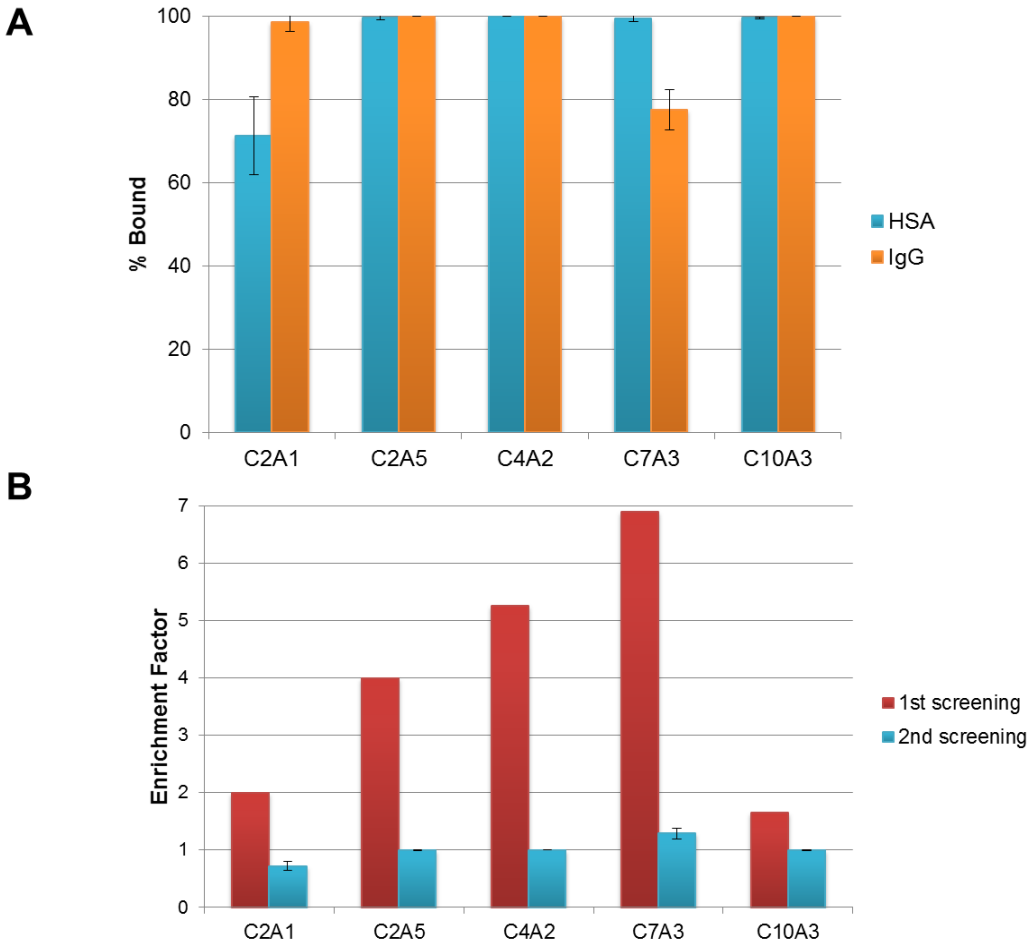


Figure IV.3 : Screening results of the putative lead ligands of Ugi against HSA and IgG at pH 7.4 (n=3). Screenings were performed by adding a loading with pure HSA (250µg/ml) and pure IgG (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 7.4, to each well of a 96-well filtration block in triplicate containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Percentage of HSA and IgG bound on each ligand. (B) Results of the Enrichment factor of each selected ligand (2nd screening), and the previous results obtained (1st screening).

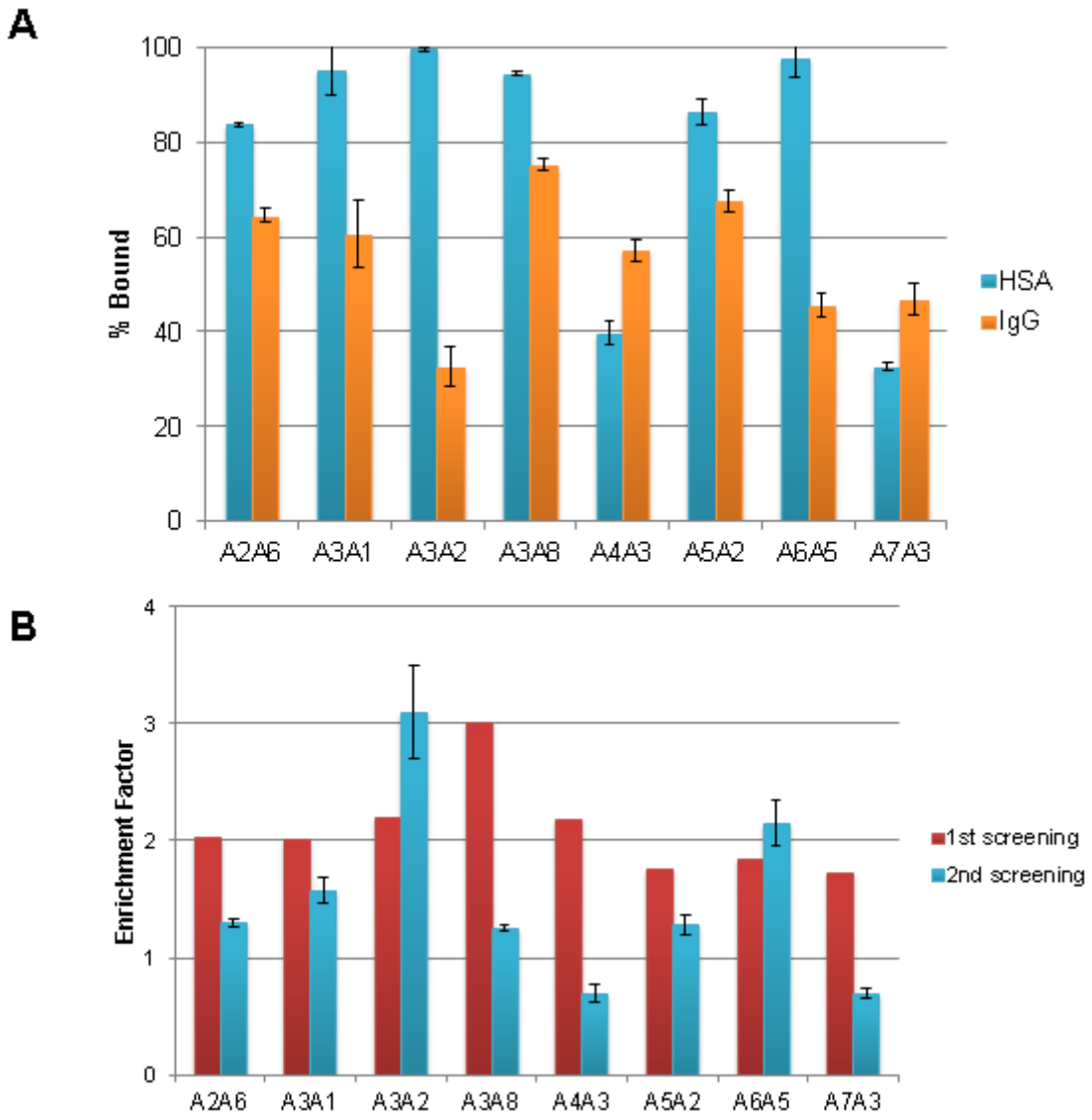


Figure IV.4: Screening results of the putative lead ligands of Triazine against HSA and IgG at pH 7.4 (n=3). Screenings were performed by adding a loading with pure HSA (250µg/ml) and pure IgG (250µg/ml) in PBS (10mM Sodium Phosphate, NaCl 150mM) at pH 7.4, to each well of a 96-well filtration block in triplicate containing 0.25g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Percentage of HSA and IgG bound on each ligand. (B) Results of the Enrichment factor of each selected ligand (2nd screening), and the previous results obtained (1st screening).

In order to confirm the results with the ligands A3A2 and A6A5, a new screening in on-column format was carried out with HSA and IgG. When making the screening in block, it is first necessary to incubate the proteins with the adsorbents for 1 hour and subsequently centrifuge to collect the samples. When employing the screening on column, the adsorbents are packed in the column, thus allowing the flow of the solutions through the action of gravity.

The results obtained with the two independent screenings are shown in Figure IV.5, together with the results previously obtained from the screening on the block. Ligand A3A2 revealed 100% binding to HSA while for IgG the binding was only 43%, which corresponds to 252µg/g resin and 133µg/g resin, respectively. For ligand A6A5, it was obtained 99% binding to

HSA and 53% for IgG binding was only. This corresponds to 250µg/g resin and 163µg/g resin, respectively. The amount of protein bound in µg/g of resin was determined through the Equation 4.2. The results obtained in column when compared with the results obtained in the screening in block (previous experiment) were similar, being therefore consistent results.

A single elution condition was tested, employing a typical elution in affinity chromatography assays (low pH).

$$Amount\ of\ protein\ bound\ (\mu g/g\ resin\ ) = \frac{Amount\ of\ protein\ loaded\ (\mu g)\ -\ \sum amount\ of\ protein\ washed\ (\mu g)}{0.5g\ resin}$$

(Equation 4.2)

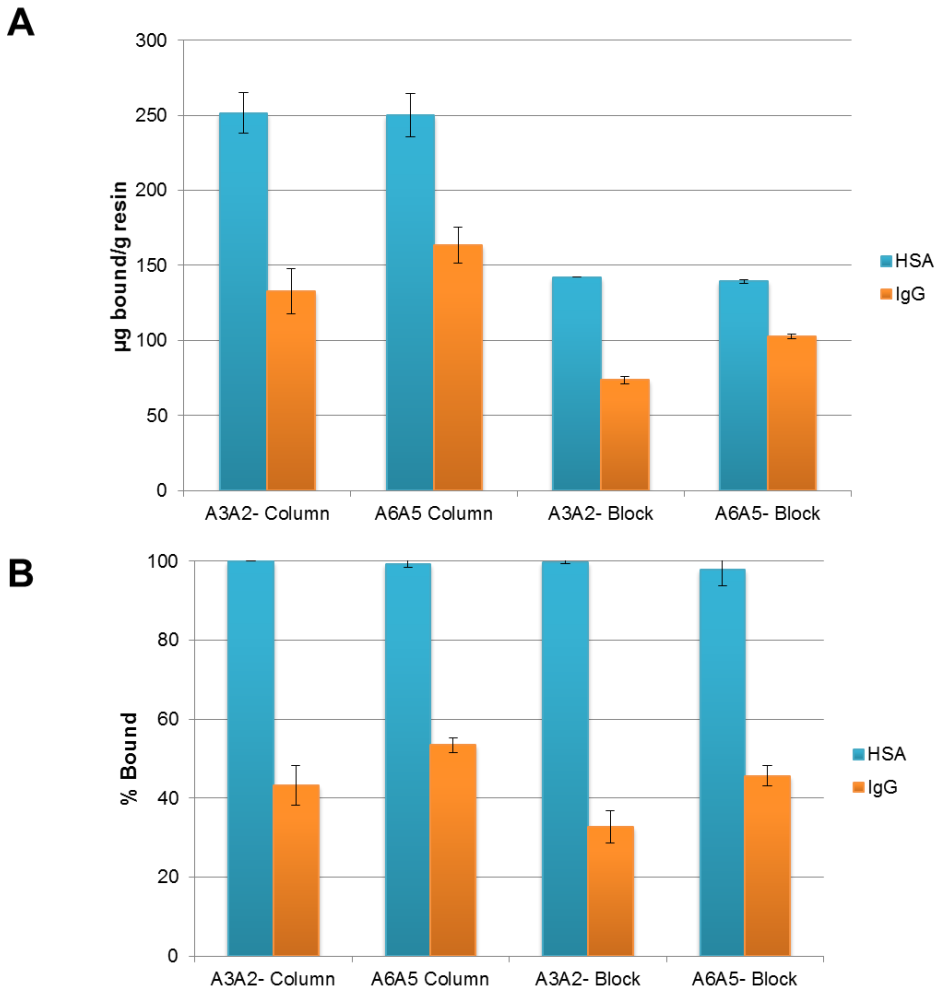


Figure IV.5: Screening results of triazine lead ligands on-column (n=2). Screenings were performed by adding a loading with pure HSA (250µg/ml) and pure IgG (250µg/ml) in PBS (10mM Sodium Phosphate, 150mM NaCl) at pH 7.4, to each column containing 0.5g of ligand-functionalized resin. The unbound protein from flow-through and washes was collected in 96-well transparent microplates and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in µg/g resin of HSA and IgG on-column (n=2) and in block of each lead ligand (n=3) (B) Percentage of HSA and IgG bound on-column (n=2) and in block (n=3) of each lead ligand.

The quantification of the protein eluted from the columns was determined by QuantiPro™ BCA method in the microplate reader at 560nm, using Equations 4.3, 4.4 and 4.5.

$$Amount\ of\ protein\ eluted\ (\mu g) = \sum amount\ of\ protein\ washed\ (\mu g) \quad \text{(Equation 4.3)}$$

$$\%\ Eluted = \frac{Amount\ of\ protein\ eluted\ (\mu g)}{Amount\ of protein\ bound\ (\mu g)} \times 100 \quad \text{(Equation 4.4)}$$

$$Amount\ of\ protein\ eluted\ (\mu g/g\ resin) = \frac{\sum amount\ of\ protein\ washed\ (\mu g)}{0.5\ g\ resin} \quad \text{(Equation 4.5)}$$

The percentage of protein eluted from A3A2 ligand were only 17% HSA and 22% IgG (46µg/g resin and 33µg/ g resin, respectively) (Figure IV.6). For A6A5 ligand, it was possible to elute 2% HSA and 15% IgG (6µg/g resin and 26µg/g resin, respectively). As the optimization of the elution profile from an affinity is usually a trial and error procedure, further studies need to be performed to selectively recover the bound HSA.

Ligand A3A2 is composed by 3.4-Dimethylaniline as amine R1 and the compost Isopentylamine as amine R2, which mimic the leucine amino acid. Ligand A6A5 has the drug Sulfisoxazole as amine R1 and the compost Phenethylamine as the amine R2, which mimic the phenylalanine amino acid. Both ligands possess aromatic rings (Figure IV.7), which can establish hydrophobic interactions with HSA and IgG.

Therefore, it is important to consider elution conditions that promote a decrease in hydrophobic effects. In Table IV.5 are listed the common elution buffers employed during HSA recovery from the commercial adsorbents, which also possess hydrophobic structures.

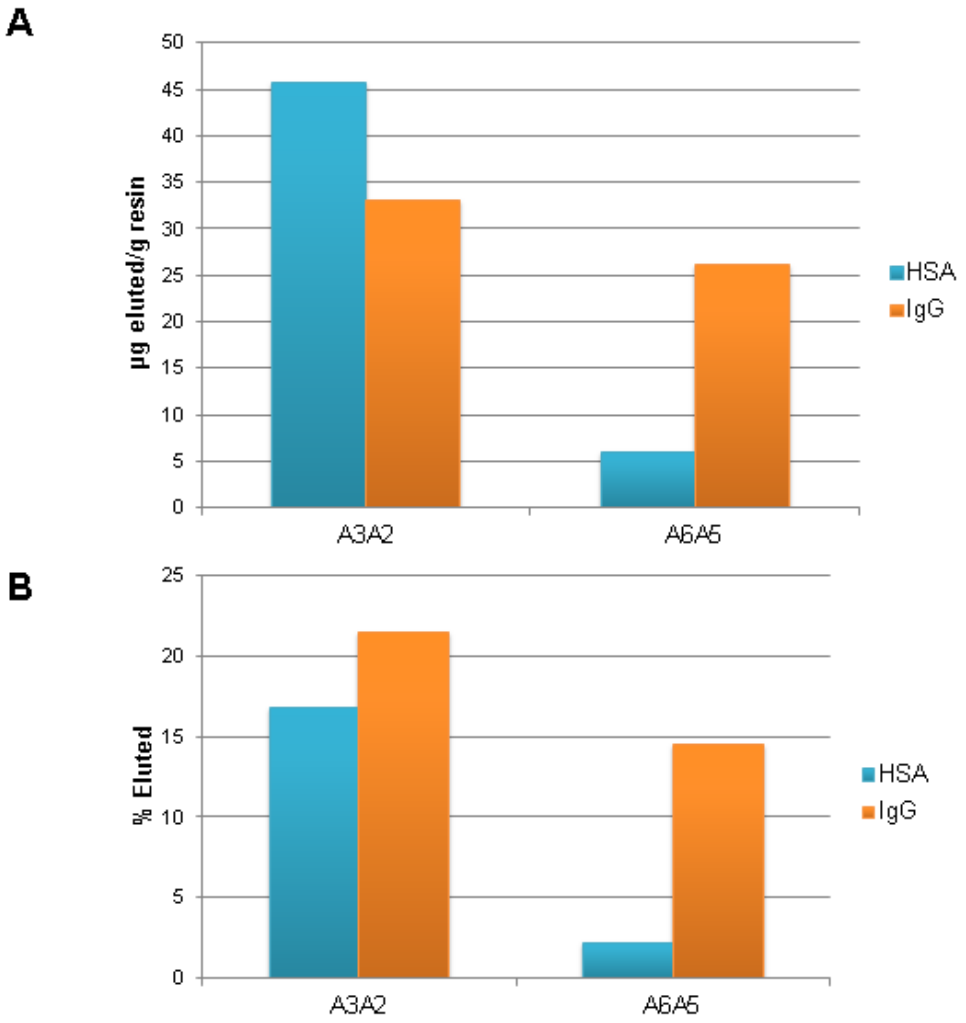


Figure IV.6: Elution results of triazine lead ligands. After screening, the HSA and IgG bound to ligand-functionalized supports were eluted by adding 0.5ml 0.1M Glycine- HCl at pH 2.5 to each column. This procedure was performed 5 times. The eluted proteins were collected in 1.5ml tubes and after, it was necessary add 1M Tris (hydroxylmethyl) aminomethaneid at pH 9.0 to neutralize the samples. Posteriorly, the samples were analyzed in 96-well transparent microplate and quantified by the QuantiPro™ BCA method, measured at 560nm. (A) Amount in µg/g resin of HSA and IgG eluted in each ligand. (B) Percentage of HSA and IgG eluted on each ligand.

Table IV.5: List of the elution buffers used in kits to purify HSA existing in the market

Product	Elution Buffer	Company
AlbuPure®	150mM Ammonium acetate, 10mM Sodium caprylate, pH 6.0.	ProMetic
Mimetic Blue® P6XL	25mM Sodium phosphate, 150mM NaCl, 30mM Sodium caprylate, pH 6.0.	ProMetic
Cibacron Blue 3GA	0.01M Tris-Chloride acid pH 7.5-8.0, 1.5M NaCl.	Sigma Aldrich
Capto Blue	50mM Sodium phosphate, 1.5M Potassium chloride (KCl), pH 6.0.	GE Healthcare

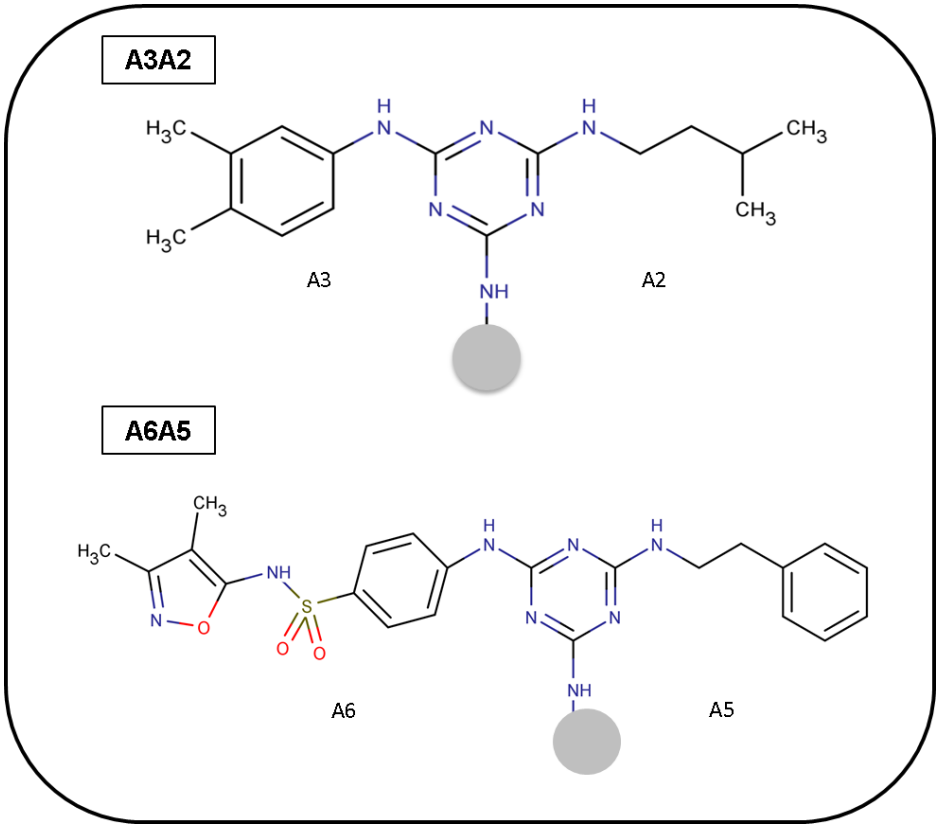


Figure IV.7: Lead ligands structure immobilized on agarose. The grey sphere is a schematic representation of an agarose bead. The structures were designed in MarvinSketch, ChemAxon.

4.4 Conclusions

A total of 17 ligands were selected in chapter 3 on the basis of a high binding to HSA at both pH 6.0 and 7.4, being 4 from the Ugi library and 13 from the Triazine library.

In this chapter, the possibility to further develop these ligands as affinity reagents for HSA purification was explored. Therefore, the 152 ligands from the two libraries were screened for binding IgG at physiological conditions.

In general, ligands with a high affinity towards HSA tend to be hydrophobic, it is necessary to test the selectivity of the ligands. Human IgG was used, because it is also found in plasma in high amounts.

After the screening against IgG, a selectivity criterion was used in this work, and 13 ligands were chosen to proceed, as they presented the highest enrichment factors, i.e., preferential binding to HSA. Those were re-synthesized and re-screened in the block against HSA and IgG and two ligands were selected on the basis of the consistent results achieved.

The two ligands, A3A2 and A6A5, were packed in a small column and a preliminary single elution condition was tested, showing that further optimization of buffers is needed.

However, such small synthetic ligands appear as a promising alternative to purify HSA, due to its high stability and strength and can be reused. Moreover, it can be produced at a lower cost compared to the existing options.

V. General conclusions

With an average consumption in the developed world of 200-400kg/million inhabitants, and almost $6.9 billion in the industry supplies products to more than one million inhabitants each year, HSA is emerging in the pharmacokinetic industry, due its fantastic capacity to carry a large and wide variety of compounds (Curling, 2004).

In biotechnology and biomedical applications, HSA can be founded in three different ways, namely HSA alone, HSA in fusion with biological proteins/peptides, or HSA complexes, being of much interest in the therapeutic area.

HSA is an important biomarker of many diseases, such as cancer, rheumatoid arthritis, and ischemia, and is also very used clinically to treat several diseases, like hypovolemia, shock, burns, trauma, hemorrhage, nutrition support and many others. Moreover, many therapeutic proteins or drugs can interact with HSA (by fusion or complexes) as part of its daily round through the circulation blood, increasing its half-life time, due the pH-dependent binding, between the FcRn receptor and the domain III of HSA (Fanali *et al*, 2012; Nilvebrant and Hober, 2013).

HSA has innumerous applications in the biomedical market, and can be found as HSA alone, HSA fused with biological proteins/peptides or HSA complexes, due to its high demand HSA has a great interest in the therapeutic area.

Due to high variability applications to HSA, is an effective strategy improving the pharmacokinetic properties of the drugs, increase the half-life of many therapeutic proteins, and also develop new alternatives for the purification of the HSA. For this reason, the aim of this work was the development of biological and synthetic affinity ligands towards HSA.

The first part of the work was to optimize the expression of a biological ligand called WW Clone 3, a small protein previously selected by phage display to present affinity towards HSA. This ligand is of biological origin, and for that reason has low toxicity and having a small size can be synthesized biologically and chemically.

The gene codifying the protein of interest was cloned into the vector expression (vector pET 21c, Amp^R), and then expressed in *E. coli* strain BL21 (DE3), at three different temperatures (18ºC, 25ºC and 30ºC) with IPTG induction. It was found that the best temperature for cell growth was 30ºC, producing a larger amount of total protein with approximately 45mg/ml in the soluble fraction and 10mg/ml in the insoluble fraction. However, it was not possible to detect the protein in any of the above fractions This might indicate that the protein was produced in a very small amount, or in fact protein was not expressed. With these results, it was not possible to continue this work because the result was not sufficient to achieve the goal initially proposed. For that reason, it is necessary a new strategy to optimize the expression of this protein.

The second part of the work was to develop synthetic ligands with affinity for HSA, based on the synthesis of two solid-phase combinatorial libraries. These ligands are more resistant and are more easily produced, and can be used for various applications. However, in

order to develop these ligands as affinity reagents for HSA purification, this application was explored.

The first step was to design two libraries based on amino acids of the protein PAB and drugs that bind naturally to the domain II of HSA. We focused on targeting domain II, because it is important have the domain III of HSA available to bind to FcRn.

The libraries were synthesized using the Ugi and Triazine reactions, producing 152 different ligands, 88 ligands from the Ugi library and 64 ligands from the Triazine library.

The libraries were screened against pure HSA in 10mM Sodium Phosphate, NaCl 150mM at pH 7.4 and at pH 6.0. A total of 17 ligands were selected on the basis of a high percentage of binding to HSA (>50%) at both pH 6.0 and 7.4, being 4 of the Ugi library and 13 of the Triazine library. The synthetic ligands showed a percent bound to HSA higher at pH 7.4, in this way, this pH was chosen to continue the studies to test the selectivity of the ligands, in order to re-synthesize and re-screen the lead ligands to HSA and confirm the reproducibility of the results.

Therefore, the 152 ligands from the two libraries were screened for binding IgG at physiological conditions. After the screening against IgG, a selectivity criterion was used in this work, and 13 ligands (5 of Ugi library and 8 of Triazine library) were chosen to proceed. The 13 ligands were re-synthesized and re-screened against HSA and IgG, and two ligands showed consistent results.

The two ligands, A3A2 and A6A5, were packed in small column and re-screened in individual assays to bind HSA and IgG at pH 7.4. The results remained consistent; the A3A2 obtained a percentage of 100% and 43% of bound HSA and IgG, respectively. The A6A5 ligand obtained a percentage of 99% and 53% of bound HSA and IgG, respectively.

In conclusion, the synthetic ligands based on the Ugi and Triazine reactions have been successfully used to bind to HSA at pH 7.4, and the 96-well screening format proved to be a reproducible, easy to perform and time saving protocol. Although the binding conditions were very efficient using 10mM Sodium Phosphate 150mM NaCl at pH 7.4, it is necessary to optimize the means to reduce the binding to IgG, in order to increase selectivity. The elution step also needs to be optimized to recovery HSA, through a strategy utilizing conditions that promote a decrease of hydrophobic interaction between the ligand and the target.

These synthetic ligands appear as a promising alternative to purify HSA, due to its resistance, long life, and low cost.

VI. Bibliography

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