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Multimethodological study of molecular recognition phenomena

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Chapter 1

INTRODUCTION

1.1 General remarks

Understanding the basis of molecular recognition phenomena plays a very important role in various fields of chemical research, but surely it becomes of primary importance in the context of pharmaceutical research.

The model of “lock and key” can be assumed in the case of specific biological complex formation. When a particular chemical species is located in the neighbourhood of another complementary chemical species, the two entities will interact with each other to form a biological complex in a selective and dynamic fashion. Many examples can be observed among the biological systems, such as DNA-protein, RNA-ribosome, enzyme-substrate, antigen-antibody, protein-protein, drug-receptor, etc.

All the physiological processes are governed by proteins in association with other entities. Protein-protein complex formation is the basis of the complex network that regulates not only the physiological mechanisms but also the pathological pathways: incorrect protein–protein interaction is the molecular switch of numerous diseases [1]. These findings explain how the study of the bio-recognition phenomena behind a biological process is nowadays considered a useful tool to deeply understand physiological mechanisms allowing the discovery of novel biological target and the development of new lead candidates [2].

Furthermore, in order to be successful, a drug candidate should also possess an excellent pharmacokinetic profile, defined by its ADMET parameters. Absorption, Distribution, Metabolism, Excretion and Toxicity represent five crucial steps of drug life in human body that significantly influence its pharmacological efficacy. In fact, in order to reach its site of action a drug must first of all be absorbed into the circulatory system from its site of administration; distributed throughout the peripheral tissues; metabolized through interaction with specific enzymes located in different body districts (liver, intestines, kidneys, lungs, skin, plasma and central nervous system); then eliminated completely from the body. In addition, the given drug dose (and its metabolites) must not be toxic for our body.

Recent market surveys have revealed that about half of all drugs in development fail to make it to the market because of ADMET deficiencies [3]. So it is clear how the interest in the evaluation of pharmacokinetic and toxicological drug properties in early

stages of drug discovery is increasing significantly, in order to eliminate as soon as possible the looser compounds. To this purpose multiple in silico, in vitro and in vivo ADMET experimental approaches have been developed both in industry and academia.

Surely, the physicochemical parameters of a new chemical entity, such as ionization constant, lipophilicity and solubility, dramatically affect the drug bioavailability and therefore its toxicological effects [4]. In addition, the plasma proteins binding is also closely correlated to the final pharmacological and toxicological success of the new drug, as It represents a crucial step for the drug to reach its site of action.

The monitoring of drug binding to plasma proteins is becoming essential in the field of drug discovery to characterize the drug distribution in human body. Human serum albumin (HSA) is the most abundant protein in plasma, with a concentration of about 600 μM [5]. This protein represents the major serum carrier being responsible for the transport of numerous endogenous factors (non-esterified fatty acids, bilirubin, bile salts, steroid hormones, hematin, thyroxin, vitamins and metal ions) and various xenobiotics (drugs and metabolites), through the formation of non-covalent complexes at specific binding sites. The knowledge of drug-HSA interactions is of great importance for the pharmacokinetic, pharmacodynamic and toxicological evaluation of a new drug candidate [6]. In fact, the unbound drug fraction substantially affects the concentration of the drug at its specific receptor site: higher is the binding percentage for the serum carrier, lower is the amount of drug available for the pharmacological effect. From this point of view this could be considered a drawback, but we have to underline that the binding with HSA become a great advantage in presence of poorly soluble compounds: their binding to HSA results in improving solubility and sometimes in decreasing toxicity [7]. Furthermore, the bound drug fraction acts as a reservoir for a longer drug action, increasing its half-life in the human body and maintaining its therapeutic level.

Because of the ability of this protein to bind numerous different molecules, another aspect to be considered is the possible competition between more than one drug or endogenous factors that interact with the same HSA binding site: in this case the free concentration of the displaced drug changes significantly, thus adversely affecting its pharmacokinetics and pharmacological activity [6].

Since animal models are often used for pharmacokinetic in vivo experiments, the interest in studying the binding of new compounds to albumins from different species is

growing. Despite the great similarity between the amino acidic sequences of albumins from different animal species, it has already been demonstrated the existence of significant interspecies differences. Hence this kind of investigation is very useful to evaluate the reliability of extrapolating the distribution data obtained in animals to humans [8,9].

Therefore, it is clear how the characterization of interactions between proteins and drugs determines a growing need of methodologies to study any specific molecular event as well as the structural elements that drive the process.

A wide variety of biochemical techniques have been applied to this purpose. Among all, high-performance liquid affinity chromatography (HPALC), circular dichroism (CD) and optical biosensor technology represent three techniques that can be able to elucidate the interaction of a new drug with its target and with other proteins that could interfere with ADMET parameters.

HPALC is a particular type of affinity chromatography where a biological ligand immobilized on the chromatographic support is used as stationary phase.

In 1990, the HSA-based HPLC columns were first used for the enantiomeric resolution of chiral drugs [10]; afterwards there was an increased interest in the use of these columns for the characterization of chiral and achiral compounds binding processes. Moreover, a large body of literature has extensively demonstrated that the HSA maintains the same binding characteristics held in solution also after the immobilization process [10-13]. This evidence has made HPALC a reliable technique for the study of the interaction between small molecules of pharmacological interest and albumins from different species.

In HPALC zonal elution conditions, the retention of the analyte is a direct evidence of the degree of binding for the protein: more strongly bonded compounds show elution times longer than those obtained with the weaker bonded compounds. A rapid determination of the bound fraction of large libraries of novel synthesized molecules can be obtained with this technique, allowing ranking analysis in relatively short times. Moreover, in frontal analysis conditions the HPALC technique allows also the determination of the association equilibrium constants of the compounds under investigation. In this case the analyte passes over the HSA column continuously, utilizing a large range of analyte concentrations directly in the mobile phase.

The main advantage of this technique is that the chromatographic system can be automated in order to obtain reproducibility, precision and speed. The resolution of chiral entities obtained with these columns allows also the investigation of the enantioselectivity of the binding process. Furthermore, the immobilized HSA column can be reused for even long time (almost 1000 injections), resulting in low protein consumption [13]. On the other hand, the biochromatographic system detects all the interactions that occur with the stationary phase, so the retention time will also be affected by the non-specific interactions with the silica matrix itself. The study of compounds with very high affinity for the protein (bound fraction > 99%) represents another drawback of this technique. This circumstance requires the presence of an organic modifier in the mobile phase, which can lead to variations in the protein conformation and in the interaction with the drug under investigation [14]. The problem could be solved by using very short columns [15].

Both Circular Dichroism and Biosensor technique represent two different analytical approaches based on the monitoring of variations of specific physicochemical properties of the protein or of the ligand, when interacting with each other.

Circular Dichroism (CD) is a spectroscopic technique based on the different absorption of the two components of plane polarized light (right and left-hand circularly polarized light) by an optically active compound. This phenomenon arises not only when the compound under investigation hold chromophores with intrinsic chirality, but also when It is located in a chiral environment [16]. This explains why CD is the technique of choice for the determination of the absolute configuration of chiral compounds, as well as for the characterization of molecular recognition phenomena in solution [6, 17]. The literature is full of applications that highlight the value of this technique at different phases of drug discovery process [9, 16, 18]. First of all, CD is employed for the stereochemical characterization of new drug candidates and therapeutic peptides, in order to reliably establish the relationship existing between stereochemistry and pharmacological activity [6, 19, 20]. This technique is also widely used to monitor the conformational transitions of proteins and peptides, due to secondary structural changes functional in various physiological and pathological processes [21-23]. The interest towards this type of study has grown after the recent evidence that conformational changes of physiological peptides and proteins represent the start

button of different neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease amyotrophic lateral sclerosis and prion diseases [24].

The CD provides direct information on the binding interaction between a new drug candidate and its target protein. It is possible to detect the conformation of the bound drug from the CD induced signal obtained upon binding to the target protein and to measure also the binding constant of the specific drug-protein interaction, without interfering with the equilibrium process. Furthermore, competition experiments carried out with this technique provide useful information about the drugs binding sites on the target protein and about different co-binding situations that can occur at specific binding sites [16].

The main advantage of the CD technique is surely the possibility to obtain all these information directly in solution, thus avoiding the immobilization step that could compromise a reliable investigation of the interaction. Moreover, this technique allows us to study the behavior of both enantiomers of chiral drugs. Despite that a huge drawback is the relatively large amount of protein necessary to perform a complete set of experiments.

Concerning the Biosensor technology, this technique is responsible for the big steps forward seen in the biomolecular interaction analysis field over the last decade. The use of Optical Biosensor to characterize target macromolecules and biopharmaceuticals is well documented in the current literature [25-27]. The Optical Biosensor is based on the optical phenomenon, called Surface Plasmon Resonance (SPR). This device consists of three main components: the sensor surface, where one of the interacting compounds is attached to; the microfluidic system, to ensure reproducible sample consumption and an SPR detector, which measures the change in the refractive index close to the surface of the sensor chip, during complex association and dissociation. The SPR response observed is proportional to the mass of the analyte that is bound to the immobilized ligand. This instrument provides qualitative information about the specificity of the binding (Yes/No answer), but also many quantitative data, such as the amount of active analyte in the sample, kinetic parameters of the interaction (k_{on}, k_{off}) and the affinity constants that characterize the complex formation. Thus, it is clear how this technique represents a useful tool in advanced drug discovery stages,

where it becomes crucial to have a clear and complete view of the binding mechanisms to understand different biological and pathological phenomena [14]. Since the sensitivity of this technique is directly related to the mass of the analyte under investigation, it is obvious that it will be the technique of choice to study protein-protein interactions. Recent improvements in the SPR biosensor have made possible the use of this technology also to detect small molecule drugs (< 200 Da).

The main advantage of this technique is the lack of a labeling step for both the ligand and the analyte, thus avoiding extra time and cost or misinterpretation of the collected data due to the occlusion of specific binding sites [2]. However, the immobilization remains always a critical step, because the immobilized compound may take different behavior from that observed in solution. On the other hand the chip can be reused even for a long time after the immobilization, depending on the stability of the immobilized ligand. This parameter can be also monitored by tracking the surface's binding capacity and the baseline stability during the analysis. Furthermore, the SPR biosensor requires short time of analysis and very low sample consumption.

All the underlined advantages and drawbacks, as well as the various fields of application and the different types of information that can be obtained from the techniques mentioned above, highlight how the multimethodological approach represents the best strategy for the study of molecular biorecognition phenomena, especially in advanced stages of drug discovery, giving a detailed and complete description of the particular recognition process.

1.2 Aim of the investigation

The main intent of this work is to highlight the importance of the elucidation of the biorecognition phenomena existing behind biological processes, since it represents a crucial step for the discovery of novel biological target and the development of new lead candidates. The investigation involved the use of three orthogonal analytical techniques, in order to obtain a clear and complete picture of the particular protein-small molecule system.

In particular:

- ✓ 5-Imino-1,2-4-thiadiazoles and quinazolines derivatives as glycogen synthase kinase 3b (GSK-3b) and phosphodiesterase 7 (PDE7) inhibitors were characterized for their human serum albumin binding, using high-performance liquid affinity chromatography and circular dichroism; in order to obtain an evaluation of the drug bound fraction to the transport protein and to get information on the high affinity binding sites of these compounds to HSA
- ✓ the species-dependent binding of tocainide analogues to two different serum albumin (HSA and RSA) were carried out by affinity chromatography and circular dichroism, in order to highlight the different binding capacity and stereoselectivity of the two similar proteins
- ✓ an optical biosensor analysis has been developed to study the binding of new synthesized bicalutamide analogues for their biological target, the androgen receptor; thus achieving the affinity constant and the kinetic parameters that drive the specific binding process
- ✓ the bicalutamide analogues were also characterized for their HSA and RSA binding, by using all the three techniques, i.e. affinity chromatography, circular dichroism and optical biosensor. Important differences in the behavior of the two proteins were highlighted also in this case.

This study underline the importance of getting in early stages of drug discovery not only information about the specificity of a new chemical entity, but also about its pharmacokinetic parameters. Moreover, big attention requires the extrapolation of the in vivo pharmacokinetic data obtained on animals to humans. The multimethodological approach represents the best way to study the biorecognition process and to develop the medium-high throughput screening needed in the early phases of drug discovery.

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Chapter 2

5-Imino-1,2-4-thiadiazoles and quinazolines derivatives as glycogen synthase kinase 3b (GSK-3b) and phosphodiesterase 7 (PDE7) inhibitors: determination of binding to human serum albumin[1]

Abstract

5-Imino-1,2,4-thiadiazoles and quinazolines derivatives as glycogen synthase kinase 3b (GSK-3b) and phosphodiesterase 7 (PDE7) inhibitors were characterized for their human serum albumin (HSA) binding using high-performance liquid affinity chromatography (HPLAC) and circular dichroism (CD). For the HPLAC investigation, HSA was previously covalently immobilized to the silica matrix of the HPLC column. This HSA-based column was used to characterize the high affinity binding sites of 5-imino-1,2,4-thiadiazoles and quinazolines derivatives to HSA. Displacement experiments in the presence of increasing concentrations of competitors known to bind selectively to the main binding sites of HSA were carried out to determine their possible binding site. The same drug–protein system was studied by CD.

The analysed compounds showed a high affinity to HSA. Competition experiments showed an anticooperative interaction at sites I and II, and an independent binding at bilirubin binding site on HSA.

2.1 Introduction

Chronic age related neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease represent a huge and increasing disease burden to society. Alzheimer's disease (AD) is the leading cause of dementia and is predicted to become even more prevalent as the proportion of elderly in the population increases over the next few decades. There are currently an estimated 5.3 million people with Alzheimer's disease in USA and 5.5 in Europe. It is expected by the year 2050 that the people with AD will be four times more. On the other hand, the number of persons with Parkinson's disease (PD) over age 50 in Western Europe's five most populous nations was up to 4.6 million in 2005, and this will double to up to 9.3 million by 2030 [2].

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase with a critical role in neurodegeneration and memory formation. The family consists of two isoforms, a and b, which are 98% identical within their kinase domains but differ substantially in their N- and C-terminal sequences. GSK-3b is particularly abundant in the central nervous system (CNS) and directly phosphorylates several neuronal microtubule-associated proteins involved in microtubule stabilization. Different studies have suggested the central role of glycogen synthase kinase 3b (GSK-3b) in the pathogenesis of both sporadic and familial forms of AD [3, 4]. Because of these data, inhibition of GSK-3b is a promising strategy for the treatment of AD and other neurodegenerative diseases [5].

On the other hand, phosphodiesterases (PDEs) selectively degrade cyclic purine nucleotides (cAMP and cGMP) that serve as second messengers in a number of cellular pathways. PDEs are isoenzymes encoded by at least 22 different genes and organized into 11 families (PDE1–11) characterized by molecular biological techniques in different mammalian tissues [6, 7]. PDEs have proven to be drug-able targets, with compounds on the market or in late-stage clinical development for a variety of diseases [8]. Because elevation of the intracellular cAMP level impacts immunosuppressive and anti-inflammatory properties [9] treatment of different immune disorders such as Multiple Sclerosis, diseases of the central nervous system (e.g., AD, depression) and inflammatory processes employing selective inhibitors of cAMP-specific PDEs have been widely studied [10-12].

PDE7 is a cAMP-specific PDE family [13], including two members (PDE7A and PDE7B). PDE7A is highly expressed in the immune system including spleen, lymph nodes and blood leukocytes [14], while PDE7B is abundant in the brain, liver, heart, thyroid glands and skeletal muscles [15]. It has been recently published, that PDE7 inhibition enhances neuroprotection and diminishes neuroinflammation in well-characterized cellular and animal models of PD [16].

For all these reasons, GSK-3b and PDE7 inhibitors play an important role for the next future treatment of different neurodegenerative diseases [17, 18]. 5-Imino-1,2,4-thiadiazoles (Compounds 1-5) [19] and quinazolines (Compunds 6–8) [20] have been previously synthesized as potential new drugs for CNS disorders. Their chemical structures together with the value of the IC_{50} on their targets are depicted in Fig. 2.1. These compounds were able to inhibit these enzymes at micromolar concentrations.

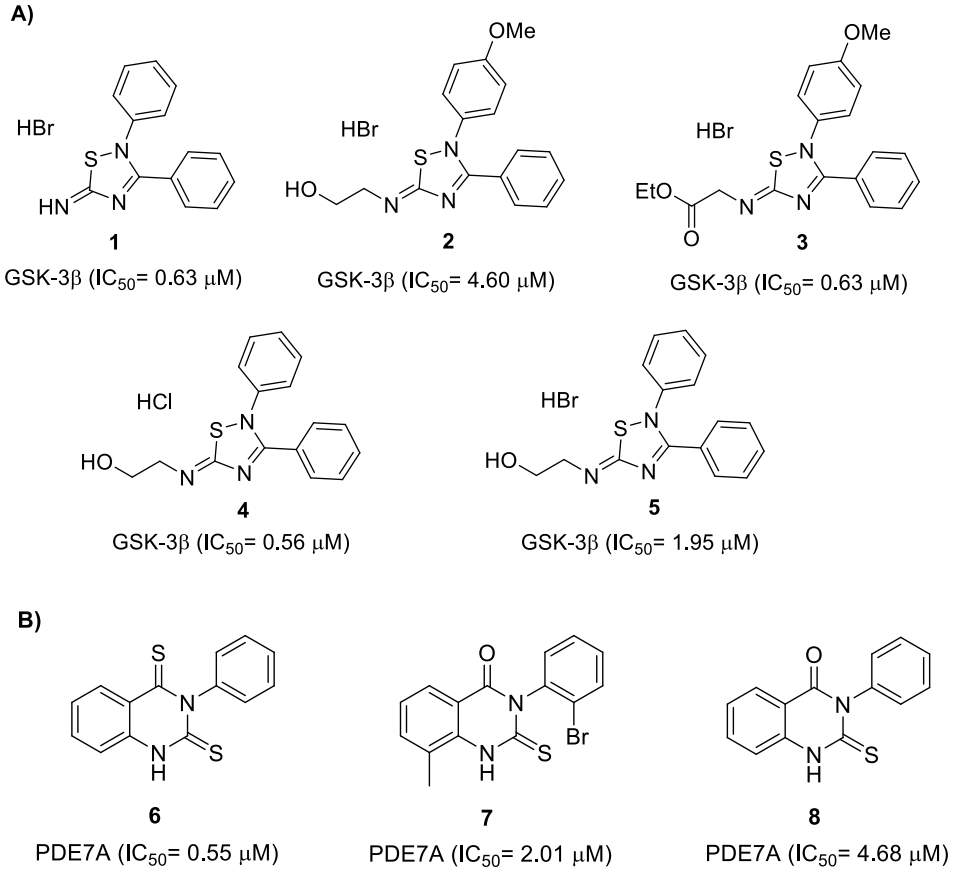

Fig. 2.1 A) GSK-3 inhibition of Iminothiadiazoles **1**-**5. B)** PDE7 inhibition of quinazolines **6**-**8**

Although the discovery of GSK-3b and PDE7 inhibitors is essential for the treatment of severe diseases, it is also important for further pharmacological development that these compounds present a favorable Absorption–Distribution–Metabolism–Excretion (ADME) profile. Determination at early steps of these properties is a crucial issue to eliminate weak candidates.

ADME properties are the major reason for the failure of drug candidates in the late stages of drug development, being a serious economic problem in the pharmaceutical industry [21].

Human serum albumin (HSA, 66 kDa) is the most abundant protein in plasma with a concentration of 0.6-0.7 mM [22]. The main role of HSA in plasma is to transport and to distribute many substances within the body such as drugs, fatty acids, heavy metals, hormones and vitamins. This flexible protein contains numerous potential drug binding sites [23]. In pharmaceutical industry it is important to determine and characterize the pharmacokinetics and pharmacological effects of drugs. Thus, analysis of the drug binding to the most abundant plasma protein is interesting not only because the unbound drug fraction affects many critical pharmacokinetic parameters including the steady-state distribution volume, but also because it provides useful information to design the administration regimen dose.

Here, the distribution behavior of the 5-imino-1,2,4-thiadiazoles and quinazolines families were studied by high-performance liquid affinity chromatography (HPLAC). A HSA-based column was used for ranking the compounds under investigation for their binding to the serum carrier, and for getting information on the binding areas of the studied drugs to the plasmatic protein. The bound fractions of 5-imino-1,2,4-thiadiazoles and quinazolines derivatives were determined by measuring their retention on the column, using the zonal chromatographic approach. Pivotal displacements experiments [24, 25] by adding selected competitors [26] into the mobile phase were carried out to individuate the possible binding sites. Results were confirmed by circular dichroism (CD) competition studies.

2.2 Materials and methods

2.2.1 Chemicals and instruments

Human serum albumin (HSA) (Cohn fraction V powder essentially fatty acid free, A1887), L-tryptophan (L-Trp), glycine, N-benzoyl-DL-leucine, rac-warfarin, sodium valproate, rac-ibuprofen sodium salt, sodium salicylate, bromophenol blue, and bilirubin were purchased from Sigma–Aldrich (Milan, Italy). Diazepam and clonazepam were kindly provided by Prof. Regina H. Costa Queiroz, Faculty of Pharmacy, University of San Paolo, at Ribeirao Preto (Brazil). Other chemicals were reagent grade from commercial suppliers and were used without further purification. Potassium phosphate buffers (PB) were prepared by adjusting the pH of solutions of K_2HPO_4 with equimolar solutions of KH_2PO_4. K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$ powders were purchased from Carlo Erba Reagenti (Milan, Italy).

The HPLC apparatus consisted of a Jasco PU-2089 plus (Jasco, Tokyo, Japan) HPLC pump, a Rheodyne injector system with a 20 µL loop and a Jasco MD-2010 plus diode array detector. The column was thermostated at 28 °C with a Column Chiller Model 7955 (Jones Chromatography Ltd., UK).

The chromatographic retention was reported as the capacity factor (k), where k is defined as $(t_{drug} - t_0)/t_0$ (t_{drug} = retention of the solute; t_0 = retention of a non-retained solute). Buffers were filtered with 0.22 µm mixed cellulose esters filter membranes (Millipore, Milan, Italy). The mobile phases were degassed prior to use by ultrasonic waves for at least 15 min. Compounds (1-8) were previously synthesized [19, 20].

2.2.2. Column preparation

The silica column (50 mm × 4 mm i.d.) packed with Kromasil 200 Å (5 µm) was derivatized with 3-glycidoxypropyltrimethoxysilane by an in situ process as previously reported [27]. HSA was covalently immobilized on the packed epoxy-silica column by the reported [28, 29] online procedure, with slight modifications. In brief, a HAS solution (10 mg/mL of protein in 50 mM PB, pH 6.5, 1 M ammonium sulphate) was circulated through the epoxy-silica column in closed circuit for 24 h. The column was then washed with 120 mL of 50 mM PB, pH 7.4; and 100 mL of 1 M glycine dissolved in PB. The

HSA-based column (50 mm × 4 mm i.d.) was employed for the determination of the HSA bound fraction and for the displacement studies.

2.2.3. Column evaluation

Rac-warfarin, and N-benzoyl-DL-leucine, two racemates known to bind HSA enantioselectively, were injected into the column in order to check that the immobilized protein maintained the binding properties of the native HSA. Stock solutions of the racemates were prepared (0.5 mg/mL, 1-propanol), and then diluted 100-fold to the appropriate concentration [30 µM] with PB (pH 7.4; 67 mM). The chromatographic runs were performed at a flow rate of 1 mL/min, injecting 20 µL of the analytes. Mobile phases were PB (pH 7.4; 67 mM)-1-propanol (97:3, v/v) in the case of rac-warfarin and PB (pH 7.4; 67 mM)-1-propanol (99:1, v/v) in the case of N-benzoyl-dl-leucine [30].

2.2.4. Affinity chromatography: bound fraction determination

Stock solutions of compounds (1-8) were prepared at a concentration of 0.5 mg/mL in 1-propanol and were diluted 100-fold with PB (pH 7.4; 67 mM) just before injection into the HSA column, to reach a final concentration of 30 µM. The mobile phase was PB (pH 7.4; 67 mM), and the flow rate 1 mL/min. The bound drug fraction (B%) was determined by measuring the capacity factor, according to a previously reported method [31].

$$B\% = 100 \times \left(\frac{k}{k+1}\right)$$

2.2.5. Affinity chromatography: displacement experiments

A series of displacement experiments were carried out to have information on the binding site(s) of 5-imino-1,2,4-thiadiazoles and quinazolines. Increasing amounts of the markers for the three major binding sites of HSA were added to a mobile phase containing PB (pH 7.4; 67 mM). Sodium salicylate, rac-ibuprofen and sodium valproate were used as markers of Sudlow's site I, II and the bilirubin site, respectively. The concentration of the marker in the mobile phase ranged from 0 to 100 µM for sodium salicylate, from 0 to 50 µM for rac-ibuprofen and from 0 to 3 mM for sodium valproate. All the compounds were injected at the same concentration used for the determination

of the bound fraction described in Section 2.2.4. The compounds solutions were injected and the capacity factors of the drugs were determined in the absence and in the presence of increasing concentrations of the competitors.

The plotting of the reciprocal of the capacity factor (1/k) versus the molar concentration of the marker gives information on the interaction mechanism. A direct competition at a single site gives a straight line with a positive slope in this plot, while a non-competitive relationship gives only random variations [24, 25, 32]. The following linear equation is satisfied in the case of direct competition, where the analyte binds to a single binding site:

$$\frac{1}{k_A} = \frac{V_M \times K_I \times [I]}{K_A \times m_L} + \frac{V_M}{K_A \times m_L} \qquad \text{(a)}$$

where k_A is the measured capacity factor of the analyte A, V_M is the column void volume, K_I is the association equilibrium constant of the competitor for the single binding site of A, [I] is the molar concentration of the competitor, K_A is the association equilibrium constant of A for its binding site, and m_L is the total moles of analyte binding sites in the column. This last parameter, m_L, corresponds to the protein moles correctly anchored to the silica matrix.

The equilibrium dissociation constant (K_D) of the displacer for the single binding site of the analyte can be then calculated by linear fitting of the data.

In particular, K_D can be obtained as

$$\frac{intercept}{slope} = \frac{1}{K_I} = K_D \qquad \text{(b)}$$

Multisite binding determines deviation from this linear behavior.

To check the dependability of the column and better estimate the entity of displacement, warfarin and diazepam were selected as controls when salycilate and ibuprofen were used as competitors, respectively.

2.2.6. Circular dichroism: displacement experiments

Circular dichroism measurements were performed with a Jasco J-810 spectropolarimeter. Displacement experiments were carried out on the compound of each family, displaying the highest affinity to HSA in the affinity chromatography study. For the 5-imino-1,2,4-thiadiazoles family compound 3 was employed as the competitor, and phenylbutazone, diazepam and bromophenol blue as selected markers for site I, site II and bilirubin binding site, respectively. The change in the induced CD signal of a solution containing equimolar concentrations of HSA and marker [15 μM in PB (pH 7.4; 67 mM)] was monitored after addition of different amounts of compound 3. For the quinazolines family, the opposite approach was employed; the selected compound 6 was used as a marker, while, warfarin, ibuprofen and valproic acid (selective for site I, site II and bilirubin site, respectively) were used as the competitors. The change in the induced CD signal of a solution containing equimolar concentrations [15 μM in PB (pH 7.4; 67 mM)] of HSA and compound 6 after the addition of different amounts of warfarin, ibuprofen and valproic acid was monitored. Stock solutions of compound 3 (4.57 mM in 1-propanol), phenylbutazone (10.37 mM in 1-propanol), diazepam (15 mM in 1-propanol), bromophenol blue (12.2 mM in 1-propanol) and stock solution of compound 6 (9.62 mM in 1-propanol), warfarin (20 mM in 1-propanol), ibuprofen (36.8 mM in 1-propanol), valproic acid (20 mM in 1-propanol) and HSA (409 μM in PB, pH 7.4, 67 mM) were prepared immediately before the experiments. All the measurements were carried out using a 10 mm pathlength cell.

5-Imino-1,2,4-thiadiazoles: the working solutions were prepared mixing HSA, marker and compound 3 at molar ratios of 1/1/0, 1/1/0.5, 1/1/1, 1/1/2, 1/1/4 and 1/1/8, respectively.

Quinazolines: the working solutions were prepared mixing the HSA, compound 6 and warfarin at molar ratios of 1/1/0, 1/1/1, 1/1/2, 1/1/4, 1/1/8 and 1/1/16. HSA, compound 6 and ibuprofen at molar ratios of 1/1/0, 1/1/4, 1/1/8, 1/1/16, 1/1/24 and 1/1/32. HSA, compound 6 and valproic acid at molar ratios of 1/1/0, 1/1/4, 1/1/8, 1/1/16 and 1/1/32.

2.3 Results and discussion

2.3.1 HSA bound fraction determination by affinity chromatography

Prior to the injection into the HSA covalently immobilized column of 5-imino-1,2,4-thiadiazoles and quinazolines compounds, the reliability of the prepared HSA-based column for affinity chromatography studies was checked by injecting racemates of molecules known to bind the protein in a enantioselective manner to verify if the protein, once anchored to the silica matrix, maintains its binding properties [30].

Compounds (1–8) were injected into the HSA column and the ranking for their HSA bound fraction was performed on the basis of the capacity factor, k, as determined in aqueous solution. Drugs with known bound fraction to HSA (clonazepam, diazepam and rac-warfarin) were analyzed under the same experimental conditions to check the dependability of the column for ranking the new tested compounds for their HSA binding (Tables 2.1 and 2.2). Acetaminophen was analyzed as well as a negative control (experimental bound fraction 6.25%, the literature bound fraction < 20%) [33]. A good correlation of the HSA bound fraction of drugs between the literature data and the experimentally obtained values was obtained. All compounds analyzed showed a high affinity to HSA (≥ 94%). In the 5-imino-1,2,4-thiadiazoles family, compounds 2, 4 and 5 which presented an ethanol chain in their structure, gave lower bound fraction values than compound 1, which has not any substituent linked to the nitrogen atom. Compound 3, with an ethyl ester chain gave the highest bound fraction (B% = 98.3%) to HSA. Note that in the quinazolines derivatives, the replacement in the structure of a carbonyl atom (compound 8), to the thiocarbonyl one (compound 6), significantly increased the bound fraction percentage (94.4% to 99.8%). All these data are valuable to design and optimize the synthesis of new compounds to optimize their distribution in the human body, giving a better balance between drug distribution and half-life drug.

2.3.2 HSA binding site investigation: displacement affinity chromatography and circular dichroism

Displacement affinity chromatography experiments were carried out by determining the capacity factor of each analyte upon increasing concentrations of selected competitors added to the mobile phase. The analysis of the chromatographic

Table 2.1
Bound fraction (B%) of the studied compounds obtained by affinity chromatography.

Compound	*B*% by chromatography[a]	*B*% from literature
Clonazepam	85.73 ± 0.55	86[b, [33]]
Diazepam	94.48 ± 0.31	93.5[c, [30]]
Rac-warfarin	97.80 ± 0.06	98.5[c, [30]]
1	96.74 ± 0.26	–
2	93.80 ± 0.20	–
3	98.28 ± 0.32	–
4	94.92 ± 0.46	–
5	94.41 ± 0.55	–
6	99.75 ± 0.01	–
7	98.04 ± 0.07	–
8	94.42 ± 0.06	–

[a] Calculated as the average of the results obtained by two or three injections from the equation obtained by linear correlation of the experimental and literature B% values of the standards (y = 0.3257x + 68.206, r^2=0.9808).
[b] In whole plasma.
[c] Measured by affinity chromatography.

Table 2.2
KI values obtained by displacement chromatography analysis. The competitor affinity values were determined by slope and intercept of the 1/k plot of the analyte upon increasing the concentration of the competitor (see equations a and b in section 2.2).

Compound	Salicylic acid	Ibuprofen	Valproic acid
Clonazepam	N.S.	----------	N.S.
Diazepam	----------	8.71×10^3	----------
Warfarin	1.01×10^4	----------	1.16×10^4
1	N.S.	N.S.	N.S.
2	N.S.	N.S.	N.S.
3	3.63×10^3	N.S.	N.S.
4	N.S.	N.S.	N.S.
5	N.S.	N.S.	N.S.
6	1.16×10^4	2.58×10^4	N.S.
7	7.78×10^3	2.76×10^4	N.S.
8	7.29×10^3	1.34×10^4	N.S.

N.S. Variation of k values not statistically significative (P>0.05)

data allowed the affinity constant of the competitors (K_I) to be determined (see Section 2.2). The same methodology described for the bound fraction determination was

employed for the competition studies. The change of the capacity factor (k) was monitored upon addition of increasing concentrations of selected competitors into the mobile phase. These displacers or competitors were selected because of their specific binding sites to human serum albumin such as sodium salicylate (site I), rac-ibuprofen (site II) and sodium valproate (site III or bilirubin site) (Table 2.2).

As an example, the results obtained for one compound of each family (5-imino-1,2,4-thiadiazole 3 and quinazoline 6) in the competitive experiments binding, together with some compounds used as controls (clonazepam, diazepam and warfarin) is represented in Fig. 2.2. A significative decrease of the retention times was found in compound 3 (5-imino-1,2,4-thiadiazole) and compounds 6–8 (quinazolines) when salicylic acid up to 100 μM was used as a displacer. The K_I values of the displacers obtained are collected in Table 2.2. When salicylate was used as a displacer, compounds 1, 2, 4 and 5 from the 5-imino-1,2,4-thiadiazoles family did not significantly change their retention time, being the decrease below the statistical significance ($P > 0.05$). However in 5-imino-1,2,4-thiadiazole 3 and quinazolines (6–8) a shortening of the retention time was observed and the change in the k value was statistically significant ($P < 0.05$). Note that in the case of quinazolines 6–8, the K_I values are of the same order of magnitude of the affinity constant obtained for the known ligand of site I warfarin, suggesting a direct competition. 5-Imino-1,2,4-thiadiazole 3, showed a lower K_I value suggesting an allosteric competition.

The employment of ibuprofen up to 50 μM as a displacer showed a decrease of the retention time and a statistically significant change in the k value ($P < 0.05$) in the case of quinazolines, while only a small effect, consistent with an allosteric competition, was observed for the 5-imino-1,2,4-thiadiazole analogues. Values of K_I of the same order of magnitude were obtained for quinazolines (compounds 6–8) compared to the affinity constant of ibuprofen measured by the known displacer, diazepam, suggesting that also site II is involved in the binding of these molecules to HSA. On the other hand, very small not statistically significant changes in retention times were found for both 5-imino-1,2,4-thiadiazoles and quinazolines when valproic acid was employed as the displacer up to 3 mM concentration (Table 2.2).

Since the data showed that both salicylate and ibuprofen compete significantly with quinazolines (6–8) for their binding to HSA we can hypothesize that both sites I and

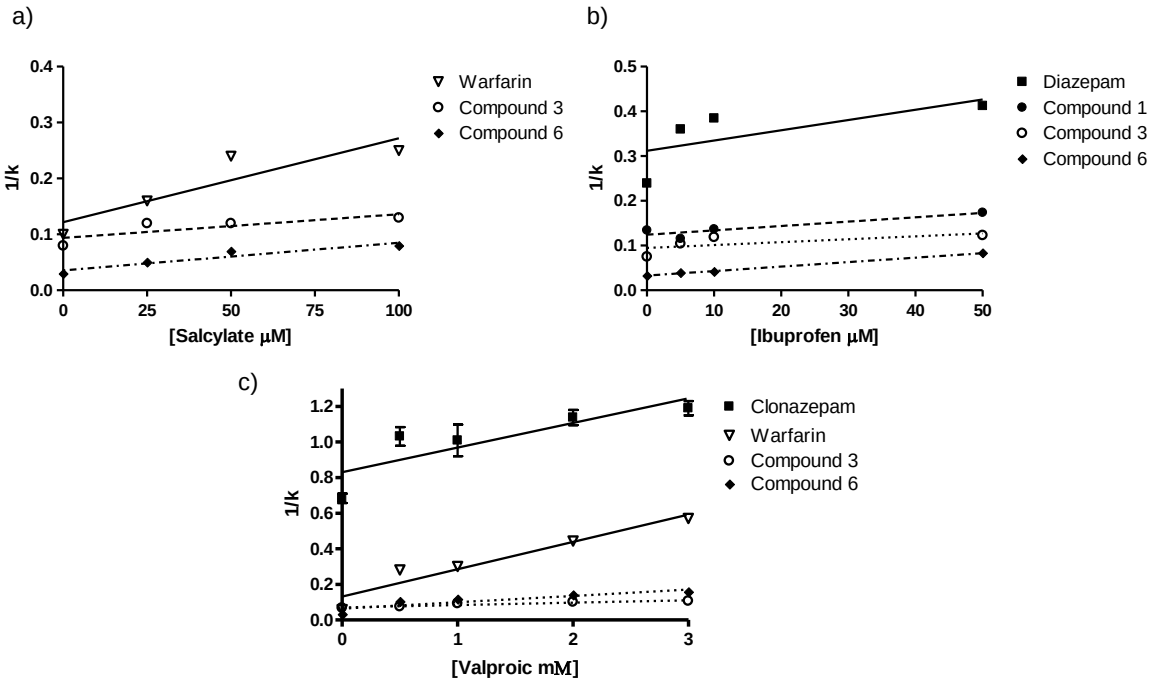

Fig. 2.2 Displacement experiments. Behaviour of 1/k values of Diazepam, Clonazepam, Warfarin and compounds **1**, **3** and **6** in the absence and in the presence of increasing concentrations of salicylate (a), *rac*-ibuprofen (b) and valproate (c).

II are primarily involved in the binding of these molecules to HSA. Among the 5- imino-1,2,4-thiadiazoles studied, only the binding of compound 3 was significantly affected by the presence of salycilate, and only a weak displacing effect was observed in the presence of ibuprofen, suggesting that none of site I and site II is the primary binding site of these molecules. An independent binding should be suggested for 5-imino-1,2,4-thiadiazoles and quinazolines analogues, and valproate, selective marker of the bilirubin binding site.

Competition experiments were also carried out by CD spectroscopy, by monitoring the change of the induced CD spectrum observed for a [HSA]/[marker] complex 1:1 (15 µM) in the presence of increasing concentrations of a selected displacer. Usually a known molecule selective for a certain binding site is used as ‘‘marker’’ of that site, while the molecule studied is used as ‘‘displacer’’. If the molecule under investigation displays an induced CD signal when bound to HSA, the opposite approach, were the molecule under investigation is used as ‘‘marker’’ (of its own site(s)), while the known molecules are used as ‘‘displacers’’ can be applied as well. The choice of which

molecules are to be used as a marker and which as a displacer is made case by case depending on the spectroscopic properties of the molecules involved. In the analysis of compound 3, the markers employed were phenylbutazone (selective binding to site I), diazepam (selective binding site II) and bromophenol blue (selective binding to site III) while compound 3 was used a displacer. In the analysis of compound 6, the opposite approach was employed and warfarin (site I), ibuprofen (site II) and valproic acid (bilirubin site) were selected as displacers and compound 6 as the marker. The experiment consists in the determination of the spectroscopic characteristics of the involved compounds or the markers-displacers by monitoring the induced CD signal in the absence or presence of the competitor [34].

In agreement with the results obtained by affinity chromatography, the addition of compound 3 showed to displace phenylbutazone (PBU) and diazepam (DZP) from site I and site II, respectively only when added at high concentrations, (Fig. 2.3), confirming the small anticooperative effect for both sites observed in the affinity chromatography displacement experiments. No change was observed in the induced CD spectrum of bromophenol blue upon addition of compound 3 up to [HSA]/[marker]/[compound 3] 1/1/8, confirming the independent binding for 5-imino-1,2,4-thiadiazoles and bilirubin site ligands.

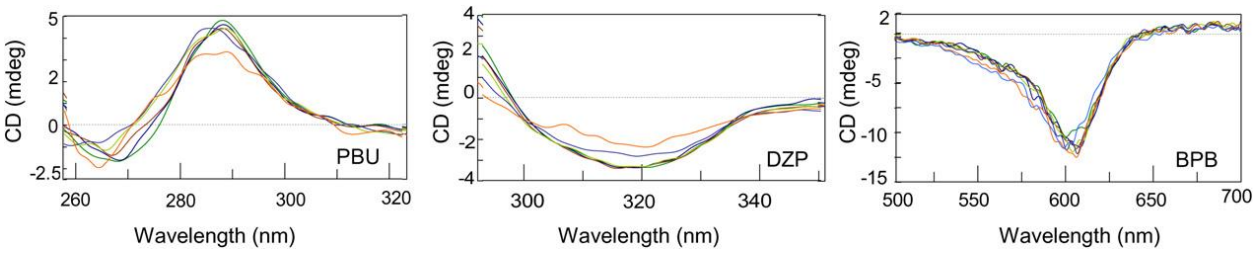

Fig. 2.3 Effect on the induced CD of phenylbutazone (PBU), diazepam (DZP) and bromophenol blue (BPB), bound to HSA upon addition of increasing concentrations of compound 3 to the [HSA]/[Marker] complexes. Stoichiometry of the [HSA]/[Marker]/[Compound 3] complexes: 1/1/0, 1/1/0.5, 1/1/1, 1/1/2, 1/1/4 and 1/1/8.

Different results were obtained for quinazoline 6 where a competitive binding at sites I and II were found. Both displacers warfarin and ibuprofen were able to change significantly the induced CD spectra of [HSA]/[Compound 6], while no significant changes were observed when valproic acid was used as displacer (Fig. 2.4). These results confirm the affinity chromatography data, indicating that both site I and site II are

involved in the binding of compound 6 to HSA, while an independent binding to bilirubin site was observed.

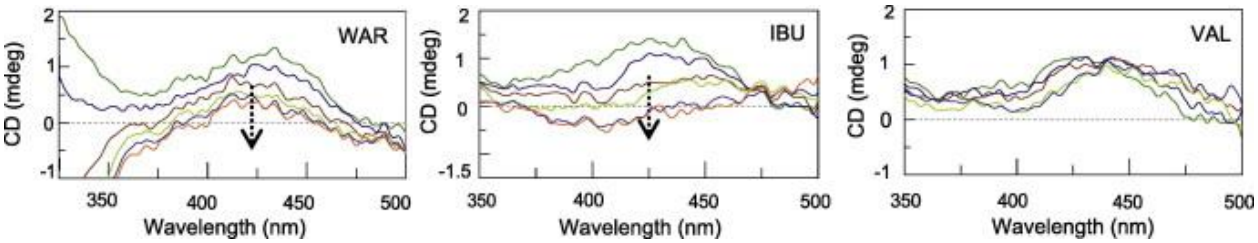

Fig. 2.4 Effect on the induced CD of compound 6 bound to HSA upon addition of increasing concentrations of rac-warfarin (WAR), rac-ibuprofen (IBU) and valproic acid (VAL) to the [HSA]/[Compund 6] complexes. Stoichiometry of the [HSA]/[Compound 6]/[WAR] complexes: 1/1/0, 1/1/1, 1/1/2, 1/1/4, 1/1/8 and 1/1/16. Stoichiometry of the [HSA]/[Compound 6]/[IBU] complexes: 1/1/0, 1/1/4, 1/1/8, 1/1/16, 1/1/24 and 1/1/32. Stoichiometry of the [HSA]/[Compound 6]/[VAL] complexes: 1/1/0, 1/1/4, 1/1/8, 1/1/16 and 1/1/32.

2.4 Conclusions

As the proportion of elderly in the population increases over the next few decades it is important to discover new drugs for the treatment of diseases whose main risk factor is aging such as Alzheimer's and Parkinson's disease. Two enzymes implicated in the pathogenesis of these diseases are the GSK-3b and the PDE7 respectively. It has been recently reported that two families of compounds (5-imino-1,2,4-thiadiazoles and quinazolines) are inhibitors of these enzymes.

Moreover, because the development of drugs for disease treatment requires many steps before the drug could be in the market, the evaluation of different pharmacokinetic properties at previous stages is recommended to avoid attrition in this process. Thus here we evaluated the determination of binding to the most abundant human protein in plasma (albumin) of these compounds. All these compounds showed a higher affinity to HSA. According to the results obtained by affinity chromatography and CD competition experiments, 5-imino-1,2,4-thiadiazoles bind to HSA with a high affinity, at a binding site other than the most characterized binding sites of HSA. Results obtained with quinazolines suggest that both site I and site II are involved in the binding site of these molecules. Both families of compounds showed an independent binding with the bilirubin site, suggesting that no interaction between these molecules and bilirubin

should be evidenced in vivo at this level. Overall the 5-imino-1,2,4-thiadiazoles and quinazolines families represent valuable leads compounds to be further studied in different preclinical assays as a possible treatment of diverse neurodegenerative diseases.

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Chapter 3

Species-dependent binding of tocainide analogues to albumin: affinity chromatography and circular dichroism study

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Abstract

A series of novel tocainide analogues were characterized for their HSA and RSA binding, by using high-performance liquid affinity chromatography (HPLAC) and circular dichroism (CD). In this HPLAC study, HSA and RSA were covalently immobilized to the silica matrix of HPLC columns, with a procedure that maintained unaltered the binding properties of the proteins. The tocainide analogues were ranked for their affinity to HSA and RSA on the basis of their bound fractions measured by the two albumin-based columns. This technique was also applied to characterize the high affinity binding sites of these tocainide analogues to the protein. For this purpose displacement experiments were carried out by means of increasing concentrations in the mobile phase of competitors known to bind selectively to the main binding sites of HSA. The results obtained with the immobilized proteins were confirmed by investigating the same drug-protein systems in solution by circular dichroism. The comparison of the data collected with both methodologies highlighted the dramatic effect of small differences in the amino acidic sequences of the two proteins. In fact, despite their similar primary and secondary structures, a small difference in the amino acidic sequence leads to significant differences in their three-dimensional structure reflecting their different binding capacity and their stereoselectivity. Therefore, this study confirms how it is crucial to consider the significant differences among the animal models when performing pharmacokinetic studies. It is also clear that the knowledge of serum carrier binding parameters at an early stage of drug discovery represents a great advantage that may help to save time and efforts.

3.1 Introduction

The early determination of the absorption, distribution, metabolism, excretion, toxicity (ADMET) parameters is fundamental in drug discovery and development [1,2]. Drug binding to plasma proteins strongly affects the pharmacokinetic parameters, because the actual volume of distribution and concentration of a drug are strictly related to its unbound fraction [3,4]. Human serum albumin (HSA) is the most abundant plasma protein and has an important role as carrier of exogenous and endogenous compounds and as depot of hydrophobic compounds [5,6]. The HSA binding can be altered by the concurrent administration of drugs that compete for common or linked binding sites, with a significant change of the free fraction, particularly for the highly bound drugs. Since this competition may result in toxic levels of free circulating drugs, any new drug candidate should be characterized for its affinity to the serum carrier as well as for its binding sites location.

Tocainide is an efficient class Ib antiarrhythmic drug targeting voltage gated sodium channels (VGSCs) [7,8]. Unfortunately, tocainide displays off-target activities that give rise to serious adverse side effects [9].

The tocainide structural framework was selected to create new potent and selective sodium channel blockers potentially useful in the treatment of myotonic syndromes. [10-12]. In a recent study, a three dimensional quantitative structure-activity relationship (3DQSAR) model was built to reveal the stereo-electronic parameters that govern the sodium channel block [13]. The resulting pharmacophore hypothesis suggested that the combination of the homologated alkyl chain, obtained constraining both the stereogenic centre and the terminal amine group of tocainide into a rigid pyrrolidine ring (corresponding to a β-prolinic derivative of tocainide, BO3), would result in a compound active in the sub micromolar range during use-dependent block. In the light of this evidence, and according to the suggestion deriving from the chemometric analysis previously reported [13], a new series of tocainide analogues bearing crucial modifications at the pharmacophore moieties were synthesized to identify novel compounds with a combined increase of both potency and use-dependent behaviour.

Preliminary pharmacological in vitro data were obtained by testing the effect of these compounds on sodium currents of adult skeletal muscle fibers measured by voltage clamp (data not shown) [14].

In a previous work, high performance affinity liquid chromatography (HPALC) and circular dichroism (CD) were used to study the binding of a small series of tocainide analogues to HSA [15]. The two techniques resulted well suited to determine the binding parameters, as previously reported by several authors [16-21]. In the present work the same techniques were employed to characterize the binding of a larger series of tocainide analogues to rat serum albumin (RSA) and the results were compared to those obtained using HSA as the target protein. The data were summarized in a model describing the binding sites location of the tocainide analogues on RSA, relatively to other drugs (ibuprofen, ketoprofen, diazepam) and bilirubin, which are better characterized for their binding sites on HSA. Since the binding of drugs to RSA is yet not fully characterized, the data obtained within this work might spread some light over the topography of the binding sites of this protein.

The interest in studying the binding of compounds to albumins from different species is related to the wide use of animals, and in particular rats, to perform activity and distribution experiments in vivo. A better knowledge of the binding of drugs to different albumins will be helpful to evaluate the reliability of extrapolating the experimental data obtained with animals to humans [22-25].

3.2 Experimental

3.2.1 Chemicals and instruments

Human serum albumin (HSA, essentially fatty acid free, A1887),rat serum albumin (RSA, essentially fatty acid and globulin free,A6414), l-tryptophan (l-Trp), glycine, N-benzoyl-dl-leucine, rac-warfarin, sodium valproate, rac-ibuprofen (IBU), rac-ketoprofen (KETO), rac-indoprofen, sodium salicylate (SAL), furosemide, phenylbutazone (PBU) and bilirubin (BIL) were purchased from Sigma–Aldrich (Milan, Italy). Diazepam (DZP) and rac-oxazepam were kindly provided by Professor Regina H. Costa Queiroz, Faculty of Pharmacy, University of San Paolo, at Ribeirao Preto (Brazil). KH_2PO_4, K_2HPO_4, and $(NH_4)_2SO_4$ powders were purchased from Carlo Erba (Milan, Italy). Other chemicals

were reagent grade from commercial suppliers and were used without further purification. Potassium phosphate buffers (PB) were prepared by adjusting the pH of solutions of K_2HPO_4 with equimolar solutions of KH_2PO_4.

The HPLC apparatus consisted of a Jasco PU-2089 plus (Jasco,Tokyo) HPLC pump, a Rheodyne injector system with a 20 µL loop and a Jasco MD-2010 plus (Jasco, Tokyo) diode array detector. The column was thermostated at 28°C with a Column Chiller Model 7955 (Jones Chromatography Ltd., UK). Buffers were filtered through 0.22 µm mixed cellulose esters filter membranes (Millipore, Milan, Italy). The mobile phases were degassed prior to use by ultrasonic waves for at least 15 min.

CD measurements were performed with a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature control system set at 25°C. The spectra were recorded using 1 cm pathlength cell at a scan rate of 20 nm/min, time constant 2 s, resolution 0.2 nm, and spectral bandwidth 1 nm.

3.2.2 Tocainide analogues, synthesis and physico-chemical properties

Synthesis and physicochemical characterization of the tocainide (Toc) analogues BO1, BO2, BO3, Toc (Tocainide), BO5, BO6, BO7,BO8, BO9, BO10, BO11, BO12, BO13, BO14 have been described elsewhere [14,15]. The structures of the compounds are reported in Fig. 3.1.

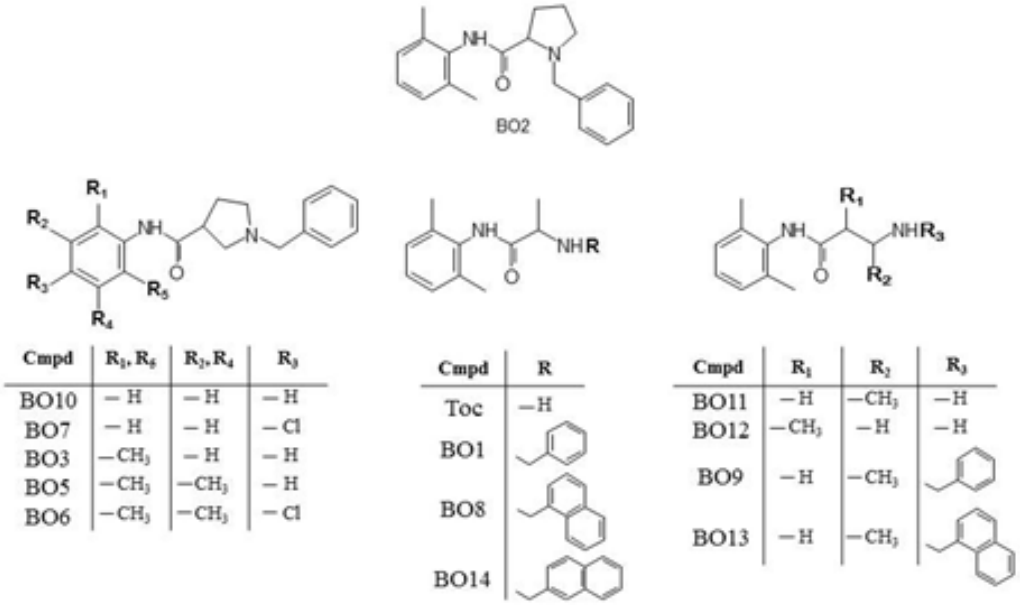

Fig. 3.1 Structures of tocainide and its analogues.

3.2.3 Columns preparation

The silica monolithic columns (50 mm × 4.6 mm i.d.), 20 nm mesopores, were silanized by an in situ process as previously reported [15] with slight modification. In brief, the monolithic columns encased in a PEEK plastic cover were dried at 120°C for 6 h under helium. After cooling, the columns were flushed with dry toluene (10 mL) at 0.5 mL/min. The columns were then heated up to 100°C, and derivatized in situ by pumping through a solution of 3-glycidoxypropyltrimethoxysilane (10 mL in 90 mL of toluene) at 0.5 mL/min to give an epoxide silica column. After cooling the columns were washed with anhydrous toluene (10 mL) and anhydrous acetonitrile (20 mL) at 0.5 mL/min.

HSA and RSA were covalently immobilized online on the monolithic epoxy-silica columns following a previously reported procedure [26]. In brief, either HSA or RSA solutions (10 mg/mL of protein in PB (50 mM, pH 6.5), 1 M ammonium sulphate) were circulated through the epoxy-silica columns in closed circuit for 24 h. The columns were washed with 120 mL of PB (50 mM, pH 7.4), and 100 mL of 1 M glycine dissolved in PB (50 mM, pH 7.4). The columns were then washed with PB (67 mM, pH 7.4)/1-propanol 95:5 and stored at 4°C until use. The two albumin-based columns were employed for the determination of the bound fractions of the studied compounds. The RSA-based column was also used for the displacement studies on the compounds under investigation.

3.2.4 Columns characterization

The columns were characterized in terms of amount of albumin correctly immobilized by the frontal analysis of l-tryptophan as previously reported [26,27] for the HSA-based column and of lidocaine for the RSA-based column. A series of solutions of l-Trp in PB (67 mM, pH 7.4) ranging from 12.5 µM to 100 µM (HSA column), and of lidocaine in PB (67 mM, pH 7.4) ranging from 5 µM to 50 µM (RSA column), were continuously applied to the columns as mobile phases. The relevant breakthrough volume ($V - V_0$) of each solution, where V is the breakthrough volume and V_0 is the system void volume, was determined and the following linear equation was applied [27]:

$$\frac{1}{m_{L_{app}}} = \frac{1}{K_A \cdot m_L \cdot [analyte]} + \frac{1}{m_L} \qquad (3.1a)$$

or

$$\frac{1}{(V-V_0)\cdot[analyte]} = \frac{1}{K_A \cdot m_L} \cdot \frac{1}{[analyte]} + \frac{1}{m_L} \quad (3.1b)$$

where [analyte] is the molar concentration of L-tryptophan or lidocaine, $m_{L_{app}}$ represents the moles of analyte required to reach the mean position of the breakthrough curve at a given concentration of applied analyte (so it is equal to $(V - V_0) \times [L - Trp]$), K_A is the association equilibrium constant of the analyte for the binding to its single site, and m_L represents the moles of binding sites available for the binding of L-Trp or lidocaine. The equation assumes that the analyte binds to a single site on the protein and that bound and free species are constantly in equilibrium (i.e. the association and dissociation kinetics of the complex are sufficiently fast). If the equation is satisfied (i.e. the relationship is linear) m_L is equal to the moles of correctly immobilized proteins present in the columns [27]. The y-axis intercept corresponds to the reciprocal of the moles of immobilized protein, while the ratio of the intercept to the slope yields the K_A of the analytes. These affinity values may be compared to the data present in literature as a first evaluation of the goodness of the immobilization.

A series of racemates reported to bind the proteins in a stereoselective manner (rac-oxazepam, rac-warfarin, rac-indoprofene and N-benzoyl-DL-leucine) were used to check the dependability of the albumin-based columns. Stock solutions of the racemates prepared 0.1 mg/mL in 1-propanol were diluted with PB (67 mM, pH7.4) and injected into the columns. Mobile phases were mixtures of PB (67 mM, pH 7.4)/1-propanol.

3.2.5 Bound fraction determination

Stock solutions (1 mM) were prepared in 1-propanol. Working solutions were prepared just before injection into the columns, by diluting the stock solutions with PB (67 mM, pH 7.4). The concentration of working solutions was 20 µM, but it has been increased up to 30 µM when peak broadening (due to high retention into the column) combined with weak UV absorbance lowered the signal to noise ratio below 3.

The chromatographic retention was reported as the capacity factor (k') and its logarithm (log k'), where k' is defined as

$$k' = \frac{t_{drug} - t_0}{t_0} \quad (3.2)$$

(t_{drug}= retention of the solute; t_0= retention of an unretained solute). Samples were analyzed in duplicate or in triplicate. The flow was set at 1 mL/min. The k' values were determined in absence or presence of 1-propanol, added to the mobile phase at several concentrations. Mobile phases were composed of PB (67 mM, pH7.4)/1-propanol ranging from 90:10 to 100:0. The retention values were plotted as log k versus the organic modifier concentration. The bound fraction (B%) was determined from the capacity factor k' measured in absence of 1-propanol, according to a previously published method [28].

$$B\% = \frac{k'}{k'+1} \cdot 100 \quad (3.3)$$

The method should be validated by analysing drugs with known bound fraction to the protein.

3.2.6 Displacement experiments

The capacity factor k' of the studied compounds was measured in presence of increasing concentrations in the mobile phase (PB 67 mM, pH 7.4) of sodium salicylate (SAL), rac-ibuprofen (IBU) and sodium valproate (VAL), which are markers of Sudlow's site I, II and the bilirubin site, respectively on HSA. The concentration of the marker in the mobile phase ranged from 0 to 80 µM for SAL, from 0 to 20 µM for IBU and from 0.25 to 1.5 mM for VAL. All the compounds were injected at the same concentration used for the determination of the bound fraction described in Section 3.2.5.

By plotting the reciprocal of the capacity factor (1/k') against the molar concentration of the marker, the relationships between the analyte and the marker can be determined: direct competition at a single site yields a straight line with a positive slope, whereas anon-competitive relationship lacks of correlation [3,16,29]. In the

simplest case of direct competition, where the analyte A has a single binding site, the following linear equation is satisfied [17]:

$$\frac{1}{k'_A} = \frac{V_M \cdot K_I \cdot [I]}{K_A \cdot m_L} + \frac{V_M}{K_A \cdot m_L} \quad (4)$$

where kA' is the measured capacity factor of the analyte A, V_M is the column void volume, K_I is the association equilibrium constant of the displacer I for the single binding site of A, [I] is the molar concentration of the displacer, K_A is the association equilibrium constant of A for its binding site, and m_L is the total moles of binding sites of A in the column. It follows that the equilibrium association constant of the displacer for the analyte binding site, KI, can be calculated by linear fitting of the data from the ratio of the slope to the intercept. Deviations from this linear behaviour can be seen for multisite binding.

3.2.7 Circular dichroism: displacement experiments

The displacement experiments were carried out using compounds BO3 and BO14 as the competitors, and phenylbutazone (PBU), diazepam (DZP), rac-ketoprofen (KETO) and bilirubin (BIL) as markers for Site I, Site II and bilirubin binding site, respectively. The change in the CD signal after addition of different amounts of compounds BO3 and BO14 to a solution containing equimolar concentrations of RSA and marker [15 µM in PB (pH 7.4; 67 mM)] were monitored. Stock solutions of compounds BO3 and BO14 (10 mM in 1-propanol), PBU (15 mM in 1-propanol), DZP (15 mMin 1-propanol) and RSA (150 µM in PB, pH 7.4, 67 mM) were prepared immediately before the experiments. The stock solution of BIL (1 mL) was prepared by dissolving the powder in KOH (1/5 of the final volume), then adding sequentially K_2HPO_4 0.1 M (1/5 of the final volume) and water (3/5 of the final volume). The final concentration of BIL stock solution was 7.5 mM and was prepared immediately before use. All the measurements were carried out using a 10 mm path length cell. The working solutions were prepared by mixing RSA, marker and displacers at molar ratios from1:1:0 up to 1:1:10.

3.3 Results and discussion

3.3.1 Column characterization

The HSA- and RSA-based columns were characterized by frontal analysis in order to determine the amount of protein immobilized onto the column. The plot of the bound analyte versus the reciprocal of its concentration (see Section 2.4) was used to determine the amount of albumin correctly anchored to the silica matrix, and the affinity constants of the analytes for the albumins. As previously observed, L-Trp showed to bind to a single site on albumin (r^2= 0.9988) [27] with a K_A of 0.99 (±0.15) × 10^4 M^{-1},in agreement with the value of 1 × 10^4 M^{-1} reported in the literature [5]. Lidocaine demonstrated to bind to a single site on RSA (r^2= 0.9985) as previously observed for HSA [30]. The K_A for lidocaine binding to RSA was 1.21 (±0.20) × 10^4M^{-1}, which was consistent with the relatively weak binding reported earlier for HSA [30]. The amount of immobilized albumin was 18.4 (±2.3) mg for the HSA column and 20.2 (±3.0) mg for the RSA column. Considering an amount of silica of about 0.25 g per column the amount of protein immobilized resulted in about 74 mg/g silica and 81 mg/g silica for HSA and RSA, respectively.

By injecting racemates of drugs or amino acids reported to bind the protein in a stereoselective manner (rac-ketoprofen, rac-ibuprofen, rac-indoprofen, rac-oxazepam, rac-warfarin and N-benzoyl-DL-leucine) we also checked that the immobilization procedure preserved the native binding properties of the proteins. In fact the enantioresolution was obtained for all the analyzed racemates (see, as an example, Fig. 3.2).

3.3.2 Bound fraction determination

Tocainide and its analogues were injected into the columns containing HSA and RSA covalently immobilized. The ranking of the tocainide analogues for their HSA and RSA bound fractions was performed on the basis of the capacity factor, k', as determined in aqueous solution (Table 1). Only the enantiomer with higher affinity was considered for racemic compounds that showed enantioselectivity in the binding to the protein. The dependability of the columns for ranking the new synthesized compounds

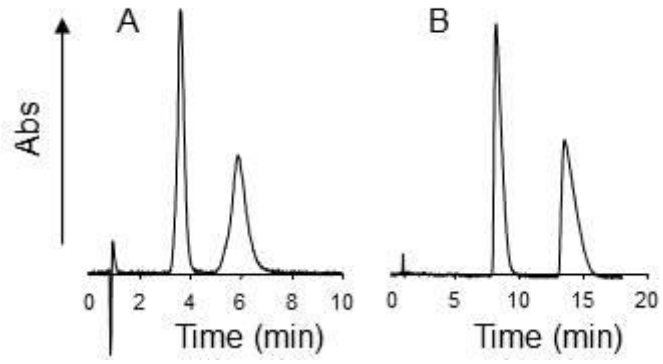

Fig. 3.2 (A) Chromatographic enantioresolution of rac-warfarin 0.01 mg/mL on HSA column; (B) chromatographic enantioresolution of rac-indoprofen 0.04 mg/ml on RSA column. Mobile phases were phosphate buffer (pH 7.4; 67 mM)/1-propanol (92:8, v/v) and (98:2, v/v), respectively. The runs were monitored at λ = 308 nm (rac-warfarin), and at λ = 260 nm (rac-indoprofen).

for their HSA and RSA binding was checked by analysing in the same experimental conditions some compounds, whose bound fraction to the serum proteins was already known. A quite good agreement was obtained in the comparison of the bound fraction of the selected drugs between experimental and literature binding data. As an example, the lower affinity (bound fraction) of diazepam to immobilized RSA compared to the immobilized HSA matched the different affinity for the two proteins measured in solution [22].

All the tocainide analogues showed a higher affinity to HSA and RSA when compared to the parent compound, Toc (tocainide,Table 3.1), and, among all the analogues, compound BO6 exhibited the highest affinity (bound fraction) to both the albumins. Tocainide (Toc), BO11 and BO12 have log P values significantly lower with respect the other analogues, thus suggesting the importance of the hydrophobicity as a parameter affecting the binding process. As already underlined in a previous study, there is a general tendency for the analogues with higher log P to bind tighter to albumin [21]. However the lack of a simple linear correlation, suggests that the overall lipophilicity is not the only parameter affecting the binding of these molecules to albumin (Table 3.1). The constriction of the amino group into a rigid pyrrolidine moiety, and the nature of the aromatic moieties, affects the affinity, each structural part giving a different contribution to the binding to the two proteins. As whole, significant differences were

Table 3.1
Ranking and bound fraction (B%) data obtained with HSA and RSA by affinity chromatography compared with literature data and predicted log P values.

Sample	Ranking HSA	Ranking RSA	B% HSA	B% RSA	Log P[a]	B% HSA Lit.
BO1	11	11	88.3	81.5	3.11 ±0.34	89.1[b]
BO2	10	8	N.D.	95.4[d]	3.42 ±0.40	94.5[b]
BO3	5	9	97.2	93.6	3.03 ±0.36	97.1[b]
Toc	14	14	N.D.	36.0	1.04 ±0.49	25.4[b]
BO5	3	4	98.2	96.7	4.00 ±0.45	98.1[b]
BO6	1	1	N.D.	99.1[d]	4.57 ±0.47	99.3[b]
BO7	2	2	98.3	97.9	3.39 ±0.07	98.3[b]
BO8	8	5	95.4	96.9[d]	4.22 ±0.31	-
BO9	9	10	95.3	87.9	3.34 ±0.39	-
BO10	4	7	N.D.	95.6	2.80 ±0.07	97.7[b]
BO11	13	12	34.7	43.5	1.28 ±0.52	-
BO12	12	13	38.0	43.5	1.24 ±0.52	-
BO13	6	6	96.7	96.0	4.42 ±0.41	-
BO14	7	3	95.8[d]	97.7	4.22 ±0.31	-
R-warfarin	-	-	98.3[b]	97.2	-	99±1[c]
S-warfarin	-	-	98.7[b]	98.4	-	98.6±0.4[c]
Furosemide	-	-	N.D.	98.8	-	93.5[b]
Diazepam	-	-	94.8	89.5	-	98.7±0.2[c]

N.D. Not determined.
a [35].
b [15].
c [36].
d Higher affinity enantiomer.

observed in the binding of tocainide and its analogues to HSA and RSA (Table 1), being the binding to HSA generally stronger than to RSA. These differences were even more impressive when the free fractions were considered instead of the bound fractions

(Table 3.2). However some similar patterns for the affinity to the two proteins driven by the type of substitution were recognized. In particular, the 1C-elongation of the side chain (Toc → BO11 and BO12) showed only a slightly higher binding to both HSA and RSA than the parent compound (Toc) while the substitution of the amino group with an aromatic moiety (Toc → BO1, BO8 and BO14) dramatically increased the binding to both the albumins. The combination of chain elongation and amino substitution had an additive effect when the aromatic was a benzyl group (BO1 → BO9) while the elongation gave a much lower contribution when the aromatic was a naphthyl group (BO8 → BO13), for binding to HSA and RSA. Conversely, when the amino group was constricted into a rigid ring, the chain elongation, obtained substituting the α-proline side chain with a β-proline (BO2 → BO3), gave a contribution only on the binding to HSA. Interestingly the substitution of a α-naphthyl to a β-naphthyl on the amino group (BO8 → BO14) gave opposite effects on the binding to HSA and RSA, decreasing the affinity to the former and increasing the affinity to the latter. Since the actual volume of distribution and the concentration of a drug at the receptor site is related to the drug free fraction [3,4], the characterization of the binding process is essential to understand the pharmacokinetic, pharmacodynamics, and toxicological profile of the drug. This aspect is particularly relevant for relatively highly albumin bound drugs, where small changes in the bound fractions have a significant impact on the free fractions. As an example, the bound fractions of BO5 are 98.1% for HSA and 96.7% for RSA; this corresponds to 1.7-fold difference in the free fraction of the same compound for the two albumins. Analogously we have a 2.6-fold free fraction of BO3 with RSA while we have a 2.1-fold free fraction of BO14 with HSA.

Thus these data support the importance of collecting drug binding data with albumins from different species, to ensure the reliability of extrapolating to men distribution data obtained on animals in preclinical studies.

It is known that the presence of an organic solvent may affect the drug–protein binding process, either by triggering a general change in the secondary or tertiary structure of the protein, and/or modifying the microenvironment at the binding site(s) so that low energy interactions (hydrophobic and dipole–dipole interactions, coulombic forces, hydrogen bonds) cannot take place. A careful observation of the variation of log k' values of the tocainide when changing the propanol concentration in the mobile

Table 3.2

Comparison between the free fraction (F%) obtained with HSA and RSA by affinity chromatography.

Sample	F% HSA	F% RSA	F% RSA/HSA
BO1	11.7	18.5	1.64
BO2	5.5[a]	4.6[b]	0.84
BO3	2.8	6.4	2.27
Toc	74.6 [a]	64.0	0.86
BO5	1.8	3.3	1.73
BO6	0.67	0.87[b]	1.30
BO7	1.7	2.1	1.26
BO8	4.6	3.1[b]	0.67
BO9	4.7	12.2	2.56
BO10	2.3 [a]	4.4	1.90
BO11	65.3	56.5	0.86
BO12	62.0	56.5	0.91
BO13	3.3	4.0	1.21
BO14	4.2[b]	2.3	0.55

[a] [15].
[b] Higher affinity enantiomer.

phase (Fig. 3.3) revealed some particular features: in presence of propanol all compounds showed a nearly linear relationship between log k' and the organic solvent concentration, while without propanol the log k' values suddenly increased without respecting this linear relationship (Fig. 3.3). Interestingly the binding of some of the compounds seemed to be slightly more affected by the presence of 2%propanol, than others. This behaviour allowed a subdivision of the compounds into two groups: the compounds whose binding was less sensitive to the presence of propanol (Fig. 3.3A) and those whose binding was more sensitive to the presence of the organic solvent (Fig. 3.3B). All the compounds bearing a β-proline ring (BO3, BO5,BO6, BO7, BO10) and BO9 belonged to the group more sensitive to the presence of propanol, while all the

others (BO1, BO2, Toc, BO8,BO11, BO12, BO13, BO14) and DZP showed a lower sensitivity. A strikingly similar variation of log k' passing from 0 to 2% of propanol was exhibited by all the compounds belonging to the same group (Fig. 3.3A and B). Besides, a significant difference was observed in the behaviour of the two groups (Fig. 3.3C), even though this difference was extremely low. This strikingly similar sensitivity to propanol between molecules, which was already observed for some HIV protease inhibitors binding to HSA [26], clearly indicate that the molecules belonging to the same group share common properties related to their binding to the protein and that this property is affected by the presence of organic solvent. The sensitivity to the organic solvent may be indicative of the solvent accessibility to the binding site(s), the overall pattern of bonds created between the molecules and the protein, small conformational changes of the protein at the binding site level, or all of them at the same time. Thus molecules showing the same sensitivity might share the same binding site(s) and the same type of bonds. This hypothesis, supported by the results obtained from the displacement experiments (see next section), suggests that the compounds within the same group bind to the same region(s) on the protein, and that they have one or more binding properties that differs from the molecules of the other group.

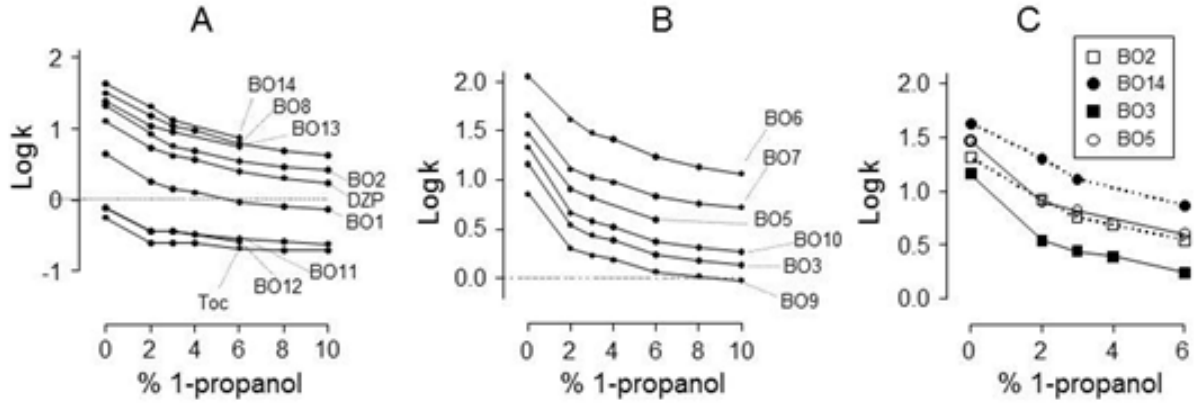

Fig. 3.3 Relationship between log k' values of diazepam and tocainide analogues obtained with the RSA-based column at various concentrations of 1-propanol in the mobile phase. (A) Compounds showing a binding less sensitive to the presence of 2% organic solvent in the mobile phase. (B) Compounds showing a binding more sensitive to the presence of 2% organic solvent in the mobile phase. (C) Comparison between the profiles of log k' versus propanol concentration of selected compounds belonging to the two different groups; the picture highlights the similar slope between 0 and 2% of organic solvent showed by compounds belonging to the same group and the difference to the other group: the similar higher slope showed by BO3 and BO5 compared to BO2 and BO14, indicate a similar higher reduction of affinity caused by the organic solvent for the formers compounds and a similar lower reduction of affinity for the latters.

3.3.3. Albumin binding site investigation by displacement affinity chromatography

In a previous work, displacement affinity chromatography and CD experiments carried out for some of tocainide analogues showed a non-cooperative binding at site I and site II and an almost independent binding at bilirubin binding site on HSA [21]. Thus these sites are not the primary binding site on HSA for the tocainide analogues studied. The same chromatographic investigation was carried out in this work for their binding to RSA, by determining the capacity factor (k') of each compound upon increasing concentrations of selected competitors added to the mobile phase. The analysis of the chromatographic data allowed the affinity constant of the competitors for the binding site(s) of the analytes (K_I) to be determined (see Section 3.2). The competition studies were performed using the same system described for the bound fraction determination. The selected displacers were the same used for HSA, i.e., sodium salicylate (SAL, site I), rac-ibuprofen (IBU, site II) and sodium valproate (VAL, site III, known also as bilirubin site). The displacements of some of the analyzed compounds (BO1, BO7, BO8, BO10, BO13, BO14) in the presence of increasing concentration of SAL are reported in Fig. 3.4 and in Table 3.3. The retention of tocainide analogues decreased when adding SAL in the mobile phase up to 75 µM. The K_I value for SAL, as determined on the basis of its competition effect, was always much lower than that expected in case of a direct competition (Table 3.3). Indeed the K_I measured when (S)-WAR, (R)-WAR and furosemide, which are supposed to bind to site I on RSA [31] were used as the analyte was in the order of 10^4 M^{-1}. This behaviour suggests an allosteric competition with a non-cooperative binding of tocainide analogues with SAL. A similar situation was observed when IBU was used as the competitor. The displacement of compounds BO8, BO13 andBO14 in the presence of increasing concentration of IBU is shown in Fig. 3.5A. A retention decrease upon increasing concentrations of IBU in the mobile phase was observed for the analyzed compounds. In all the cases, the affinity constant calculated for the binding of IBU to the compounds binding site(s) (K_I) was relatively low, compared to the expected value in the order of 10^6 M^{-1} [31] that should have been obtained in the case of a direct competition for the primary binding site of IBU (Table 3.3). However, the presence of secondary binding sites for IBU on RSA with a K_A in the order of 10^4 M^{-1} has been previously suggested [31], thus the K_I values obtained are consistent with either an anticooperative binding to the primary binding site of IBU or

with a direct competition to one or more secondary binding sites of IBU. In this latter case the small displacement monitored would be the effect of the small fraction of IBU that binds the secondary site(s) in the range of concentrations used in the experiments. These results also confirm that DZP binds to a site different from the primary binding site of IBU, as previously suggested [31,32]. Interestingly, the compounds belonging to the group more sensitive to the presence of propanol showed a K_I lower than the K_I measured for the components of the other group (Table 3.3), thus supporting the hypothesis that the compounds belonging to the same group have the same binding properties. To summarize, these results suggest that both Site I and the primary binding site of IBU are not primary binding sites for the analyzed compounds. However, further experiments by CD (see later in this section) confirm the differences between profens and benzodiazepines binding sites on HSA and RSA. Finally, insignificant changes of the retention times were observed when valproate was used as the displacer up to 1.5 mM for most of the analogues (Fig. 3.4B). Very low K_I values were obtained only when compounds BO8 and BO14 were used as the analytes (Table 3.3). Thus, an almost independent binding should be suggested for tocainide analogues when VAL was used as selective marker of the bilirubin binding site.

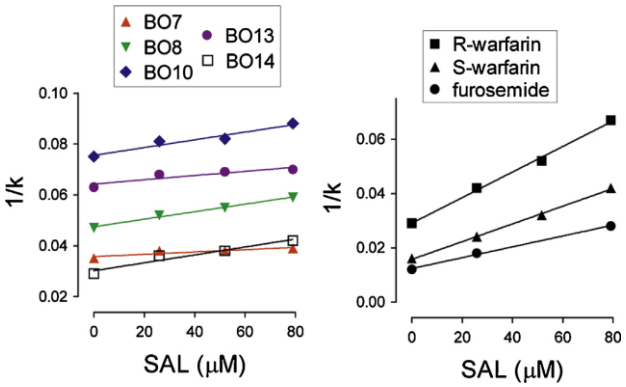

Fig. 3.4 Affinity chromatography displacement experiments carried out in the presence of increasing concentration of salicylate (SAL) on the RSA column. (left) Displacementprofiles of compounds BO7, BO8, BO10, BO13, BO14. (right) Displacement profiles of standard compounds.

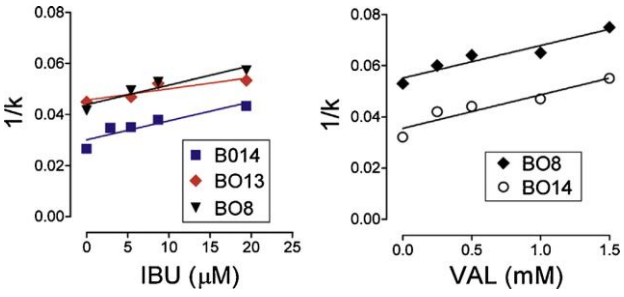

Fig. 3.5 Affinity chromatography displacement experiments on the RSA column. (left) Displacement profiles of compounds BO8, BO13 and BO14 in the presence of increasingconcentration of rac-ibuprofen (IBU). (right) Displacement profiles of compounds BO8 and BO14 in the presence of increasing concentration of valproate (VAL).

Table 3.3
The competitor affinity values (KI) values obtained by displacement chromatography analysis.

Sample	K_I Salicylate	K_I Ibuprofen	K_IValproate	1-propanol sensitivity
BO1	2.75×10^3	1.30×10^4	Not significant	–
BO2	N.D.	2.70×10^4	N.D.	–
BO3	N.D.	5.35×10^3	Not significant	+
Toc	N.D.	N.D.	N.D.	–
BO5	N.D.	7.39×10^3	Not significant	+
BO6	N.D.	N.D.	N.D.	+
BO7	1.27×10^3	4.27×10^3	Not significant	+
BO8	3.12×10^3	1.47×10^4	2.31×10^2	–
BO9	N.D.	5.36×10^3	Not significant	+
BO10	2.01×10^3	Not Significant	Not significant	+
BO11	N.D.	N.D.	N.D.	–
BO12	N.D.	N.D.	N.D.	–
BO13	Not significant	9.69×10^3	Not significant	–
BO14	5.16×10^3	2.49×10^4	3.69×10^2	–
Diazepam	1.76×10^3	2.21×10^4	1.52×10^2	–
R-warfarin	1.62×10^4	N.D.	1.79×10^3	
S-warfarin	2.1×10^4	N.D.	2.73×10^3	
Furosemide	1.62×10^4	N.D.	1.45×10^3	

3.3.4. Albumin binding site investigation by circular dichroism

Competition experiments were also carried out with RSA in solution by CD spectroscopy. The experiments consisted in monitoring the change of the induced CD spectrum observed for a [RSA]/[marker] complex 1:1 (15 μM) in the presence of increasing concentrations of the competitors. In general CD competition experiments for protein binding studies can be carried out using competitors known to bind to specific binding sites, as it was done for the affinity chromatography experiments. Alternatively the compound under investigation can be used as the competitor, and analyzed against specific markers known to bind to specific binding areas. In our experiments compounds BO3 and BO14, selected as representative of each group of the tocainide analogues, were used as the competitors. The strategy of the experiment is determined by the spectroscopic characteristics of the involved compounds, being the measurements based on the monitoring of the induced CD signal in the absence and in the presence of the competitor [18]. In order to make easier the analysis of the data, the marker should present absorption and an induced CD signal at lower energy with respect to the absorption of the competitor and of the protein [18]. The markers employed were phenylbutazone (PBU, selective binding to site I on HSA), diazepam and rac-ketoprofen (DZP, KETO, selective binding to site II on HSA) and bilirubin (BIL, selective binding to bilirubin site).

The addition of increasing concentrations of compounds BO3 and BO14 up to a molar ratio [competitor]/[marker] 8/1 determined a blue-shift of the induced CD spectrum of [RSA]/[PBU] 1/1 complex (Fig. 3.6). This result suggests an allosteric interaction between the site I marker and competitors, without a significant displacement of the marker, consistently to the results obtained by affinity chromatography. When rac-ketoprofen (KETO) was used as the marker, the induced CD spectrum of [RSA]/[KETO] 1/1 complex was unaffected by the presence of BO3 up to [competitor]/[marker] 10/1 molar ratio, while the presence of compound BO14 decreased the intensity of the induced CD signal proportionally to the added concentration, indicating a direct competition of BO14 for the stereoselective binding site of ketoprofen (Fig. 3.7). Both BO3 and BO14 were able to partially displace diazepam from its stereoselective site (Fig. 3.8). In the case of BO3, the maximum displacement was obtained at [competitor]/[marker] 8/1, while in the case of BO14 a similar maximum displacement

was obtained at [competitor]/[marker] 1/1. The higher displacement efficiency showed by BO14 might reflect its higher affinity, as suggested by the higher bound fraction measured in the affinity chromatography experiments. However, since the small induced CD signal characteristic of the complex [DZP]/[RSA] 1:1 is significantly affected by background noise, this information should be considered only qualitative. The different competition observed at the profens and benzodiazepines binding sites indicated that the site II on RSA is different from that of HSA, with a probable different location of diazepam and ketoprofen binding sites in the same binding area [32]. To better clarify the binding site locations, rac-ibuprofen (IBU) was tested as competitor against [RSA]/[DZP] and [RSA]/[KETO] 1:1 complexes. IBU showed to competitively displace diazepam from its stereoselective binding site and the induced CD signal of [RSA]/[DZP] complex at 320 nm was null at [competitor]/[marker]4:1 (Fig. 3.9A). At the same time a small induced CD signal of the complex [RSA]/[IBU] proportional to the concentration of IBU added to the solution became evident at wavelengths below 305 nm, especially at higher concentrations of IBU (Fig. 3.9B). Considering that the K_A of IBU for its primary site [31] is about 40-fold higher than the K_A of DZP for its stereoselective binding site [22], a much more efficient displacement was expected in case of a direct competition for the binding at the same primary site. Thus these results are consistent with the partial displacement of DZP observed when IBU was used as competitor in the affinity chromatography displacement experiments. When added to the complex [RSA]/[KETO] 1:1 up to [competitor]/[marker] 2:1, IBU showed only a small effect on the stereoselective binding of KETO, while a further addition up to [competitor]/[marker] 4:1 was responsible of a significant displacement of KETO from its stereoselective binding site that continued in a competitive trend upon further increase of the concentration of IBU (Fig. 3.9C). However, even the addition of [competitor]/[marker] 12:1 did not displace completely ketoprofen from its stereoselective binding site. As observed for the displacement of DZP, a small induced CD signal of the complex [RSA]/[IBU] proportional to the concentration of IBU was visible at wavelengths below 305 nm. These results suggest that ketoprofen and ibuprofen do not share a common primary binding site on RSA, but they have still a binding site in common, drawing a furthermore complex picture of the location of profens/benzodiazepines binding sites on this albumin.

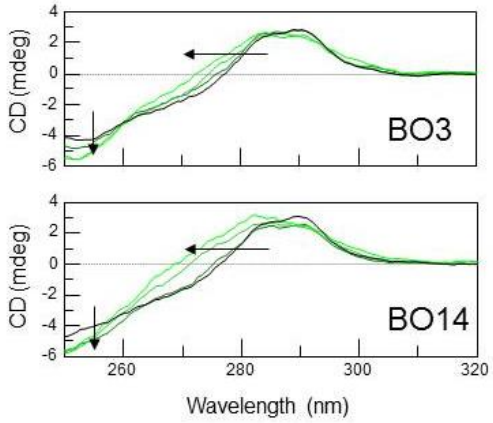

Fig. 3.6 Blue-shift of the induced CD spectrum of phenylbutazone (PBU) bound to RSA (15 µM) upon addition of increasing concentration of compounds BO3 (top) and BO14 (bottom) to the [RSA]/[PBU] complex at stoichiometry [RSA]/[PBU]/[competitor]: 1/1/0 (black), 1/1/1, 1/1/4, 1/1/8, green colour scale. The arrows indicate the direction of the shift caused by the increase of competitor concentration.

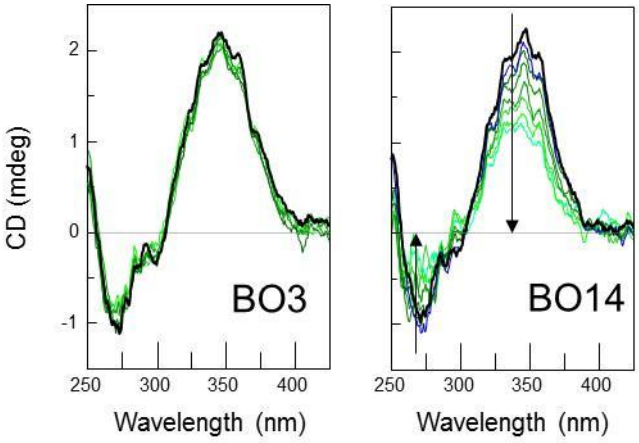

Fig. 3.7 Effect on the induced CD spectrum of ketoprofen (KETO) bound to RSA (15 µM) upon addition of increasing concentration of compounds BO3 (left) and BO14 (right) to the [RSA]/[KETO] complex. Stoichiometry of the [RSA]/[KETO]/[competitor] complexes are: 1/1/0 (black), 1/1/1 (blue), 1/1/2, 1/1/4,1/1/6, 1/1/8, green colour scale, for BO3 and 1/1/0 (black), 1/1/0.5 (blue), 1/1/1, 1/1/2,1/1/4, 1/1/6, 1/1/8, 1/1/10, green colour scale, for BO14. The arrows indicate the direction of the shift caused by the increase of competitor concentration.

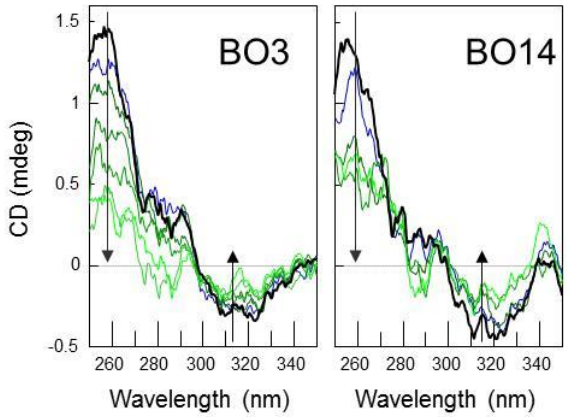

Fig. 3.8 Effect on the induced CD spectrum of diazepam (DZP) bound to RSA (15 µM) upon addition of increasing concentration of compounds BO3 and BO14 to the [RSA]/[DZP] complex. Stoichiometry of the [RSA]/[DZP]/[competitor] complexes are:1/1/0 (black), 1/1/0.5 (blue), 1/1/1, 1/1/2, 1/1/4, 1/1/8, 1/1/10, green colour scale, for BO3 and 1/1/0 (black), 1/1/0.5 (blue), 1/1/1, 1/1/6, 1/1/8, green colour scale for BO14. The arrows indicate the direction of the shift caused by the increase of competitor concentration.

Finally, a contemporary reduction of the intensity and a blue-shift were observed in the induced CD spectrum of BIL upon addition of both compounds BO3 and BO14 up to a [competitor]/[marker] 8/1 and 10/1 molar ratios, respectively (Fig. 3.10A), followed by a small but linear reduction of the UV of BIL (Fig. 3.10B). Differently from the complex HSA/BIL, where only the intensity of the induced CD signal change when varying the

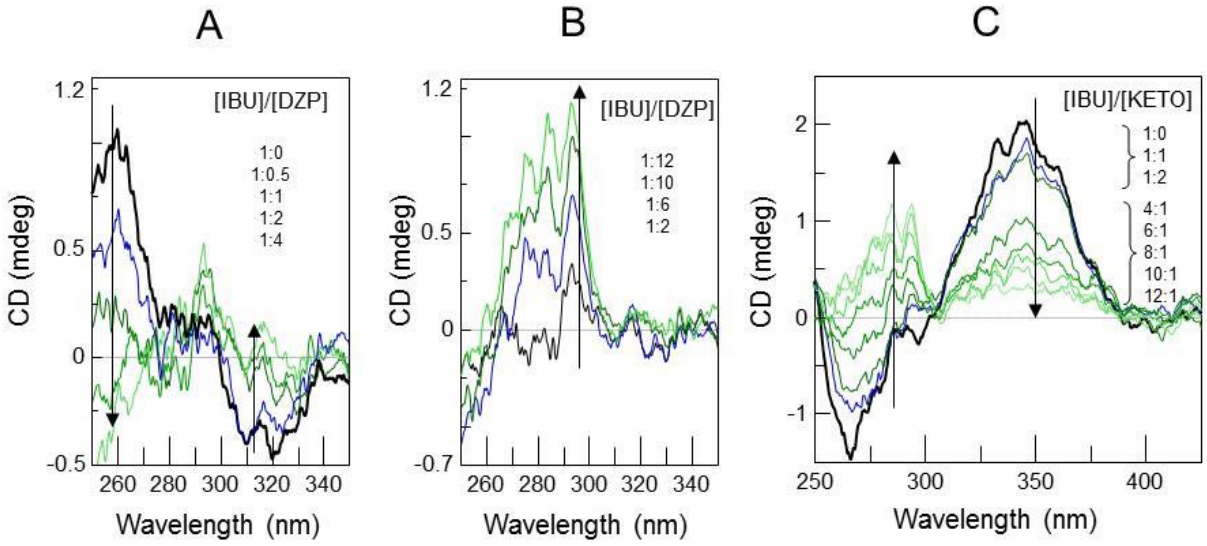

Fig. 3.9 Effect on the induced CD spectra of diazepam (DZP, A, B) and ketoprofen (KETO, C) bound to RSA (15 µM) upon addition of increasing concentration of rac-ibuprofen (IBU) to the [RSA]/[marker] complex. The arrows indicate the direction of the shift caused by the increase of competitor concentration. (A) The induced CD signal of the complex [RSA]/[DZP] disappeared completely at [IBU]/[DZP] 4:1. (B) A further increase of IBU concentration on the [RSA]/[DZP] complex revealed the induced CD spectrum of [IBU]/[RSA] complex at wavelengths below 305 nm. (C) IBU did not show a significant displacement of KETO at [IBU]/[KETO] up to 2:1, while higher concentrations of IBU caused a significant reduction of CD signal of [RSA]/[KETO] with the contemporary increase of the induced CD of [IBU]/[RSA] complex at wavelengths below 305 nm.

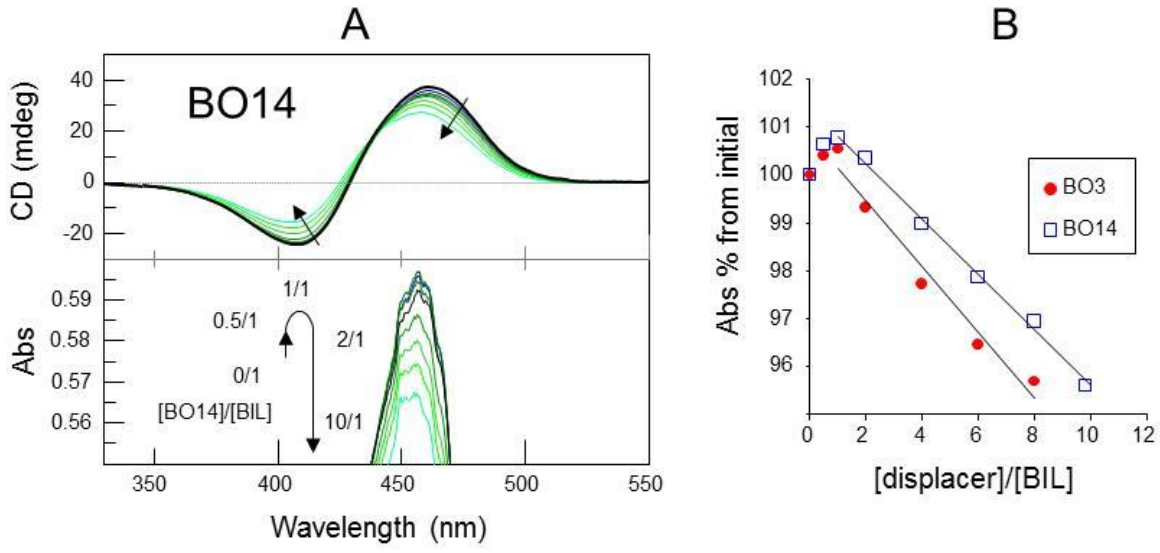

Fig. 3.10 (A) Effect on the induced CD (top) and UV (bottom) spectra of bilirubin (BIL) bound to RSA upon addition of increasing concentration of compound BO14 to the [RSA]/[BIL] complex. The arrows indicate the direction of the shift caused by the increase of competitor concentration. Stoichiometry of the [RSA]/[BIL]/[BO14] complexes:1/1/0, 1/1/0.5, 1/1/1, 1/1/2, 1/1/4, 1/1/6, 1/1/8, 1/1/10. (B) Change in the UV signal of bilirubin upon addition of increasing concentration of BO3 and BO14.

complex concentration, the induced CD spectrum of the complex RSA/BIL shows a change of both intensity and position of the spectrum, probably reflecting the presence of multiple stereoselective binding sites. Furthermore the addition of IBU to the complex RSA/BIL cause a similar effect on the CD spectrum to those observed with BO3 and BO14 [33]. Taking all these information together, the effect of BO3 and BO14 on RSA/BIL complex might reflect the direct competition of the two tocainide analogues to a secondary stereo-selective binding site of BIL that could be also a binding site for IBU. Alternatively, the same effect could be ascribed to an allosteric interaction that affects the protein conformation at the bilirubin site level, decreasing its affinity and/or modifying the bound conformation.

3.3.5. Tocainide analogues binding location

From displacement experiments by both affinity chromatography and CD it appears clear that the binding site of SAL and PBU, which should be located on subdomain IIA of RSA [31] is not a binding site of tocainide analogues. The results obtained with the markers of site II and bilirubin site of HSA describe a much more complex pattern of interactions between tocainide analogues, profens, diazepam and bilirubin on RSA, much different from that of HSA. An attempted model for the binding sites of tocainide analogues, ibuprofen, diazepam, ketoprofen and bilirubin that summarize the results described in this paper is depicted in Fig. 3.11. None of the molecules investigated seems to bind the primary binding site of ibuprofen, not even ketoprofen which is known to share the same binding site of ibuprofen on HSA. This might be consistent with the opposite stereoselectivity observed for the binding of ketoprofen on HSA and RSA [22]. The tocainide analogues could be divided in two groups. The binding of one group (BO3, BO5,BO6, BO7, BO10, BO9) showed a higher sensitivity to the presence of propanol, a lower binding affinity of ibuprofen (of about 6×10^3 M^{-1} as average) for their binding site, an independent binding with ketoprofen, a competitive behaviour with diazepam and bilirubin. The other group (BO1, BO2, Toc, BO8, BO11, BO12, BO13,BO14) showed a lower sensitivity to the presence of propanol, a higher binding affinity of ibuprofen (of about 2 × 104M−1as aver-age) for their binding site that matched the affinity of ibuprofen for the diazepam binding site, a competitive binding with ketoprofen, a competitive behaviour with diazepam and

bilirubin. The higher and lower sensitivity to the presence of propanol could reflect the location of the binding site in an area more or less exposed to the solvents. The binding of diazepam seems to be affected by tocainide analogues and ibuprofen, thus its binding site should occupy a central position with respect the others, but separated from ketoprofen binding site. Finally, the effect of ibuprofen and tocainide analogues on the induced CD arising from bilirubin bound to the protein might reflect either a displacement at a common binding site, or an allosteric effect on the bilirubin affinity and/or conformation at the primary binding site of bilirubin.

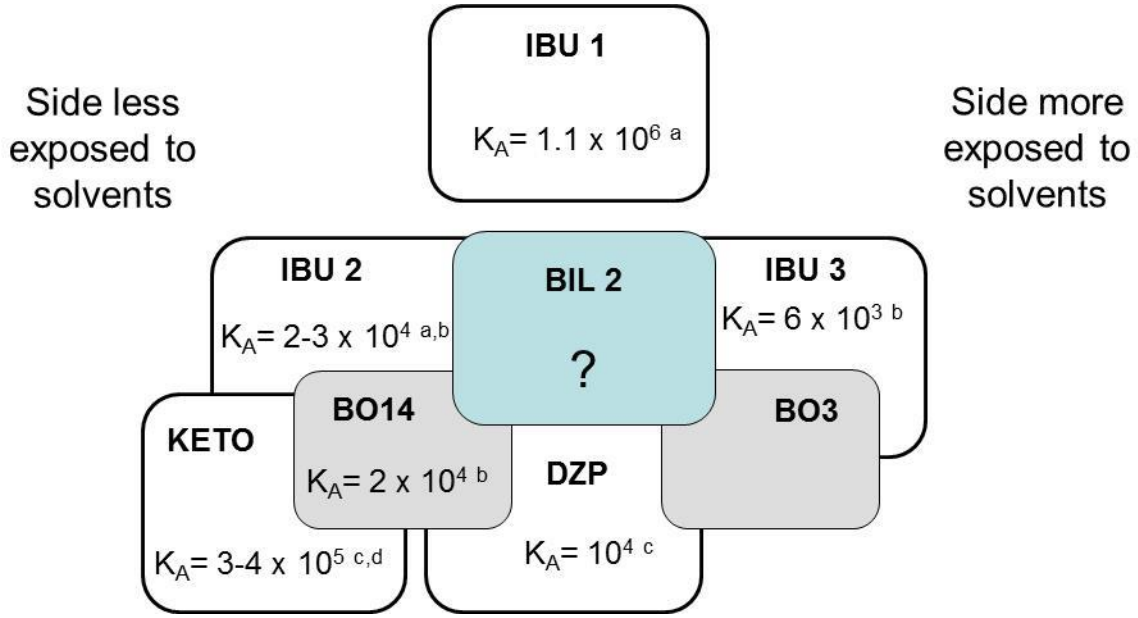

Fig. 3.11 Interaction model of compounds BO3, BO14, ibuprofen, diazepam, ketoprofen and bilirubin on RSA summarizing the results of this work. The affinity values (K_A) measured in this work or found in other literature sources are reported. [a]Value obtained by equilibrium dialysis [31]. [b]Present work. [c]Value obtained by circular dichroism [22]. [d]Value obtained with photolabeled albumins [34].

3.4 Conclusions

All the tocainide analogues showed a higher affinity to HSA and RSA than the parent compound. Lipophilicity is not the only parameter affecting the binding of these molecules to albumins.

Significant differences in the bound fraction of the analyzed compounds to HSA and RSA were observed, being the binding to HSA generally stronger than to RSA. The affinity of these compounds to RSA showed characteristic sensitivities to the presence

of small concentration of propanol added to the mobile phase that suggested the existence of two main binding families. The higher and lower sensitivity to the presence of propanol could reflect the location of the binding site in an area more or less exposed to the solvents. HPALC and CD competition experiments confirm a different behaviour for the two groups of tocainide analogues. The group presenting higher sensitivity to the presence of propanol, showed a lower binding affinity of ibuprofen for their binding site, an independent binding with ketoprofen, and a competitive behaviour with diazepam and bilirubin. The other group showed a higher binding affinity of ibuprofen for their binding site that matched the affinity of ibuprofen for the diazepam binding site, a competitive binding with ketoprofen, and a competitive behaviour with diazepam and bilirubin. The binding of diazepam seems to be affected by tocainide analogues and ibuprofen, thus its binding site should occupy a central position with respect to the others, but separated from ketoprofen binding site.

Drug binding data to albumins from different species are useful to evaluate the reliability of extrapolating to human's distribution data obtained on animals in preclinical studies.

Acknowledgements

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Chapter 4

Optical biosensor analysis in studying new synthesized bicalutamide analogues binding to androgen receptor

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Journal of Pharmaceutical and Biomedical Analysis, accepted for publication.

Abstract

Bicalutamide (Casodex®) is a non-steroidal anti-androgen drug used in the treatment of prostate cancer, which represents the second most common malignancy diagnosed in men worldwide. In this work, we analyze the ability of some novel bicalutamide analogues to bind the androgen receptor, by using an optical biosensor. Androgen receptor was covalently immobilized on a carboxy methyl dextran matrix. The immobilized receptor chip was then used for the binding experiments of the bicalutamide analogues. The (R)-bicalutamide dissociation constant was in good agreement to the value reported in literature obtained by using radiolabeled targets. Most of the new synthesized compounds showed higher androgen receptor binding level, when compared to the reference. Our results clearly indicate that the surface plasmon resonance (SPR) technique offers many advantages with respect to other available technologies in terms of studying biomolecular interactions. Moreover, this study provides an effective methodology for determining the binding affinity of novel chemical entities for the isolated androgen receptor, thus excluding possible off-target interactions occurring in conventional cell-based techniques.

4.1 Introduction

The study of the biorecognition phenomena represents a fundamental step of the early phases of drug discovery and development, for both characterizing new potential targets and identifying new lead candidates.

A wide variety of analytical, affinity based and spectroscopy, techniques are available to define the binding parameters of interacting molecules, as well as to determine the structural features driving the process [1-7]. However, most of them require high amounts of compounds [2,3] or the use of target molecules labeled with a fluorescent [5] or radioactive tag [8].

Among all the available analytical techniques, surface plasmon resonance (SPR) biosensor technology has been recognized as a powerful tool for drug discovery and development applications giving a great improvement to the biomolecular interaction analysis field [9-14].

Real time and label-free assay are the main advantages of the SPR technique. Biosensor experiments involve the immobilization of one reactant on the biosensor surface and the monitoring of its interaction with a second component in solution. The instrument measures the change in the refractive index that occurs during complex association (or dissociation) close to the surface [15]. This real time analysis allows the determination of (i) the amount of bound analyte, (ii) its affinity for the receptor and (iii) the kinetic rates of the interaction (k_{on} and k_{off}) [16]. The response obtained is directly proportional to the mass of the analyte, which binds to the surface. In the past, this has limited standard SPR analyses to the study of analytes with molecular masses of several thousand daltons. Nowadays considerable improvements have been introduced in this technique in terms of hardware, experimental design and data processing, thus also low molecular weights compounds can be monitored by using the optical biosensor technique [17].

In this paper we describe the use of current SPR technology for characterizing the binding process between the androgen receptor (AR) and nine novel synthetic AR ligands (400-600 Da).

The AR is a member of the steroid receptor superfamily and its ligands play important roles in several biological processes such as the development and

maintenance of secondary sexual organs, bone, muscle, and others [18,19]. However, it has been widely demonstrated that approximately 90% of prostate cancers (PCa) are dependent on androgens at initial stages and that AR is expressed throughout cancer progression. Endocrine therapy of PCa is directed towards the reduction of serum androgens and/or the AR inhibition with anti-androgens. The latter occurs by competitively blocking the interaction between the AR and the endogenous ligands. Among the AR antagonists, non-steroidal antiandrogen (R,S)-bicalutamide (Casodex®, 1), has been chosen as the routine clinical treatment due to its superior pharmacokinetic profile and minor side-effects [20]. Nevertheless, after its long-term use, a subset of patients benefit of the anti-androgen discontinuation (anti-androgen withdrawal syndrome, AWS), indicating that these drugs serve as agonist under resistance circumstances [21]. Indeed, there is an urgent need to find novel and more active non-steroidal antiandrogens with superior affinity for the receptor and able to act on resistant PCa stages.

In this work the amine-coupling procedure was used to covalently immobilize the AR on a carboxy methyl dextran matrix. The immobilized receptor chip was then used to determine and compare the binding properties of (R)-bicalutamide (the most active enantiomer) and of the novel synthesized derivatives (Fig. 4.1) [22-25]. The androgen receptor binding level of all the analogues under investigation was ranked, allowing the identification of the higher bounded compounds, compared to the reference, e.g. (R)-bicalutamide, which is the active enantiomer of the commercial drug [26]. Kinetic binding studies of (R)-bicalutamide and some of the analogues were carried out using single cycle and multi cycle kinetics approaches. Moreover, the reliability of the binding parameters obtained was verified by repeating the experiments over time.

4.2 Materials and methods

4.2.1 Chemicals

Compounds (R)-bicalutamide, (S)-BIC14, (S)-BIC20, (R)-BIC33, (S)-BIC32, (R)-BIC30, (R)-BIC51, (R)-BIC59 were prepared as previously reported [22-25]. BIC60 synthesis was performed according to reported procedure [22]. BIC60 characterization 1HNMR (400 MHz, C6D6) δ:9.31 (s, 1H, NH), 7.56-7.50 (m, 3H, ArH), 6.97 (d, J=2.0Hz,

1H, ArH), 6.88-6.85 (m, 2H, ArH), 6.78 (d, J=8.4Hz, 1H, ArH), 6.60 (t, J=8.4Hz, 2H, ArH), 6.43 (t, J=8.4Hz, 2H, ArH), 4.18 (d, J=14Hz, 1H, CH2), 3.12 (d, J=14.4Hz, 1H, CH2), 2.27 (bs, 2H, NH2); 13CNMR (100 MHz, C6D6) δ: 170.6, 165.5 (d, J=305.7), 163.0 (d, J=298Hz), 141.1, 136.8, 135.5, 130.8, 130.7, 127.1, 127.0, 121.3, 116.8 (q, J=4.7Hz), 116.4, 116.2, 115.9, 115.7, 115.4, 104.9, 64.3, 62.0.

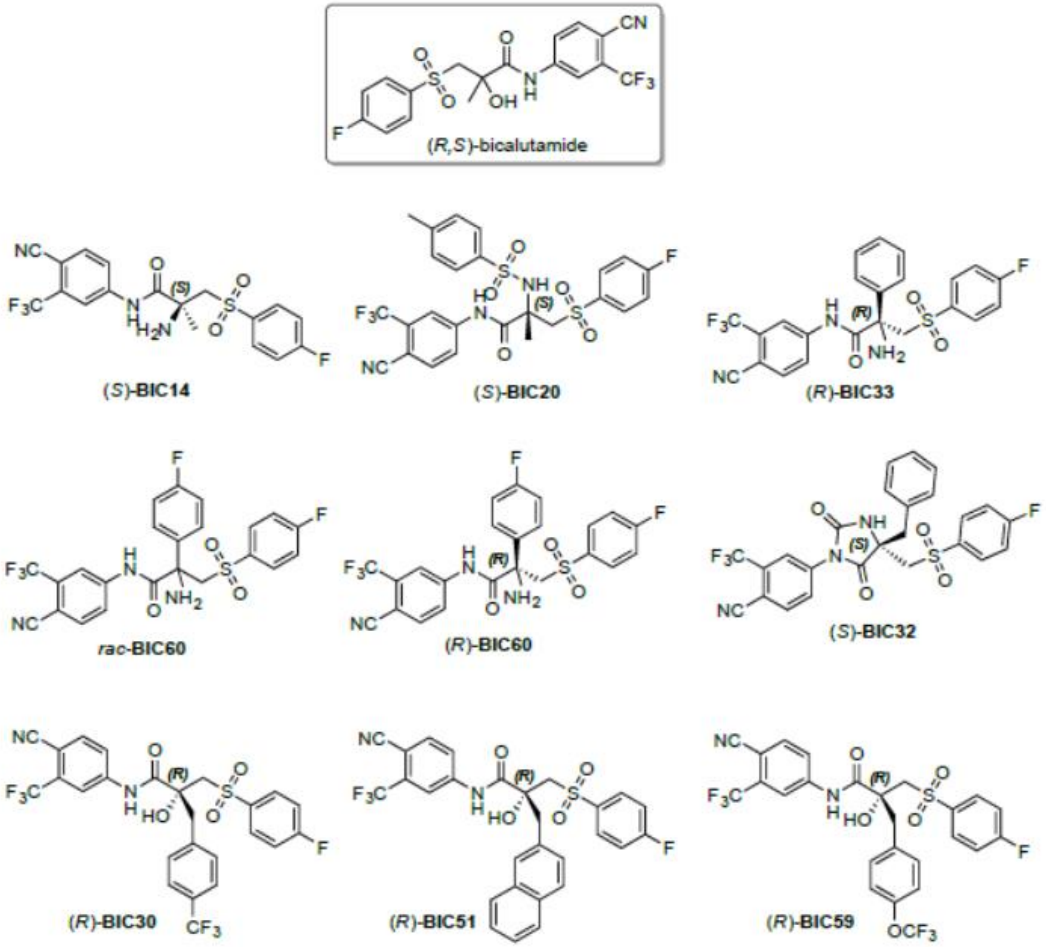

Fig. 4.1 Structures of (R)-bicalutamide and its analogues.

Androgen Receptor (recombinant, expressed in insect cells) was purchased from Sigma-Aldrich (Swiss) at a concentration of 0.1 mg/mL in 20 mM Tris-Cl (pH 8.0), 20% Glycerol, 100 mM KCl, 1 mM DTT and 0.2 mM EDTA. Monosodium phosphate (NaH_2PO_4 x $2H_2O$) was purchased from Merck (Darmstadt, Germany). Sodium chloride, potassium chloride, Tween-20 and N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic

acid) (HEPES) were from Sigma–Aldrich (Buchs, Switzerland). Sodium acetate and sodium hydroxide were from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Switzerland). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine-HCl, HBS-P+ (0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20) and the sensor chip with carboxymethylated matrix CM5 were from Biacore (GE Healthcare). Twice distilled and degassed water deionized and filtered with Millipore Elix 3 was used for mobile phase's preparation. The buffer solutions were filtered through a 0.45-µm membrane filter and degassed before use.

4.2.2 Instrumentation

Surface plasmon resonance analyses were performed by using Biacore X100 optical biosensor, thermostated at 25°C and equipped with research-grade CM5 sensor chips (Biacore). The Biacore X100 evaluation software was used for evaluation of sensorgrams.

Biosensor data were analyzed using Biacore™ X100 2.0 Evaluation Software. Data processing was performed in BIA evaluation software.

4.2.3 Androgen receptor immobilization

The androgen receptor surface was prepared by using standard amine-coupling procedure [27]. The androgen receptor was coupled through his surface amine groups via amine bonds with the CM5 sensor chip of the Biacore X100 instrument. The running buffer was PBS (Phosphate buffered saline: 20 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.005% Tween-20, pH 7.4) and the temperature was set at 25 °C. The receptor was dissolved at 50 µg/mL in 10 mM Sodium Acetate pH 4.0. Carboxyl groups on the dextran layer of the chip were activated by injecting a 1:1 mixture of 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 0.1 M N-hydroxysuccinimide for 10 min at a flow rate of 5 µL/min. The androgen receptor solution was injected in channel 2 and was allowed to react for 7 min at a flow rate of 5 µL/min. The remaining carboxyl groups were blocked by injecting a solution 1 M ethanolamine for 7 min at a flow rate of 5 µL/min. The entire coupling procedure results in 8109 RU of androgen receptor immobilized. The receptor was immobilized only in

one channel of the sensor chip in order to monitor the possible non-specific bindings by means of the second channel. The reference control channel was only activated with 1:1 EDC/NHS mixture, and then treated with ethanolamine to block NHS-ester groups. The androgen receptor immobilization experiment is summarized in the sensorgram shown in Fig. 4.2, where the optical biosensor response (resonance units) is reported as a function of time. After the immobilization the sensor chip surface was stabilized by various injections of running buffer at 30 µL/min all over the night.

The sensor chip was stored in a refrigerator, in a 50 ml tube containing running buffer without DMSO (see section 4.2.5) so that the support was completely covered.

4.2.4 Preparation of sample solutions

All the set of analyzed compounds were stored as 1mg/mL stock solutions in 100% dimethyl sulfoxide (DMSO) and diluted to 1 mM with 100% DMSO. Immediately prior to analysis, samples were mixed with assay buffer and DMSO providing a 10 µM compound in a final buffer composition of 10 mM Hepes, 150 mM sodium chloride, pH 7 and 2% (v/v) DMSO, which was also used as the instrument running buffer and sample dilution buffer. The running buffer should contain the same DMSO concentration as the samples, since DMSO has a high refractive index contribution and small differences in DMSO concentration will significantly affect the bulk response.

4.2.5 Ranking of ligand binding to Androgen Receptor

The running buffer for AR ranking analysis consisted of 10 mM Hepes, 150 mM sodium chloride, DMSO 2% (v/v), pH 7. In the ranking format 10 µM of the compounds under investigation were injected at a flow rate of 10 µL/min over the AR surface at 25 °C. Ligands were allowed to associate with the receptor for 60 s and dissociation was monitored for 4 min. Surface regeneration was obtained by injecting two short pulse of HBS 100 mM for 30 s at a flow rate of 30 µL/min and flushing running buffer at 5 µL/min for some min before each injection. Since the dissociation of all ligate rarely occurs and the repeated cycles of analysis need to start with the biosensor surface free of residual material, the regeneration conditions are very important for experiment success. A buffer injection between every sample cycle was included in order to check the possible

carry-over of analyte to the next cycle, and to stabilize the surface. All samples were injected in duplicate.

4.2.6 Kinetic Analysis of Androgen Receptor/Ligand interactions

Kinetic studies were carried out with the following compounds: (R)-bicalutamide, (S)-BIC32 and (R)-BIC33. Kinetic and equilibrium constants were determined by injecting a wide range of ligate concentrations (approximately from 1 μM to 20 μM) at a flow rate of 30 μl/min over the androgen receptor chip at 25°C. The concentration range was chosen on the basis of the linearity of the SPR responses obtained: when concentrations below 1 μM were used the SPR signals were not detectable; on the other hand concentrations above 20 μM led to artifacts, due to crowding of interacting molecules on the surface as well as to precipitation of poor soluble compounds.

Buffer blanks were injected periodically and all sensorgrams were processed by using double referencing: the responses from the Fc1 channel control were firstly subtracted from the binding responses collected over the reaction surfaces and then the response from an average of the blank injections was also subtracted. Binding of each sample was allowed to occur for 60 s and the dissociation phases were of 180 s. The androgen receptor surface was regenerated between binding cycles with two short pulses of HBS 100 mM for 30 s. Kinetic experiments with (R)-bicalutamide and (S)-BIC32 were set up using the kinetic wizard in Biacore X100 evaluation software with the single-cycle approach; whereas the kinetic analysis of (R)-BIC33 were performed using multi-cycle approach. The kinetic fitting was carried out using 1:1 Langmuir binding model. The equilibrium dissociation constant (K_D) was obtained by the ratio of the binding on (k_{on}) and off (k_{off}) rate ($K_D = k_{off}/k_{on}$). Chi-square value ($\chi 2$) is used as a statistical measure to judge how closely the model given by the software fits with the experimental data. The DMSO calibration plot was built prior to perform each kinetic analysis. The complete kinetic analysis was performed three times over the same chip.

4.2.7 Solvent Refractive Index correction

A solvent refractive index (RI) correction procedure was included in the assay protocols to avoid variations in the bulk response between samples. As DMSO has a high refractive index, small variations in DMSO concentration between sample solutions

and running buffer often lead to significant nonspecific signals. The solvent RI correction becomes essential when working with low molecular weight compounds that give intrinsically small responses. In fact, in this case variations in the bulk RI can be of the same order of magnitude as the responses expected from the binding interaction. A series of eight buffer solutions containing concentrations of DMSO (1.5-2.8 %) were injected in sequence over reference and AR surfaces. The difference in response between AR and reference flow cells plotted as a function of the response in the reference flow cell provides us the DMSO calibration plot [28]. All the response levels obtained during the analysis were corrected with this curve.

4.3 Results and discussion

SPR biosensor technique has been used for nuclear receptor binding assays, as screening tool for identifying active compounds and to get information on the mechanism of the receptor mediated activity [29-32]. In the case of androgen receptor, the K_D of (R)-bicalutamide has been obtained by displacement measurements of a labeled marker compound in a cell culture [26].

The use of an isolated receptor can represent a valuable methodology for medium-high throughput screening of the binding properties of active compounds.

4.3.1 Androgen receptor immobilization

The most challenging step when setting up SPR experiments is immobilizing the target protein to the sensor surface without modifying its activity. Immobilization can either be direct, by covalent coupling the target protein, or indirect, through its capture by a molecule with high affinity for the target protein itself and covalently bounded to the sensor surface. Choosing the right immobilization strategy is not a simple issue, and several aspects should be taken into account. In particular, the orientation of the immobilized receptor, the local surface environment, the linkage stability, and the possible effects of the coupling chemistry on components of the system, represent critical aspects of the chosen strategy [16].

It has been already demonstrated that bicalutamide and its analogues bind androgen receptor ligand binding domain (LBD), which is located in the C-terminal

domain of the receptor [33]. The amine coupling method was used (see section 4.2.3) to immobilize the AR on the chip surface. In order to determine suitable coupling conditions, a pre-concentration test of the ligand on the surface was done. Since the receptor had an isoelectric point of 6.3 [34], we decided to use ligand solutions in different coupling buffers, covering the pH range 4-5 in steps of 0.5 pH units. In fact, it is already known that the immobilization buffer pH value should be at least 0.5-1 unit below the isoelectric point of the ligands and higher than 3.5, so that the surface and the ligand carry opposite net charges. The condition that gave adequate electrostatic pre-concentration of ligand to the dextran matrix was the use of buffer at pH 4.

The entire coupling procedure results in 8109 RU of androgen receptor immobilized. The immobilization experiment is summarized in the sensorgram shown in Fig. 4.2. The reference channel was treated as the experiment channel in order to follow nonspecific binding phenomena which could take place on the biosensor surface. The binding of (R)-bicalutamide was used to check that LBD was not involved in the immobilization process. As a result the K_D of the reference compound was in agreement to that reported in the literature [26]. The immobilized AR was stable over four weeks as demonstrated by the consistent ranking obtained from analyses carried out at different times for the same set of ligate concentrations.

4.3.2 Ranking analyses

Bicalutamide and its analogues were floated over the AR covalently immobilized chip. The ranking of the analytes under investigation for their AR binding level was performed by simply measuring the degree of binding at only one concentration value. The screening was performed at 10 μM. This concentration was low enough to minimize sample consumption and allow analysis of poorly soluble compounds, but also high enough to give a reasonable signal. The maximum binding response after a 60 s injection was determined for each compound. As the binding response in a Biacore assay is a function of the total molecular mass close to the chip surface, binding data were adjusted for the molecular weight of each drug in order to give comparable molar values. All the bicalutamide analogues showed a higher androgen affinity when compared to the reference, e.g. (R)-bicalutamide, with the exception of the (S)-BIC14 that shows a lower affinity (Fig. 4.3). The increasing of lipophilicity of the substituent at

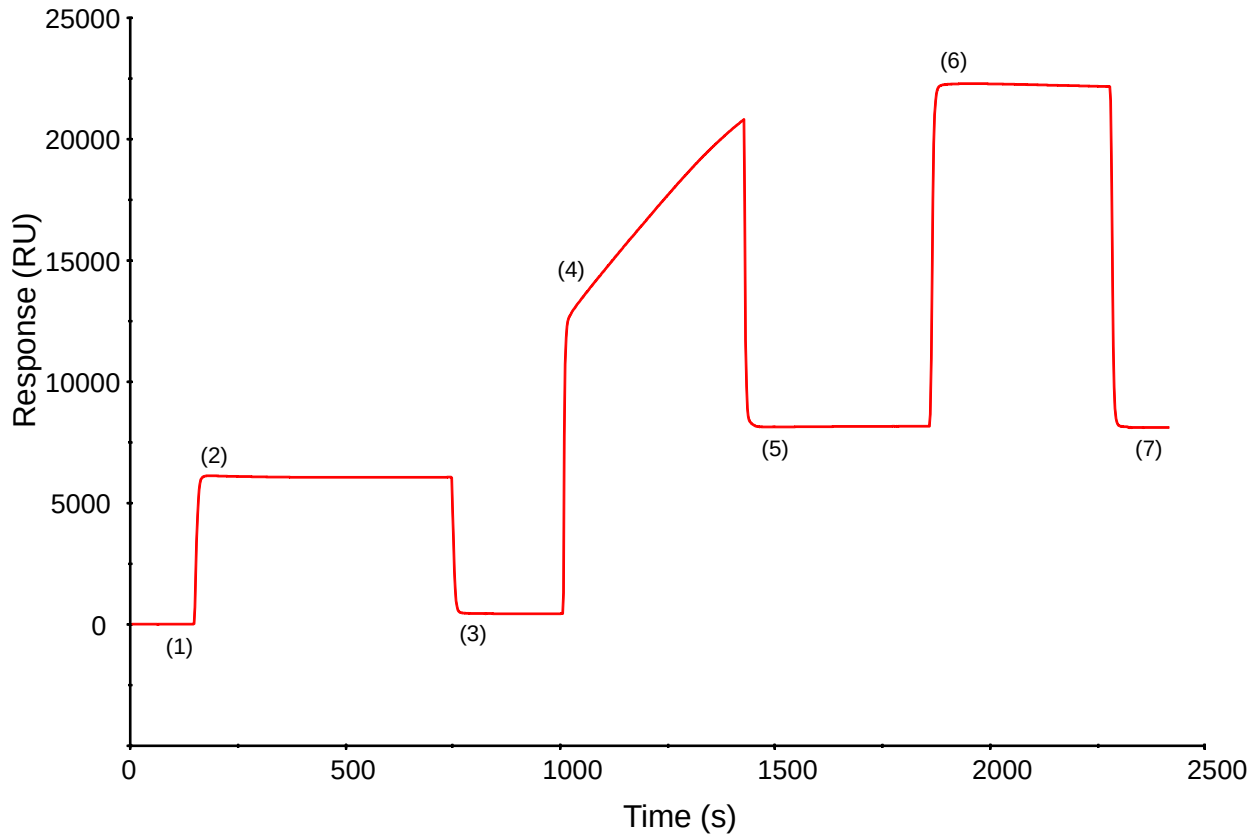

Fig. 4.2 Sensorgram of the Androgen receptor immobilization to the carboxymethyl dextran matrix: (1) buffer baseline stabilization; (2) EDC/NHS mixture add; (3) buffer re-equilibration; (4) Androgen receptor in acetate buffer add (7 minutes of reaction); (5) buffer washes; (6) block of non-coupled activated CMD sites with ethanolamine (7 min of reaction); (7) baseline stabilization.

the chiral stereocenter also increases the affinity of these compounds to AR. This behavior matches with the calculated log P of the compounds under investigation (see Table 4.1). In fact, (R)-bicalutamide and (S)-BIC14 show a lower predicted logP than the other compounds. On the other hand, (R)-BIC51, (R)-BIC59 and (S)-BIC32 hold the highest predicted log P value and produce the highest receptor binding affinity. Moreover, comparing the results of Fig. 4.3 and Table 4.1, we can appreciate that the ranking of the compounds based on the binding level reflects those based on the log P parameter, with the only exception of (R)-BIC30. These data indicate that hydrophobicity is also an important parameter in determining the interaction between the analyzed molecules and AR. In particular, the presence of two aromatic rings on the chiral center determines the increase of lipophilicity but also a better accommodation into the receptor pocket(s) with increased affinity.

It's also interesting to note that (R)-BIC60 showed higher affinity to AR than the corresponding racemate (rac-BIC60), confirming the significant influence of the stereochemistry on the binding ability, as observed for (R)- and (S)-bicalutamide [26].

These data prove that this technique provides also structure-activity relationship information useful for future rational design of potential new drugs in the treatment of androgen receptor related diseases.

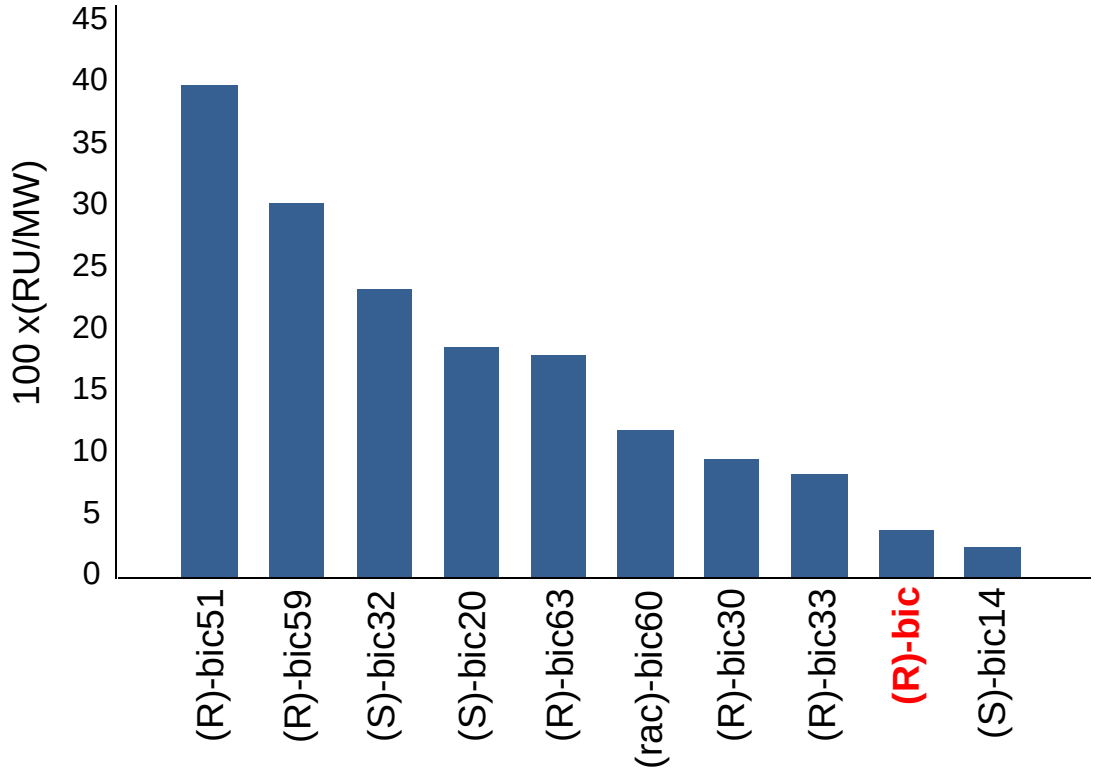

Figure 4.3 Ranking analyses of bicalutamide analogues performed at a concentration of 10 μM in HBS 10 mM + NaCl 150 mM + 2% DMSO, pH7. Binding data were adjusted for the molecular weights of each compounds, in order to give comparable molar values.

4.3.3 Kinetic analyses

A detailed kinetic analysis was performed for the following compounds: (R)-bicalutamide, (S)-BIC32 and (R)-BIC33. The binding responses obtained for concentration series of each of these compounds injected over the AR surface are reported in the Fig. 4.4. The real time detection allows to obtain kinetic rate constants of the interaction (association and dissociation rate constants) by analyzing the sensorgram shape using a mathematical model for the interaction mechanism. Each of

Sample	miLog P[a]
(S)-bic14	2.197
(R)-bicalutamide	2.154
(R)-bic33	3.416
(R)-bic30	4.477
(rac)-bic60	3.579
(R)-bic60	3.579
(S)-bic20	3.928
(S)-bic32	4.512
(R)-bic59	4.552
(R)-bic51	4.765

Table 4.1 Predicted log P values of the compounds under investigation. Compounds are tabulated according to the ascending degree of binding for the AR.
[a]Calculated using online www.molinspiration.com/cgi-bin/properties site (Molinspiration Property Engine v2009.01)

the analyzed interaction was well described by a 1:1 interaction model (Fig. 4.4). This model can be described by the following equation:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

where k_{on} is the association rate constant ($M^{-1}s^{-1}$) and k_{off} is the dissociation rate constant (s^{-1}). The equilibrium dissociation constants can be derived directly from the ratio of the rate constants obtained ($K_D = k_{off}/k_{on}$).

The (R)-bicalutamide and (S)-BIC32 kinetic experiments were set up using the kinetic wizard on Biacore X100 with the single-cycle approach (see Fig. 4.4a, 4.4b); whereas the (R)-BIC33 kinetic analyses were performed using multicycle approach (see Fig. 4.4c). In both cases several analyte concentrations were injected over the surface to determine the binding data. Multicycle kinetics runs each analyte concentration in a separate cycle, and the surface is regenerated between each cycle. On the contrary, in

the single cycle experiment, different concentrations of the analyte are injected in one cycle without regeneration between sample injections.

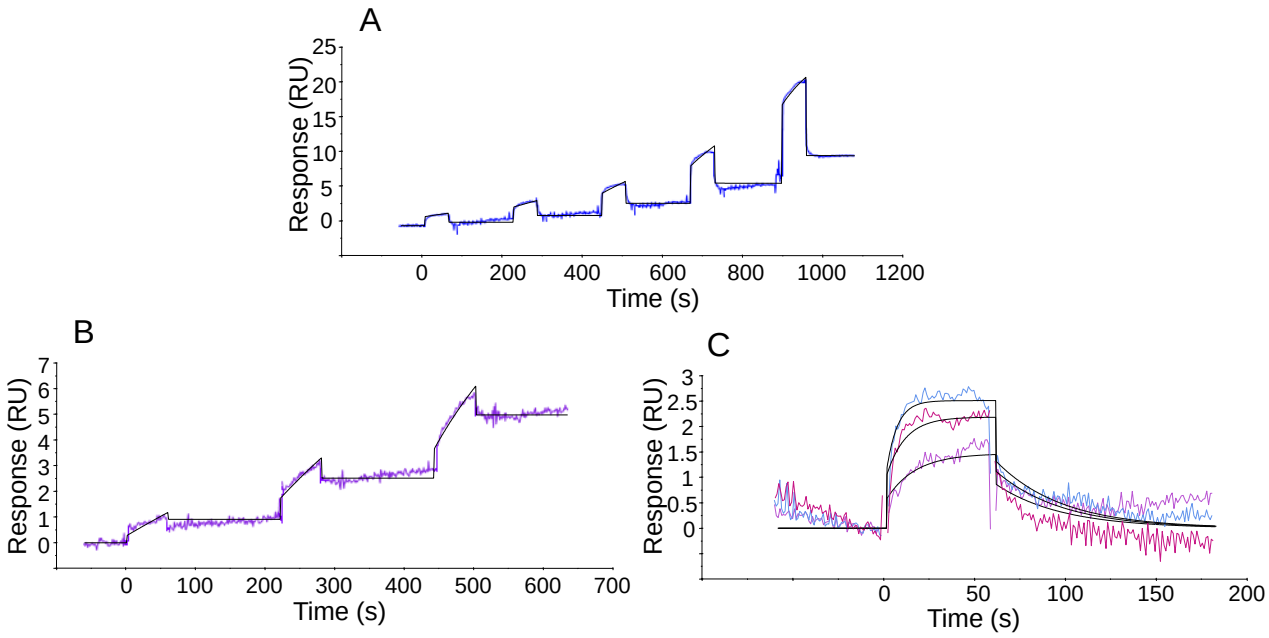

Fig. 4.4 Kinetic profiles for (R)-bicalutamide (A), (R)-BIC32 (B) and (R)-BIC33 (C) binding to AR determined using single cycle (A, B) and multi-cycle (C) kinetics. Sensorgrams show blank and reference subtracted data with kinetic fit for 1:1 interaction model overlaid in black.

Analysis of the data revealed that (R)-bicalutamide binds to the AR with an association rate constant of 371.4 $M^{-1}s^{-1}$ and a dissociation rate constant of 2.63 × 10^{-5} s^{-1}, resulting in a K_D of 70.7 nM. This value is in good agreement with the KD value of 11 Nm reported in literature for the same interaction obtained by using radiolabeled marker in cell cultures [26]. The R-bicalutamide K_D evaluation was also repeated at different moments of the sensor chip's life, following always the same protocol: the values obtained represent an evidence of the reproducibility of the employed method (37.4 nM and 43.3 nM).

The same analysis was performed with the novel synthetic derivatives. Significant differences were found for both association (k_{on}) and dissociation rates (k_{off}) (Table 4.2). Despite being the compound with lower affinity among those analyzed, (R)-bicalutamide presents the slowest association rate constant and the faster dissociation rate constant. Out of all, (R)-BIC33 exhibited the most rapid association rate constant, almost 80-fold

higher than the reference k_{on}, although it did not display a high affinity for the receptor in the ranking analysis (Fig.3). The dissociation rate constants observed for (S)-BIC32 was 149-fold slower than the reference, respectively. All these considerations should be taken into account during lead optimization studies. In fact, using just the affinity value as the ideal parameter for biological activity considerations could cancel important information about the relationship between activity and structure of new synthesized compounds [35]. At this stage of drug development, the best would be to consider both the kinetic parameters, in order to obtain a clear overview of how strong the recognition process (k_{on}) is and how stable the complex between the binders (k_{off}) is.

The equilibrium dissociation constant (KD) showed that (S)-BIC32 had an higher affinity for the receptor than the lead compound (173 pM).This datum was in accordance with the ranking study presented before.

SAMPLE	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
(R)-bicalutamide	371.4	2.63×10^{-5}	7.07×10^{-8} [a]
(S)-bic32	1024	1.77×10^{-7}	1.73×10^{-10}
(R)-bic33	2.77×10^{4}	n.d.[b]	n.d.[b]

Table 4.2 Rate constants and affinities of AR/ligand interactions. Parameters were estimated by global fit of an equation describing 1:1 binding.
[a]value in good agreement with those reported in literature obtained by usingradiolabeled target [26]
[b]not determined, because outside of instrument range

4.4 Conclusions

SPR based optical biosensors offers some advantages with respect to other spectroscopic and affinity based techniques. In fact, the drug/protein binding process is monitored in real time with the SPR based biosensor, allowing the evaluation of the kinetics parameters directly from the shape of the sensorgrams recorded. Furthermore, this technique requires small amounts of both the ligand and the ligate, and it is well suited for medium-high throughput screening applications.

In particular, the present study reports on the determination of the binding affinity and the kinetics parameters of the series of novel androgen receptor ligands. Our data indicate that this methodology can represent a very useful and reliable alternative to conventional assays based on displacement methodology with labeled markers in cell cultures. Moreover, when the proper protein-sensor conjugation set up is established, SPR can provide good approximation data of binding affinity between novel chemical entities and the isolated androgen receptor. These data might deliver important information during drug discovery and development steps, because they will account for the sole AR binding pocket-ligand affinity, purged from “off-target” interactions, which are likely to occur in all the conventional cell-based techniques.

In our work we demonstrated that the K_D value of the reference compound, e.g. (R)-bicalutamide, bound to the AR is in good agreement with the K_D value reported in literature for the same interaction obtained by using radiolabeled target [26]. This result confirmed that the AR was properly immobilized on the biosensor surface and that the assay could be reliable applied to the other compounds. Significant differences were found for both association (k_{on}) and dissociation rates (k_{off}) between all compounds under investigation. Overall, (R)-BIC33 exhibited the highest association rate constant, almost 80-fold higher than the reference, but it did not show high affinity for the receptor. The dissociation rate constant observed for (S)-BIC32 was 149-fold slower than the reference compound.

In order to obtain a clear overview of how strong the recognition process (k_{on}) is and how stable the complex between the binders (k_{off}) is, we believe that both thermodynamic and kinetics data should be taken into account during lead optimization studies.

Acknowledgements

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Chapter 5

Bicalutamide analogues interaction with human serum albumin and rat serum albumin: an affinity chromatography and circular dichroism investigation

Abstract

In this work the binding of some novel bicalutamide analogues (see section 4.1) for the main plasma protein, serum albumin, was characterized by using high performance affinity chromatography (HPALC) and circular dichroism (CD). The study was carried out with two different serum albumins, Human Serum Albumin (HSA) and Rat Serum Albumin (RSA), in order to underline the possible differences among species when performing pharmacokinetic studies. Concerning HPALC investigation, HSA was immobilized on a silica HPLC column, through the reported online procedure, allowing the binding properties of the protein to be maintained. With the HSA column the ranking of the bicalutamide analogues for their HSA bound fraction was performed, by measuring the degree of binding at only one concentration value. CD displacement experiments were carried out to characterize the high affinity binding sites of these compounds to the two plasma proteins, HSA and RSA. The change of the induced CD spectra observed for a protein:marker complex in the presence of increasing concentrations of the lead compound was monitored.

All the compounds under investigation showed high affinity to HSA. Competition experiments revealed an independent binding at benzodiazepine site and site II for RSA. An independent binding at site II and a cooperative binding at the bilirubin site was observed for HSA.

5.1 Experimental

5.1.1 Chemicals

Human serum albumin (HSA, essentially fatty acid free, A1887),rat serum albumin (RSA, essentially fatty acid and globulin free, A6414), 1-propanol, Dimethyl sulfoxide (DMSO), L-tryptophan (L-Trp), N-benzoyl-dl-leucine, S-warfarin, rac-ibuprofen (IBU), rac-ketoprofen (KETO), rac-suprofen, rac-indoprofen and bromophenol-blue were purchased from Sigma–Aldrich (Milan, Italy). Diazepam (DZP) was kindly provided by Professor Regina H. Costa Queiroz, Faculty of Pharmacy, University of San Paolo, at Ribeirao Preto (Brazil). Clonazepam, temazepam and lorazepam were kindly provided by Prof. Lucacchini, University of Pisa (Italy). KH_2PO_4, K_2HPO_4, and $(NH_4)_2SO_4$ powders were purchased from Carlo Erba (Milan, Italy). Potassium phosphate buffers (PB) were prepared by adjusting the pH of solutions of K_2HPO_4 with equimolar solutions of KH_2PO_4. Twice distilled and degassed water deionized and filtered with a MILLI-RX20 (Millipore) system was used for mobile phase's preparation.

5.1.2 Bicalutamide analogues, synthesis

Compounds (R)-bicalutamide, (S)-BIC14, (S)-BIC20, (R)-BIC33, (S)-BIC32, (R)-BIC30, (R)-BIC51, (R)-BIC59 were prepared as previously reported [1-4]. BIC60 synthesis was performed according to reported procedure [1]. BIC60 characterization 1HNMR (400 MHz, C6D6) δ:9.31 (s, 1H, NH), 7.56-7.50 (m, 3H, ArH), 6.97 (d, J=2.0Hz, 1H, ArH), 6.88-6.85 (m, 2H, ArH), 6.78 (d, J=8.4Hz, 1H, ArH), 6.60 (t, J=8.4Hz, 2H, ArH), 6.43 (t, J=8.4Hz, 2H, ArH), 4.18 (d, J=14Hz, 1H, CH2), 3.12 (d, J=14.4Hz, 1H, CH2), 2.27 (bs, 2H, NH2); 13CNMR (100 MHz, C6D6) δ: 170.6, 165.5 (d, J=305.7), 163.0 (d, J=298Hz), 141.1, 136.8, 135.5, 130.8, 130.7, 127.1, 127.0, 121.3, 116.8 (q, J=4.7Hz), 116.4, 116.2, 115.9, 115.7, 115.4, 104.9, 64.3, 62.0. The structures of the compounds are reported in Fig.5.1.

5.1.3 Instruments

The HPLC apparatus consisted of a Jasco PU-2089 plus (Jasco, Tokyo) HPLC pump, a Rheodyne injector system with a 20 µL loop and a Jasco MD-2010 plus (Jasco, Tokyo) diode array detector. The column was thermostated at 28°C with a Column

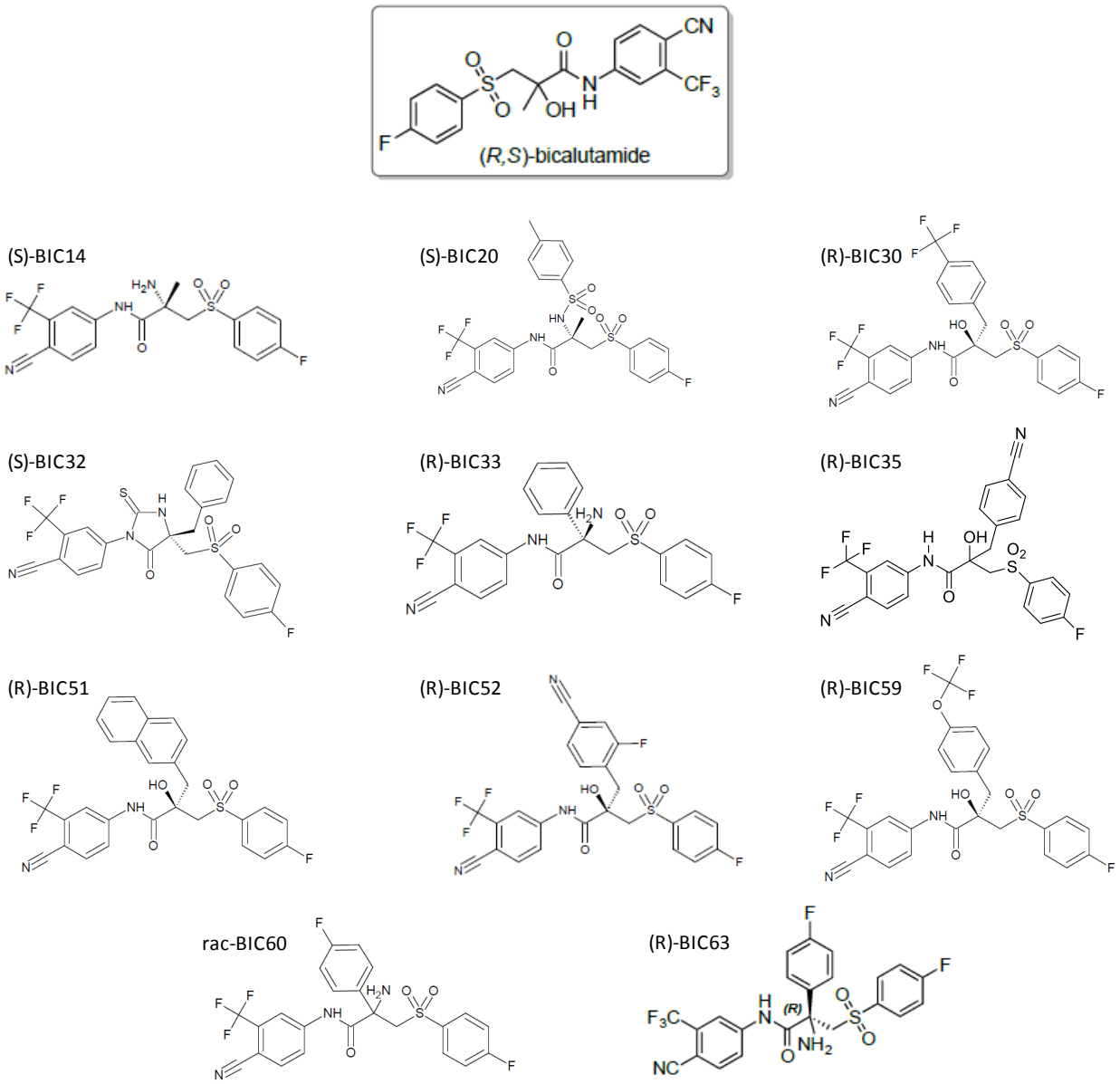

Fig. 5.1 Structures of (R)-bicalutamide and its analogues.

Chiller Model 7955 (Jones Chromatography Ltd., UK). Buffers were filtered through 0.22 µm mixed cellulose esters filter membranes (Milli-pore, Milan, Italy). The mobile phases were degassed prior to use by ultrasonic waves for at least 15 min.

CD measurements were performed with a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature control system set at 25°C. The spectra were recorded using 1 cm pathlength cell at a scan rate of 20 nm/min, time constant 2 s, resolution 0.2 nm, and spectral bandwidth 1 nm.

5.1.4 Column preparation

The silica monolithic column (50 mm × 4.6 mm i.d.), 20 nm mesopores, were silanized by an in situ process as previously reported [5], with slight modification.

HSA was covalently immobilized online on the monolithic epoxy-silica columns following a previously reported procedure [6]. In brief, HSA solution (10 mg/mL of protein in PB 50 mM, pH 6.5, 1 M ammonium sulphate) was circulated through the epoxy-silica column in closed circuit for 24 h. The column was washed with 120 mL of PB (50 mM, pH 7.4), and 100 mL of 1 M glycine dissolved in PB (50 mM, pH 7.4). The column was then washed with PB (67 mM, pH 7.4)/1-propanol 95:5 and stored at 4°C until use. The HSA-based column was employed for the determination of the bound fractions of the compounds under investigation.

5.1.5 Column characterization

The HSA-based column was characterized in terms of amount of albumin correctly immobilized by the frontal analysis of L-tryptophan as previously reported [6,7]. A series of solutions of L-trp in PB (67 mM, pH 7.4) ranging from 5 µM to 60 µM were continuously applied to the column as mobile phases. The relevant breakthrough volume ($V - V_0$) of each solution, where V is the breakthrough volume and V_0 is the system void volume, was determined and the following linear equation was applied [7]:

$$\frac{1}{m_{L\,app}} = \frac{1}{K_A \cdot m_L \cdot [analyte]} + \frac{1}{m_L} \qquad (5.1a)$$

or

$$\frac{1}{(V - V_0) \cdot [analyte]} = \frac{1}{K_A \cdot m_L} \cdot \frac{1}{[analyte]} + \frac{1}{m_L} \qquad (5.1b)$$

(see section 3.2.6).

A series of racemates reported to bind the proteins in a stereoselective manner (rac-warfarin, rac-indoprofene, rac-ketoprofen, rac-suprofen and rac-ibuprofen) were used to check the dependability of the albumin-based column. Stock solutions of the

racemates prepared 1 mg/mL in 1-propanol were diluted with PB (67 mM, pH7.4) and injected into the columns at the final concentration of 60 µM. Mobile phases were mixtures of PB (67 mM, pH 7.4)/1-propanol.

5.1.6 Bound fraction determination

For all samples a stock solution at a concentration of 0.5 mg/ml in DMSO was prepared, and diluted with the 67 mM phosphate buffer at pH 7.4 just before injection. The concentration of working solutions was 20 µM. The chromatographic retention was reported as the capacity factor (k') and its logarithm (log k'), where k' is defined as

$$k' = \frac{t_{drug} - t_0}{t_0} \qquad (5.2)$$

(t_{drug}= retention of the solute; t_0= retention of an unretained solute). Samples were analyzed in duplicate. The flow was set at 1 mL/min. The k' values were determined in absence or presence of 1-propanol, added to the mobile phase at several concentrations. Mobile phases were composed of PB (67 mM, pH7.4)/1-propanol ranging from 90:10 to 100:0. Because of the low solubility of some compounds, not all compounds have been tested without 1-propanol in the mobile phase. The retention values were plotted as log k versus the organic modifier concentration. The bound drug fraction (B%) was determined as the measured capacity factor k', according to a previously published method [8].

$$B\% = \frac{k'}{k'+1} \cdot 100 \qquad (5.3)$$

5.1.7 Circular dichroism: displacement experiments

The displacement experiments were carried out using rac-bicalutamide as the competitor. Diazepam (DZP) and rac-ketoprofen (KETO) were used as selected markers for benzodiazepines binding site and Site II, respectively; bromophenol-blue (BPB) as markers for bilirubin binding site. The change in the CD signal after addition of different amounts of rac-bicalutamide to a solution containing equimolar concentrations of HSA or RSA and marker [15 µM in PB (pH 7.4; 67 mM)] were monitored. Stock

solutions of rac-bicalutamide (15 mM in 1-propanol), DZP (15 mM in 1-propanol), KETO (15 mM in 1-propanol), BPB (15 mM in 1-propanol), HSA and RSA (150 µM in PB, pH 7.4, 67 mM) were prepared immediately before the experiments. All the measurements were carried out using a 10 mm path length cell. The working solutions were prepared by mixing the protein under investigation, marker and displacers at molar ratios from 1:1:0 up to 1:1:8.

5.2 Results and discussion

5.2.1 Column characterization

The HSA-column was characterized by frontal analysis in order to determine the amount of protein immobilized onto the column (Fig.5.2).

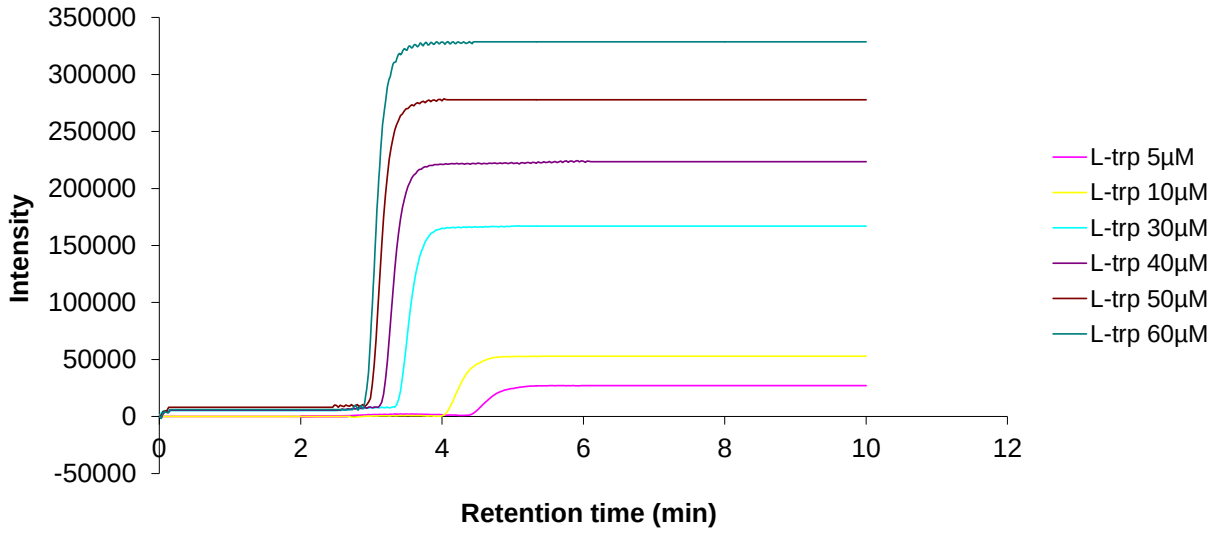

Fig. 5.2 Acquisition curves obtained by frontal analysis.

The plot of the bound analyte versus the reciprocal of its concentration (see Section 3.2.4) was used to determine the amount of albumin correctly anchored to the silica matrix and the affinity constant of the analyte for the albumin (Fig. 5.3). L-Trp showed to bind to a single site on albumin (r^2= 0.9993) with a K_A of 1.25 × 10^4 M^{-1}, in good agreement with the value of 1 × 10^4 M^{-1} reported in the literature [9]. The amount of immobilized albumin was 11.97 mg, thus considering an amount of silica of about

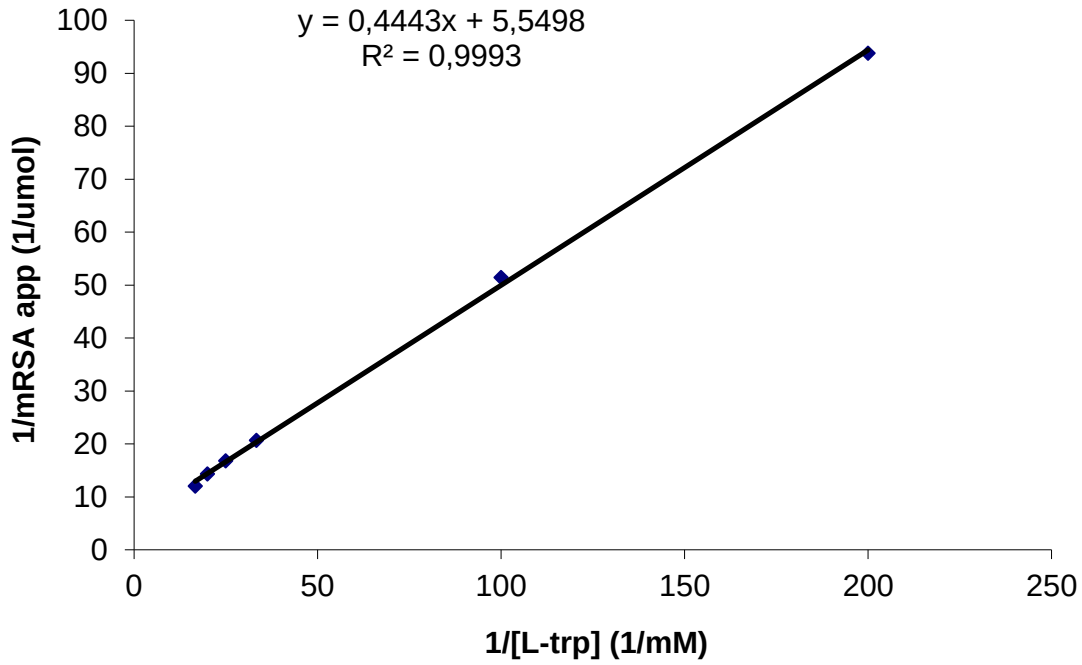

Fig. 5.3 Frontal analysis plot: m_{HSA} and K_A evaluation.

0.25 g per column the amount of protein immobilized resulted in about 48 mg/g silica.

By injecting racemates of drugs or amino acids reported to bind the protein in an enantioselective manner we also checked that the immobilization procedure preserved the native binding properties of the proteins. We chose N-benzoyl-DL-leucine, different benzodiazepines (temazepam, lorazepam and clonazepam) and various aryl-propionic acids (rac-ketoprofen, rac-ibuprofen, rac-indoprofen and rac-suprofen), which are known to bind to Site II on the HSA, as well as rac-warfarin, known to bind to Site I on the albumin. The enantioresolution was obtained for all the analyzed racemates. Moreover most of the enantiomers analyzed have also shown selectivity values ($\alpha = k_B/k_A$) higher than 1.5, demonstrating the excellent quality of this column (see Fig. 5.4).

5.3.2 Bound fraction determination

Bicalutamide and its analogues were injected into the HSA covalently immobilized column. The ranking of the bicalutamide analogues for their HSA bond fraction was performed on the basis of the capacity factor, k. The analyses were carried out gradually decreasing the propanol concentration in the mobile phase. Because of the

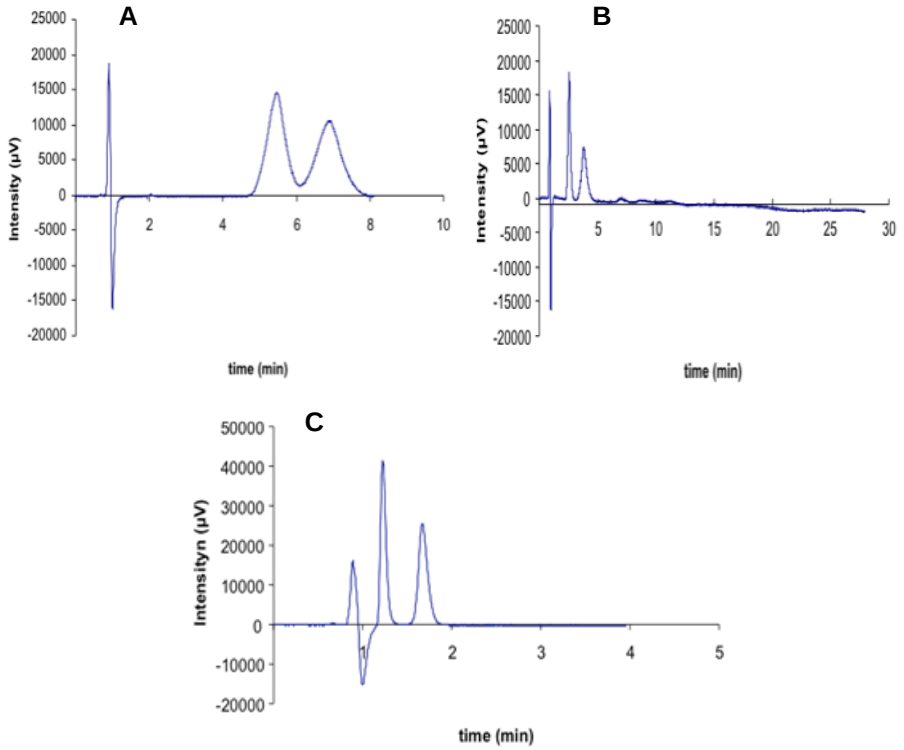

Fig. 5.4 (A) Chromatographic enantioresolution of rac-ketoprofen 60 µM; (B) chromatographic enantioresolution of rac-warfarin 60 µM; (C) chromatographic enantioresolution of N-benzoil-DL-leucine 60 µM. Mobile phase was phosphate buffer (pH 7.4; 67 mM)/1-propanol (90:10, v/v); flow rate 1 mL/min.

high affinity of these compounds for the protein, only (S)-BIC14 and (R)-BIC35 allowed the analysis in pseudo-physiological conditions (aqueous solution). Rac- and (R)-bicalutamide, (R)-BIC52 and rac-BIC60 allowed the analysis with PB (67 mM, pH 7.4)/1-propanol 98:2; (R)-BIC33 with PB (67 mM, pH 7.4)/1-propanol 97:3; (S)-BIC20 and (S)-BIC32 with PB (67 mM, pH 7.4)/1-propanol 96:4; (R)-BIC30, (R)-BIC59 , (R)-BIC51 PB (67 mM, pH 7.4)/1-propanol 94:6, as mobile phase.

It is already known that the presence of an organic modifier may cause changes in the secondary or tertiary structure of the protein; moreover also the microenvironment around the HSA binding area may be modified [10]. These variations could affect the drug-protein binding process, thus we analyzed the variation of log k' values of the bicalutamide analogues when changing the propanol concentration in the mobile phase (Fig. 5.5).

From the observed trend (Fig. 5.5) it is clear that the presence of propanol highly affect the log k' values, but it is also evident that the ranking of the compounds under

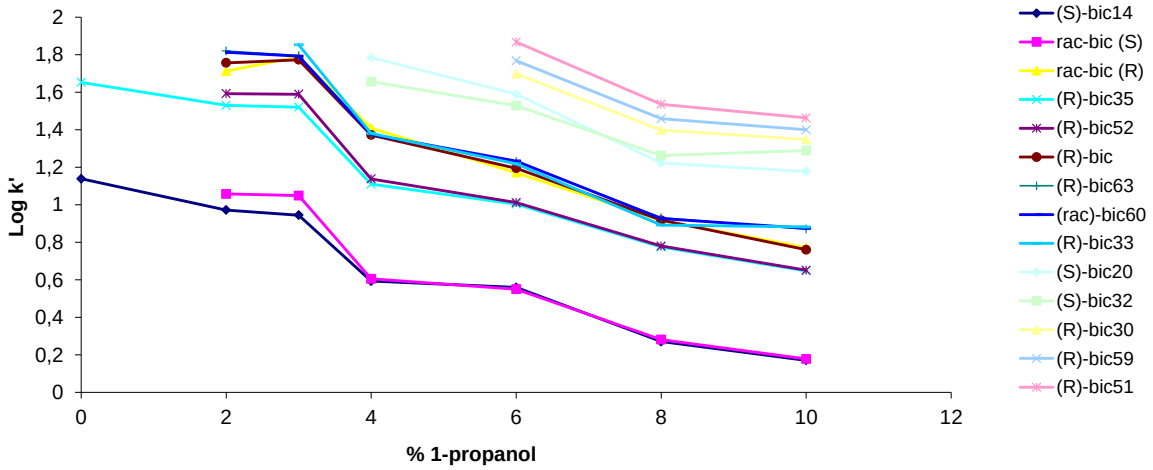

Fig. 5.5 Relationship between log k' values of bicalutamide analogues obtained with the HSA-based column at various concentration of 1-propanol in the mobile phase.

investigation it is not subject to significant variations. In order to compare all the bicalutamide analogues bound fractions, we decided to calculate this data starting from the value obtained using PB (67 mM, pH 7.4)/1-propanol 94:6 as mobile phase. Furthermore, the dependability of the column for ranking the new synthesized compounds for their HSA binding was checked by injecting in pseudo-physiological conditions additional drugs, whose bound fraction to the serum protein was already known (Table 5.1). A quite good agreement was found in the comparison of the bound fraction values experimentally obtained for the HSA binding and the literature data of these marker compounds [11].

The majority of the analogues showed a higher affinity to HSA comparing to the lead compound (Table 5.1). In particular, (R)-BIC51 and (R)-BIC59 showed the highest affinity (98.6% and 98.3%, respectively) and also the higher calculated Log P values among all the compounds under investigation (4.765 and 4.552, respectively). Since these molecules were modified with the introduction of a big substituent at the chiral stereocenter, then the increase of the lipophilicity of the α-carbon also increases the affinity of this class of compounds for the HSA. Furthermore, among all the newly synthesized analogues, (S)-BIC14 shows the lowest affinity for the protein, as well as the lowest predicted log P. In fact, this compound comes from the introduction of a polar amino group on the α-carbon of the lead compound. On the other hand, the ranking of

Sample	B%[a]	miLog P[b]
L-Trp	63.1[c]	
Clonazepam	83.1[c]	
rac-BIC(S)	71.8[d]	2.154
(S)-BIC14	72.5[d]	2.197
(R)-BIC35	90[d]	3.337
(R)-BIC52	90.3[d]	3.429
(R)-BIC	93.3[d]	2.154
(R)-BIC33	93.9[d]	3.416
(R)-BIC60	94.1[d]	3.579
rac-BIC60	94.2[d]	3.579
(S)-BIC32	97[d]	4.512
(S)-BIC20	97.4[d]	3.928
(R)-BIC30	98[d]	4.477
(R)-BIC59	98.3[d]	4.552
(R)-BIC51	98.6[d]	4.765

Table 5.1 Bound fraction (B%) obtained by affinity chromatography and predicted log P values.

[a]Calculated as the average of the results obtained by two injections.
[b]Calculated using online www.molinspiration.com/cgi-bin/properties site (Molinspiration Property Engine v2009.01)
[c]Mobile phase: PB (67 mM, pH 7.4)
[d]Mobile phase: PB (67 mM, pH 7.4)/1-propanol 94:6

the compounds based on the log P parameter does not mirror that one based on the bound fraction values. Therefore, even though the hydrophobicity is an important parameter in determining the interaction between this class of compounds and HSA, it does not appear to be the unique factor influencing this type of interactions.

The significant difference in the binding percentage between the two enantiomers of the rac-bicalutamide, (R)-BIC and (S)-BIC (93.3% and 71.8%, respectively), proves the highly enantioselectivity in the binding ability of this protein [12]. It is well known that the clinical efficacy, as well as the toxicity, of a drug strictly depends from its free fraction. It is therefore interesting to look at the free percentage of a drug derived from the following equation:

$$F\% = 100 - B\% \qquad \text{eq. 5.4}$$

In fact, small changes in the highest bound drug percentages correspond to large variations in their parallel free drug fractions, as we can appreciate in the table below (Table 5.2). Moreover, if we look at the ratio between the F% of the lead compound ((R)-bicalutamide) and the F% of each of the compounds under investigation, it is clear how the former will have a higher pharmacological activity than its analogues, unless there are other events which will balance the binding properties. (R)-bicalutamide is about five times more bioavailable than the compound with the highest bound fraction, (R)-bic51 (Table 5.2).

Sample	F%	F%bic/F%
rac-BIC(S)	28	0.24
(S)-BIC14	27.5	0.24
(R)-BIC35	9.8	0.68
(R)-BIC52	9.7	0.69
(R)-BIC	6.7	1
(R)-BIC33	6.0	1.1
(R)-BIC60	5.8	1.1
rac-BIC60	5.8	1.1
(S)-BIC32	2.9	2.3
(S)-BIC20	2.5	2.6
(R)-BIC30	1.9	3.4
(R)-BIC59	1.7	3.9
(R)-BIC51	1.3	4.9

Table 5.2 Comparison between the free fraction (F%) obtained with HSA by affinity chromatography.

5.3.3 HSA binding site investigation by circular dichroism

Competition experiments were carried out by circular dichroism, in order to identify the high affinity binding site of this class of compounds on HSA and RSA. In this way it was possible to determine the main differences between the binding properties of the two proteins. The change of the induced CD spectrum observed for a [protein]/[marker] complex 1:1 (15 μM) in the presence of increasing concentrations of rac-bicalutamide was monitored. It was not possible to study the behaviour of the newly synthesized

compounds with this technique because of their low solubility: the extremely low concentrations and the high amounts of organic modifier required have prevented form obtaining appreciable signals. The markers employed were chosen for their known ability to bind to specific binding area of these proteins: diazepam (selective binding to benzodiazepine site), ketoprofen (selective binding to site II) and bromophenol blue (selective binding to site III). Both diazepam and ketoprofen bind at site II on HSA, while the two markers bind at different binding sites on RSA. In this analysis rac-bicalutamide was used as the competitor, because of its spectroscopic characteristic. In fact, in order to make easier the experiments the marker should present absorption and an induced CD signal at lower energy with respect to the absorption of the competitor and the protein [12].

Any relevant change of the induced CD spectrum of ketoprofen and diazepam was observed upon adding increasing concentration of rac-bicalutamide up to [protein]/[marker]/[rac-bicalutamide] 1/1/6; suggesting an independent binding with site II and the benzodiazepine site on both the proteins investigated (Fig.5.6, 5.7).

When bromophenol blue was used as marker rac-bicalutamide resulted very efficient in increasing the induced CD spectra of [HSA]/[bromophenol blue] complex, suggesting a positive cooperative binding at bilirubin site, unlike the results obtained with RSA, were an independent binding was recorded (Fig. 5.8).

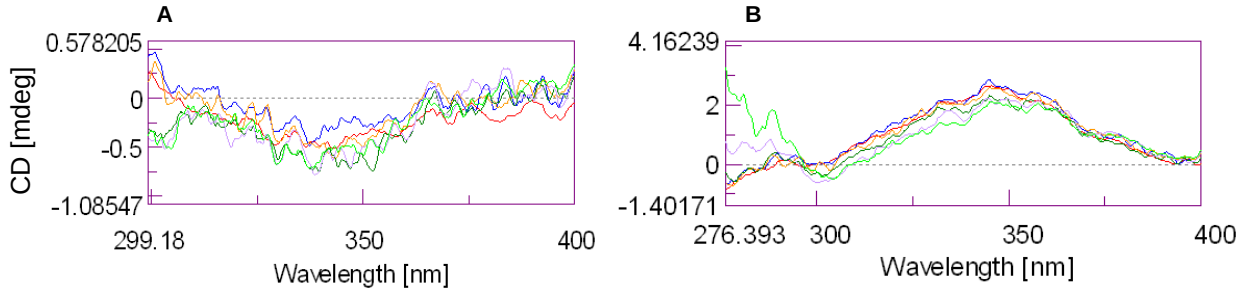

Fig. 5.6 Effect on the induced CD of ketoprofen bound to HSA (A) and RSA (B) upon addition of increasing concentration of rac-bicalutamide to the [HSA]/[marker] (A) and [RSA]/[marker] (B) complexes.

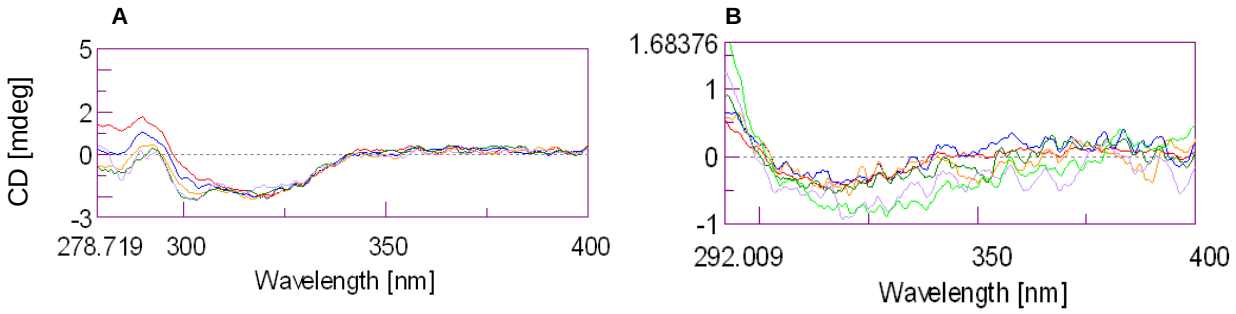

Fig. 5.7 Effect on the induced CD of diazepam bound to HSA (A) and RSA (B) upon addition of increasing concentration of rac-bicalutamide to the [HSA]/[marker] (A) and [RSA]/[marker] (B) complexes.

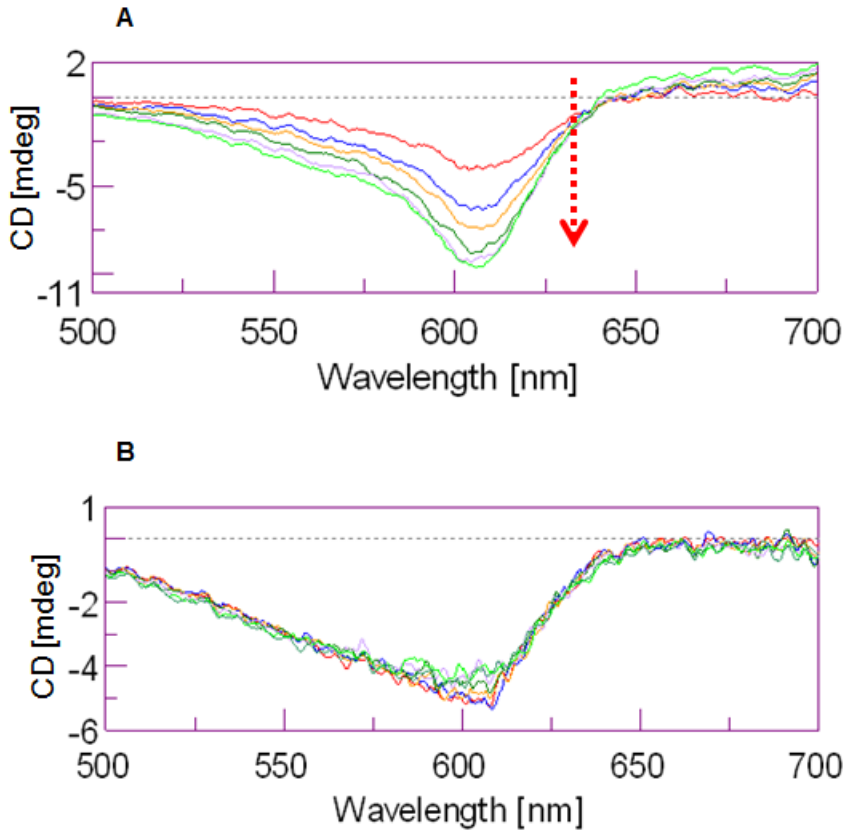

Fig.5.8 Effect of the induced CD of bromophenol blue bound to HSA (a) and RSA (b) upon addition of increasing concentration of (R)-bicalutamide to the [HSA]/[marker] (a) and [RSA]/[marker] (b) complexes.

5.4 Conclusions

A high amount of HSA was correctly immobilized onto the silica matrix, being the used method suitable to maintain the binding and enantiodiscriminating properties of the protein. The good enantioselectivity shown by the HSA-column makes it suitable for both bio-chromatographic and separative applications.

All the compounds analyzed showed high affinity for the HSA (90%≥ B% ≤ 98.6), with the exception of (S)-BIC14 and the (S) enantiomer of the racemic lead compound (rac-bicalutamide). The majority of the analogues investigated showed a higher affinity to HSA comparing to the lead compound. The hydrophobicity highly influence the protein binding level of these compounds, but it seems not to be the only factor characterizing the biomolecular interaction. From the analysis of the calculated free fraction it is clear that the (R)-bicalutamide (lead) is about five times more bioavailable in the human body than the synthesized compound with the highest bound fraction, (R)-BIC51. All these evidences should be taken into account during the design of optimized structures, in order to obtain the right balance between distribution and half-life of the new drugs.

According to the results obtained with CD competition experiments carried out with HSA and RSA, an independent binding at benzodiazepines site and site II was recorded with both the proteins. In the case of HSA, a positive cooperative binding at bilirubin site can be hypothesized, unlike RSA, which has shown an independent binding at the same binding site.

These data demonstrate the importance of getting distribution data with albumin from different species in early stages of drug discovery, in order to avoid misinterpretation of the experimental data obtained from the in vivo experiments conducted on animals.

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[2] WO/2010/116342. Non-Steroidal Compounds for Androgen Receptor Modulation, Processes for the Preparation and Uses Thereof. Applicants: Consiglio Nazionale Delle Ricerche, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) s.r.l. Inventors: Greta Varchi, Anna Tesei, Giovanni Brigliadori. Publication Date: 14.10.2010G. Varchi, A. Guerrini, A. Tesei, G. Brigliadori WO/2010/116342 Non-steroidal compounds for androgen receptor modulation.
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Chapter 6

Optical Biosensor analysis of Bicalutamide analogues interaction with human serum albumin and rat serum albumin

Abstract

The binding of some novel bicalutamide analogues (see section 4.1) for the main plasma protein, albumin, was characterized by using optical biosensor technique (SPR). The study was carried out with, Human Serum Albumin (HSA) and Rat Serum Albumin (RSA), with the aim of highlighting the possible differences among species when performing pharmacokinetic studies.

HSA and RSA were covalently immobilized on carboxymethyl dextran matrixes, via amine coupling allowing the binding properties of the protein to be maintained. The ranking of the bicalutamide analogues for their HSA and RSA bound fractions was performed, to compare the behaviour of the two albumins. The dissociation constants (K_D) of the interaction between the lead compound, (R)-bicalutamide, and the two proteins were calculated. The RSA chip allowed also the K_D evaluation for the RSA highly bounded compounds. Meaningful differences in the results obtained with the two serum proteins have been underlined.

6.1 Experimental

6.1.1 Materials and methods

Human serum albumin (HSA, essentially fatty acid free) was purchased from Calbiochem (La Jolla, CA, USA); rat serum albumin (RSA, essentially fatty acid free and globulin free) and (S)-warfarin were purchased from Sigma-Aldrich (Milan, Italy). Sodium dihydrogen phosphate dihydrate (NaH_2PO_4 x $2H_2O$) was purchased from Merck (Darmstadt, Germany). Sodium chloride, potassium chloride were from Sigma-Aldrich (Buchs, Switzerland); rac-warfarin was obtained from Sigma-Aldrich (Steinheim, Germany). Sodium acetate and sodium hydroxide were from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Switzerland). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine-HCl and the sensor chip with carboxymethylated matrix CM5 were from Biacore (GE Healthcare). Synthesis and physicochemical characterization of the bicalutamide analogues under investigation have been described previously (see section 4.2.1) [1-4]. The structures of the compounds are reported in Fig. 6.1.

The running buffer was prepared by adjusting the pH of NaH_2PO_4 x $2H_2O$ solution with the addition of 1 mM NaOH. Twice distilled and degassed water deionized and filtered with Millipore Elix 3 was used for mobile phase's preparation. The buffer solutions were filtered through a 0.22-µm membrane filter and degassed before use.

6.1.2 Instrumentation

Surface plasmon resonance analyses were performed by using Biacore X100 optical biosensor with Biacore X100 Plus Package (Biacore AB, Uppsala, Sweden). The system was thermostated at 25°C and equipped with research-grade CM5 sensor chips (Biacore). Biosensor data were analyzed using Biacore™ X100 2.0 Evaluation Software. Data processing was performed with BIAevaluation software.

6.1.3 Serum albumin immobilization

The human serum albumin surface was prepared by using standard amine-coupling method [5]. The HSA was coupled through his surface amine groups via amine

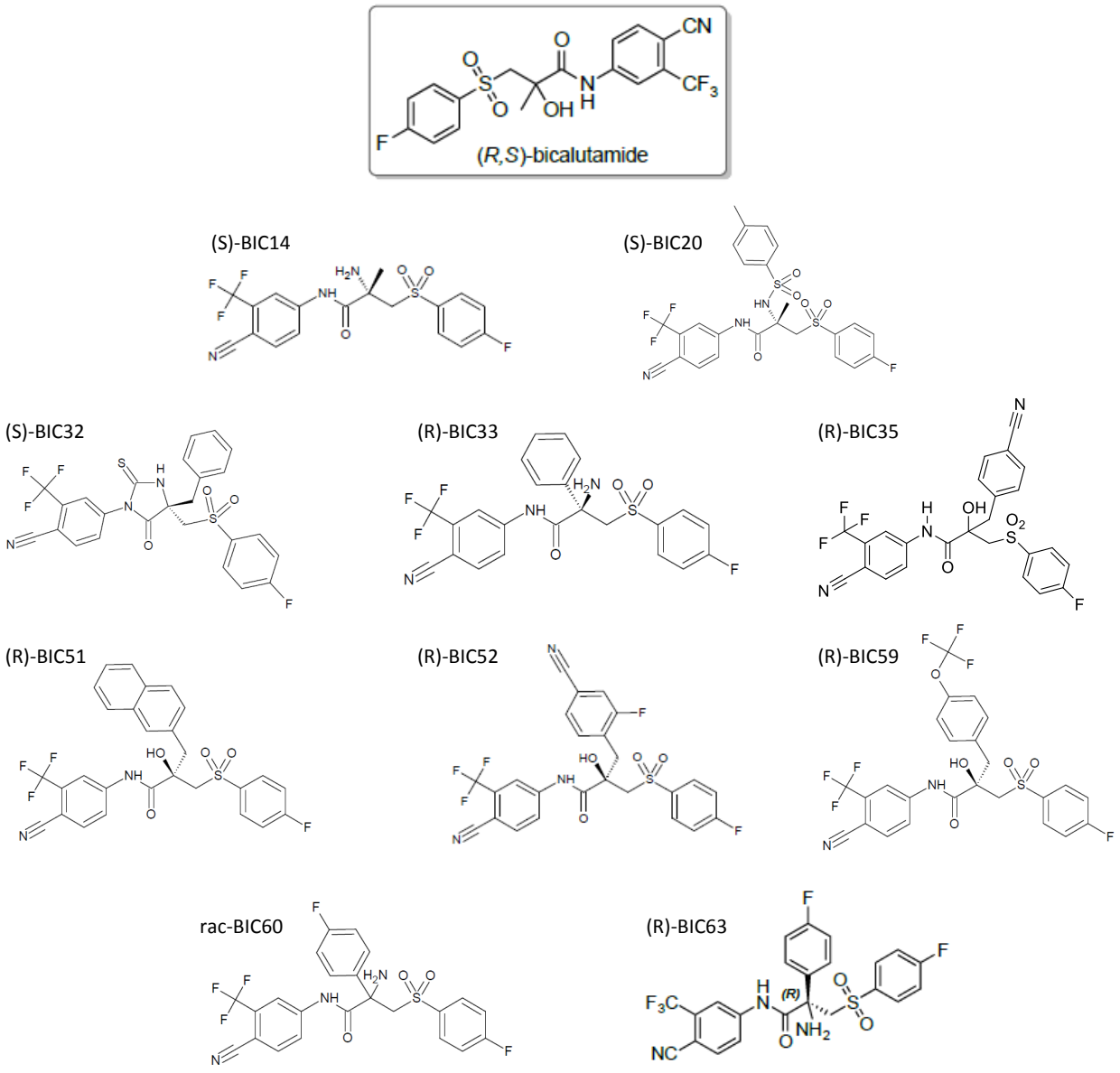

Fig. 6.1 Structures of (R)-bicalutamide and its analogues.

bonds with the CM5 sensor chip of the Biacore X100 instrument. The running buffer was PBS (Phosphate buffered saline: 10 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4) and the temperature was set at 25 °C. The protein was dissolved at 50 µg/mL in 10 mM Sodium Acetate pH 5.0. Carboxyl groups on the dextran layer of the chip were activated by injecting a 1:1 mixture of 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 7 min at a flow rate of 10 µL/min. The HSA solution was injected in channel 2

and was allowed to react for 7 min at a flow rate of 10 µL/min. The remaining carboxyl groups were blocked by injecting a solution 1 M ethanolamine for 7 min at a flow rate of 10 µL/min. The protein was immobilized only in one channel of the sensor chip in order to monitor the possible non-specific bindings by means of the second channel. The reference control channel was only activated with 1:1 EDC/NHS mixture, and then treated with ethanolamine to block NHS-ester groups. The HSA immobilization experiment is summarized in the sensorgram shown in Fig. 6.2, where the optical biosensor response (resonance units) is reported as a function of time.

The protein was immobilized on a second CM5 chip, in order to determine the reproducibility of the assays at different immobilization levels of the protein. The second immobilization experiment was carried out in manual run conditions, in order to control the run interactively. The protein was dissolved at 25 µg/mL in 10 mM Sodium Acetate pH 5.0. The chip was activated by injecting a 1:1 mixture of EDC/NHS for 7 min at a flow rate of 5 µL/min. The HSA solution was injected in channel 2 and was allowed to react for 12 min at a flow rate of 5 µL/min (6 injections of 2 min each). The remaining carboxyl groups were blocked by injecting a solution 1 M ethanolamine for 7 min at a flow rate of 5 µL/min. Two pulse of 12 s of 50 mM NaOH were injected to wash non covalently bound protein and stabilize the baseline.

Rat serum albumin was also immobilized on the dextran matrix surfaces of two different sensor chips, following the same procedure as the first HSA chip. The concentration of RSA solutions utilized for the immobilizations were 50 µg/mL and 20 µg/mL for the first chip and the second one, respectively.

After the immobilization the sensor chip surfaces were stabilized by various injections of running buffer at 30 µL/min all over the night.

All the sensor chips were stored in a refrigerator every night after the experiments, in a 50 ml tube containing running buffer so that the support was completely covered.

6.1.4 Preparation of sample solutions

Rac-warfarin and S-warfarin were freshly prepared as 1 mg/mL stock solutions and diluted with running buffer (PBS) immediately prior to use. A suitable range of concentrations (1 µM - 300 µM) was obtained by diluting the stock solutions with the

running buffer. In order to avoid disturbance of the baseline, all the dilutions were done directly into the analysis tubes.

All the compounds under investigations were analyzed in the presence of DMSO (5% v/v) in the running buffer as well as in the sample solutions. The running buffer for these analyses was prepared as already described: firstly, a 1.05 x PBS was prepared and the pH was adjusted to 6.85 by addiction of NaOH so that after adding the 5% v/v of DMSO the pH value became 7.4 [6]. R-bicalutamide, rac-warfarin and S-warfatin stock solutions were prepared in DMSO at 2 mg/mL, whereas all the bicalutamide analogues were stocked in DMSO at 1 mg/mL. Immediately prior to analysis, stock samples were diluted 1:20 with 1.05 x PBS; further dilutions were made by diluting the previous solutions with the running buffer containing 5% of DMSO. The running buffer and the samples solutions should contain the same percentage of DMSO, because of the high refractive index contribution of DMSO which may cause important variations in the final SPR signals.

6.1.5 Solvent Refractive Index correction

A DMSO calibration procedure was included in the assay protocols, in order to eliminate the bulk responses caused by the presence of a high refractive index solvent (DMSO). Especially when working with small molecules, the solvent refractive index correction becomes essential. In fact, in this case the expected analytes binding responses and those due to the bulk response could have the same order of magnitude and lead to big misunderstanding of the final data. So that a series of eight buffer solutions containing concentrations of DMSO ranging from 4.5 - 5.8 % (v/v) were injected in sequence over reference and HSA surfaces. A DMSO calibration plot was built by plotting the difference in response between HSA and reference flow cells versus the response in the reference flow cell [7]. The DMSO calibration plot was built every day, prior to start with the binding analysis. All data collected were fixed with this curve.

6.1.6 Ranking analysis

The running buffer used was PBS 10 mM with 5% (v/v) DMSO, pH 7.4. In the ranking format the compounds were injected over the HAS and RSA surfaces at a concentration value of 25 µM, with the flow rate set at 10 µL/min. Ligands were allowed

to associate with the protein for 60 s and dissociation was recorded for 10 min. After each injection a buffer injection was included to check the possible carry-over of the analyte to the next cycle and to re-stabilize the chip surface. All samples were injected in duplicate.

6.1.7 Equilibrium dissociation constant determination

Rac-warfarin, S-warfarin and the lead compound of this class of molecules, (R)-bicalutamide, were investigated for their HSA and RSA equilibrium dissociation constants (K_D) determination. (R)-BIC51, (S)-BIC20 and (S)-BIC32 were also analyzed for the determination of their RSA dissociation constants. A wide range of analyte concentrations were tested over the albumin chips at a flow rate of 30 µL/min, at 25 °C. Buffer blanks were injected periodically in order to clean the chip surface preventing the accumulation of the analyte on the sensor chip. All sensorgrams were processed by using double referencing method (see section 4.2.6). Rac-warfarin and S-warfarin were analyzed in the presence and in the absence of DMSO in the running buffer, instead (R)-bicalutamide and its analogues were always analyzed in the presence of 5% (v/v) of DMSO in the running buffer. The association phase was followed for 60 s, being the kinetic of the interaction very fast. The dissociation phase was followed for 60 s when rac-warfarin, (S)-warfarin and (R)-bicalutamide were the analytes; 10 min were needed when the bicalutamide analogues were used as analytes. Equilibrium responses at different concentrations were plotted as a function of drug concentrations and then fitted with the specific interaction model. Concerning (R)-bicalutamide and its analogues, a single-site interaction model was applied. This approach leads to a unique K_D which corresponds to the higher affinity site constant:

$$R_{eq} = \frac{R_{max} \times [L]}{K_D + [L]} \qquad \text{eq. 6.1}$$

where R_{eq} is the SPR response at equilibrium; R_{max} the maximum capacity of the ligand for the ligate; [L] the analyte concentration and K_D the dissociation constant.

Rac-warfarin dissociation constant determination was carried out by fitting the equilibrium responses obtained with an independent two site interaction model, in order

to identify the K_D values of its primary and secondary affinity binding sites on the protein:

$$Y = \frac{R_{max1} \times [L]}{K_{D1} + [L]} + \frac{R_{max2} \times [L]}{K_{D2} + [L]} \quad \text{eq. 6.2}$$

A four-site model was used to calculate the affinity of (S)-warfarin with RSA, in order to find not only the K_D value corresponding to its primary binding site on the protein, but also the K_D of the lower affinity binding sites where weak interactions contribute to the realization of the drug-protein complex. This was possible by using the following equation:

$$Y = \frac{R_{max1} \times [L]}{K_{D1} + [L]} + \frac{R_{max2} \times [L]}{K_{D2} + [L]} + \frac{R_{max3} \times [L]}{K_{D3} + [L]} + \frac{R_{max4} \times [L]}{K_{D4} + [L]} \quad \text{eq. 6.3}$$

6.2 Results and Discussion

6.2.1 Immobilization of serum albumin

HSA and RSA were immobilized on a CM5 chip. This carboxymethylated dextran matrix represents the most versatile chip available; it holds an excellent chemical stability and provides a high capacity to immobilize a wide range of ligands, such as proteins, carbohydrates, lipids, nucleic acids and small organic molecules. The amine coupling method was used to immobilize the two proteins. A typical sensorgram of the serum albumin immobilization procedure is shown in Fig. 6.2. Since the covalent binding occurs trough primary amine groups on the proteins, the running buffer and the buffer used to dissolve the protein should not contain primary amine groups. Moreover, in order to maximize the efficiency of the immobilization step a suitable electrostatic pre-concentration of the proteins on the sensor chip must be achieved. To this purpose the pH of the immobilization buffer was 5, a value between the pk_a of the surface (pH > 3.5) and the isoelectric point (pI) of the ligand (pI = 5.67 [8]); so that the ligand acquires a positive charge and results in an efficient pre-concentration into the negatively charged carboxymethyl dextran matrix.

The reference channel was reacted as the active channel, without anchoring the

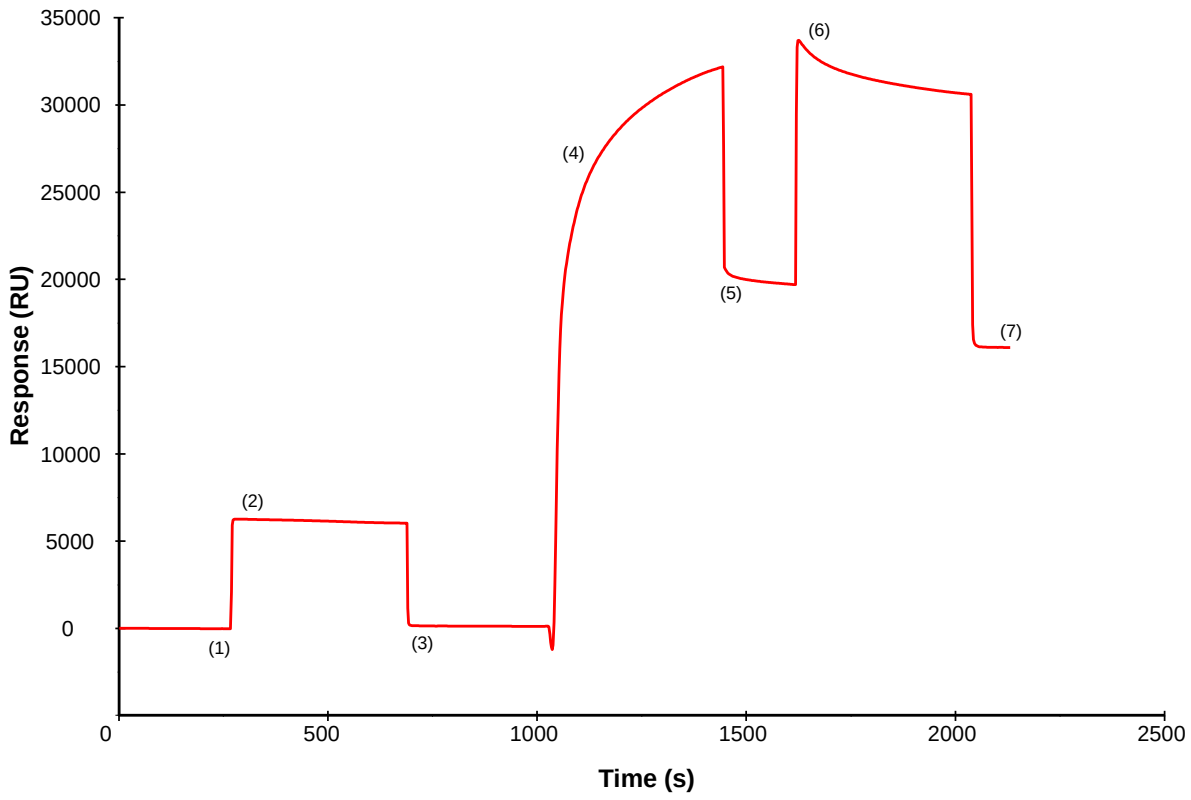

Fig. 6.2 Sensorgram of the Human Serum Albumin immobilization to the carboxymethyl dextran matrix: (1) buffer baseline stabilization; (2) EDC/NHS mixture add; (3) buffer re-equilibration; (4) HSA in acetate buffer add (7 minutes of reaction); (5) buffer washes; (6) block of non-coupled activated CMD sites with ethanolamine (7 min of reaction); (7) baseline stabilization.

protein. In this way all the non-specific binding phenomena on the biosensor surface were monitored. After the first HSA immobilization, the amount of immobilized protein results in 16114 RU.

In order to determine the reproducibility of the assays, another HSA immobilization was performed following the same chemical strategy, but using a lower HSA concentration and the manual run windows of the Biacore^{TM} X100 2.0 Control software, in order to check in real time the amount of protein immobilized. The final amount on the second sensor chip corresponds to 5572 RU (Fig. 6.3). The lower immobilization levels reached ensure to avoid artefacts due to high ligand density on the chip and mass-transport limited binding analysis. Moreover, it was possible to assess whether different protein densities could influence the data collected.

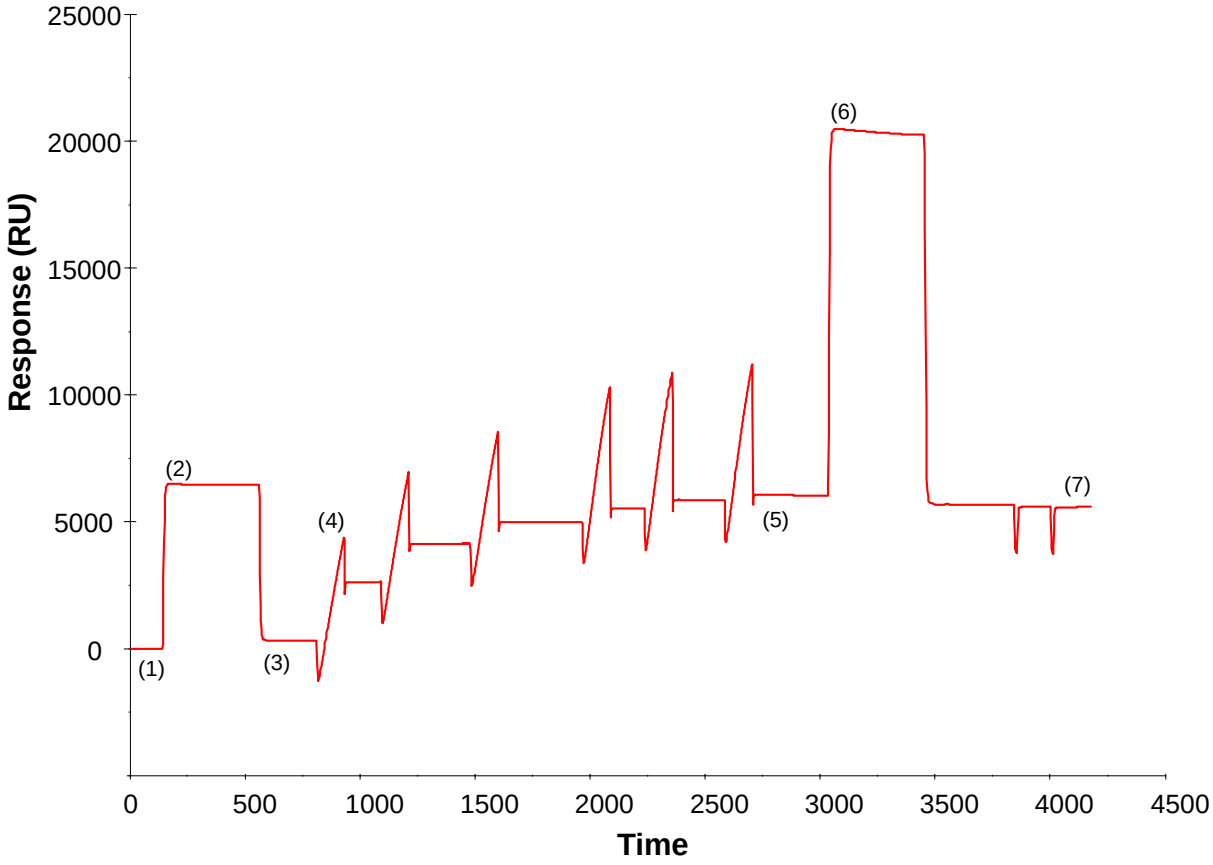

Fig. 6.3 Sensorgram of the Human Serum Albumin immobilization to the carboxymethyl dextran matrix performed in manual run mode: (1) buffer baseline stabilization; (2) EDC/NHS mixture add; (3) buffer re-equilibration; (4) HSA in acetate buffer add (7 minutes of reaction); (5) buffer washes; (6) block of non-coupled activated CMD sites with ethanolamine (7 min of reaction); (7) baseline stabilization.

The binding of rac-warfarin was used to check that the two immobilization processes did not involve the principal binding site on the protein. As a result the experimental K_D of the reference compound obtained with both two chips were in agreement to that reported in the literature (see section 6.2.3) [9, 10].

Concerning RSA, the two coupling procedures result in 12000 RU and 8910 RU of protein immobilized, with the two different CM5 chips. Also in this case S-warfarin was used as reference to check the dependability of the RSA sensor chip (see section 6.2.3) [11 - 13].

6.2.2 Ranking analysis

HSA investigation

(R)-bicalutamide and seven of its analogues, (S)-BIC14, (S)-BIC20, (S)-BIC32, (R)-BIC33, (R)-BIC51, (R)-BIC59 and rac-BIC60, were ranked for their HSA binding level. To this purpose only one concentration value of ach compound were utilized. The analytes were floated over the HSA surface at 25 µM, at low flow rate (10 µL/min) in order to promote the small molecule-protein interactions. The concentration chosen was high enough to give a reasonable signal but also low enough to avoid misinterpretation of data due to interaction with several binding sites on the protein [7]. The SPR maximum response after a 60 s injection was recorded for each of the compounds analyzed. Being the SPR response directly correlated with the specific molecular mass of the analytes, all the data collected from this analysis were divided for this value, in order to obtain comparable molar values (Fig. 6.4).

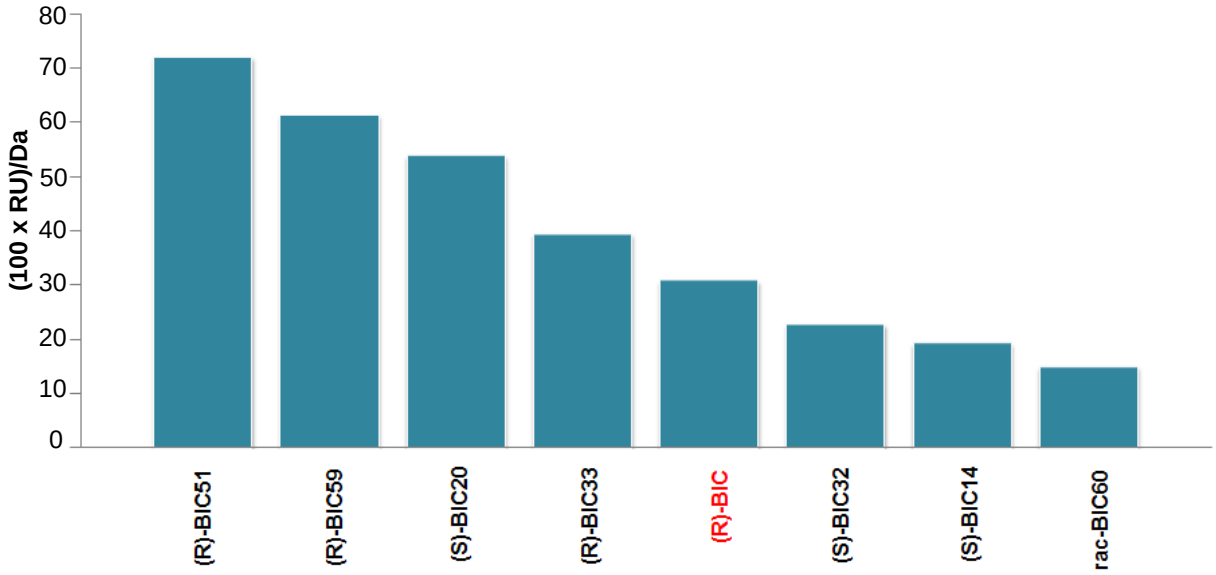

Fig. 6.4 Ranking analyses of bicalutamide analogues performed with the HSA chip at a concentration of 25 µM in PBS 10 Mm + 5% DMSO, pH 7.4. Binding data were adjusted for the molecular weights of each compounds.

(R)-BIC51, (R)-BIC59, (S)-BIC20 and (R)-BIC33 showed higher HSA affinity than the lead compound, (R)-bicalutamide; whereas (S)-BIC32, (S)-BIC14 and rac-BIC60 possess lower affinity for the protein. All the strongly bound compounds show a

voluminous and lipophilic substituent in their structure; on the contrary the lowest binders have hydrophobic (as in the case of (S)-BIC14) or slightly voluminous substituents ((S)-BIC32 and (rac)-BIC60). Therefore we can hypothesize that the presence of a large, lipophilic substituent on the chiral stereocenter of the reference compound allows a better stabilization of the newly synthesized molecule in the binding pocket of the protein, thus increasing the affinity for the protein, as well as the stability of the specific complex.

It was possible to compare the HSA affinity data of this class of compounds achieved with both SPR and HPALC techniques (see Chap. 5). What immediately stands out is that in both cases (R)-BIC51, (R)-BIC59, (S)-BIC20 and (R)-BIC33 showed the highest affinity for the protein; as well as in both cases (S)-BIC14 is one of the compounds with the lowest affinity for the HSA. On the other hand, (S)-BIC32 and rac-BIC60 showed different behavior with the two techniques.

The achieved results have proven the suitability of the SPR technique as a high-throughput screening method in the target binding level identification of new classes of pharmaceutical compounds. The comparison of this technique with the more conventional affinity chromatography highlights at once the major advantages of SPR biosensor technique. First of all, only few micrograms of protein are required to perform a chip immobilization and little volumes of buffers and samples are needed during all the experiments. Furthermore, there is a huge improvement in the analysis time: usually 20-25 min are needed for ligand immobilization (against the 2 days needed to immobilize a ligand on the silica matrix support in HPALC) and only 4-5 min are required for a complete characterization of a ligand-analyte interaction (60 min for the most bounded compounds in HPALC). Therefore this technique resulted well-suited to classify rapidly newly synthesized compounds in low-, medium- and high-level binders.

RSA investigation

All the compounds showed in Fig. 6.1 were tested for their RSA affinity on the basis of the binding level achieved after injection of a single concentration (25 µM), in the same experimental condition used for the previous HSA experiments. The RSA ranking results are shown in the figure below (Fig. 6.5). It was possible to compare these data with the SPR and HPALC data acquired with human serum albumin.

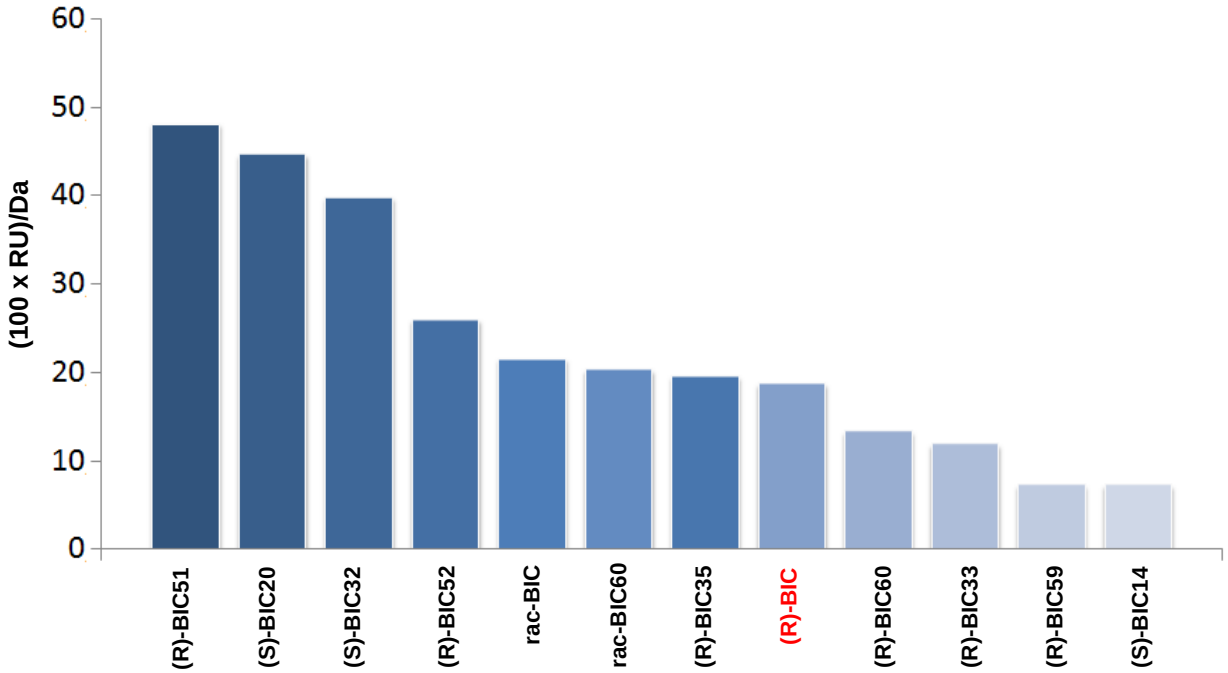

Fig. 6.5 Ranking analyses of bicalutamide analogues performed with the RSA chip at a concentration of 25 μM in PBS 10 Mm + 5% DMSO, pH 7.4. Binding data were adjusted for the molecular weights of each compounds.

(R)-BIC51 and (S)-BIC20 showed the highest affinity for RSA, whereas (S)-BIC14 represents the more weakly bound analogue. Therefore also in the case of RSA the presence of a big, lipophilic substituent on the chiral stereocenter of the reference molecule allows a better accommodation of the analogues in the binding pocket of the protein, as already seen with HSA.

One of the major differences involves the binding ability of (R)-BIC59, which is one of the strongly bound compounds to HSA, but it shows very low affinity for RSA. The same result was found for (R)-BIC33, characterized by a low binding ability for RSA, unlike the results obtained with HSA, by using both SPR and HPALC techniques. These molecules have substituents characterized by a relatively good flexibility, compared with the RSA highly bounded analogues. This characteristic probably helps the stabilization of the HSA-drug complex, but not the RSA-drug interaction. Another interesting difference is the influence of the benzonitrile substituent, which gave opposite effects on the binding to the two proteins. This is clear if we look at the binding level reached with (R)-BIC35 and (R)-BIC52, when both RSA and HSA were used. In the first case, the

presence of this substituent increases the affinity for the target protein, whereas no significant changes were found in the case of HSA. In both cases, the adding of a fluorine atom to the structure of (R)-BIC52 determines the increase of the affinity. This last effect is more evident with RSA, than with HSA.

RSA demonstrates a huge enantioselectivity effect in its binding ability and also in this case big differences with the HSA were found. The racemate form of the lead compounds results in a higher affinity for the RSA than its (R) enantiomer; the investigation of rac-BIC60 and (R)-BIC60 led to the same results. We can therefore hypothesize a higher interaction of the (S) enantiomers of both the compounds investigated with rat albumin. The HSA investigation of the same analogues by means of HPALC resulted in very different conclusions: concerning the lead compound, the (R) enantiomer showed a bound percentage much higher than those obtained with the (S) enantiomer; instead (R)-BIC60 and rac-BIC60 possess quite the same binding affinity for the protein.

These data confirm that the extrapolation of values obtained with albumin from different species should be done carefully, since these very similar proteins often show big differences in their binding ability.

6.2.3 Equilibrium dissociation constants determination

The compounds under investigation bound reversibly to both HSA and RSA, so that the association and dissociation phases of the interaction were followed.

First of all, rac-warfarin was investigated for its binding to the human serum protein, in order to check that the immobilization step does not impact the obtained binding results when compared to the results obtained in solution. The interaction was monitored firstly in pseudo-physiological condition (PBS 10 mM, pH 7.4) and after in the presence of 5% (v/v) of DMSO in the running buffer. During the injection of all the set of concentrations under investigation (about 1 µM - 300 µM) a steady state was reached in few seconds; also the dissociation occurred very rapidly when the analyte solution was replaced with the running buffer. The Figure 6.6 showed the overlaid SPR responses plotted as a function of time. It is well known that rac-warfarin binds to two different classes of binding sites on the HSA, so using a two sites binding equation on Biacore's

software (eq. 6.2) it was possible to fit equilibrium responses at different concentrations to obtain the two equilibrium dissociation constants (Fig. 6.7).

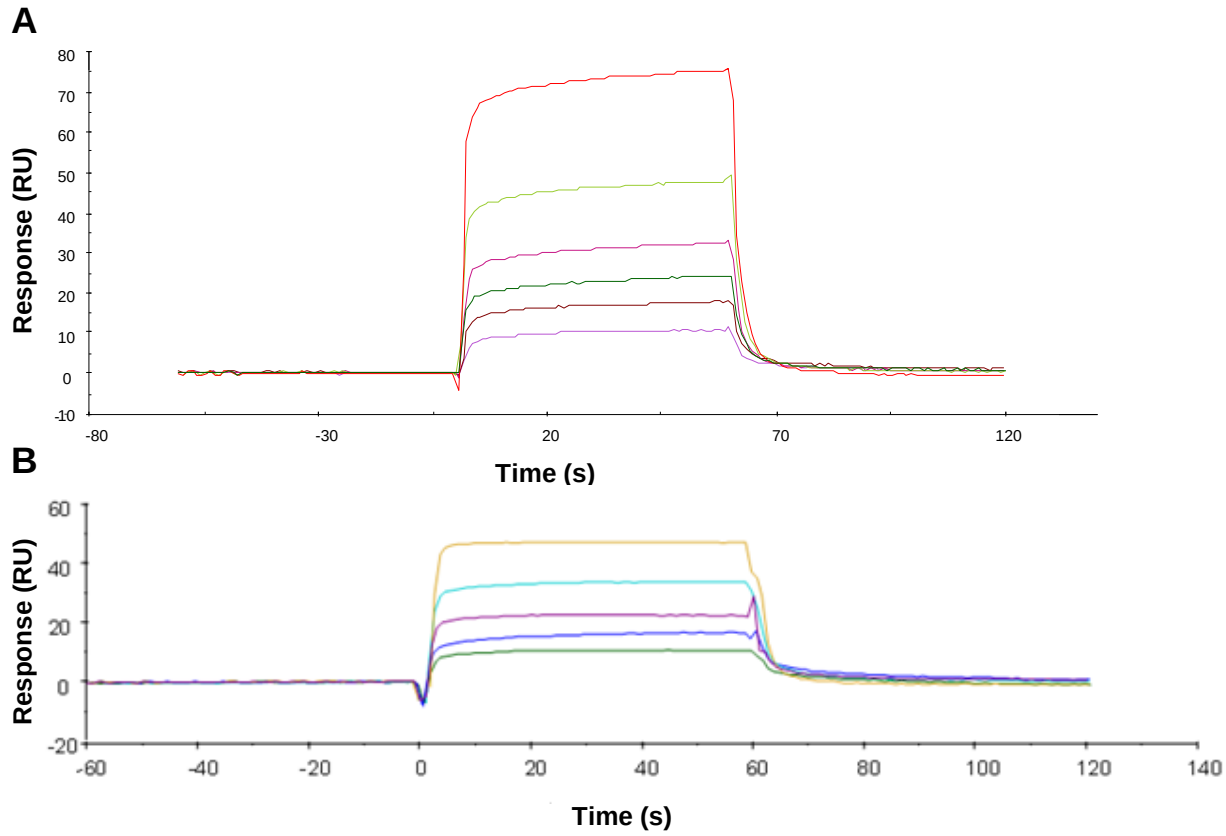

Fig.6.6 Corrected overlays sensorgrams for rac-warfarin binding to human serum albumin, obtained using different rac-warfarin concentrations (about 1 µM - 300 µM). (A) Buffer: PBS 10 mM, pH 7.4 (B) Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

The experimental K_D values (Table 6.1) are in good agreement to those reported in literature obtained by using the same technique [9], as well as with those obtained by independent techniques [10]. These data prove that the protein was correctly immobilized on the sensor chip and that the presence of DMSO does not disturb the binding ability of the anchored protein.

In order to establish the reliability of the RSA sensor chip, (S)-warfarin was used as reference compound. The same experimental condition and samples preparation as for the HSA chip validation were adopted for the RSA chips. The concentrations used ranged from 1 µM to 300 µM for the analysis in pseudo-physiological conditions (PBS 10 mM, pH 7.4), and from 4 µM to 600 µM for the analysis in the presence of 5% of

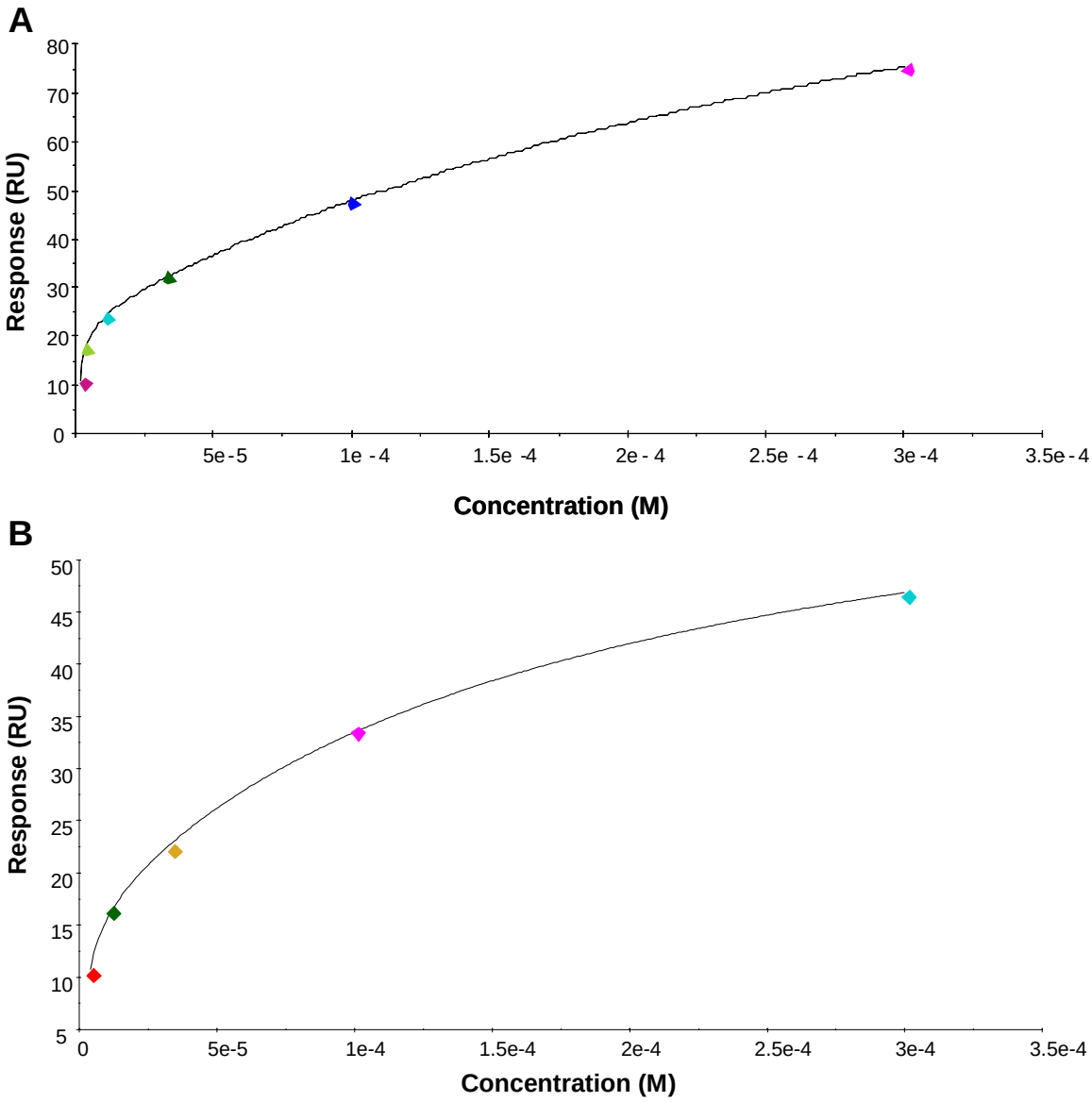

Fig. 6.7 Response data of HSA binding at equilibrium versus rac-warfarin concentration. (A) Buffer: PBS 10 mM, pH 7.4 (B) Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

	HSA (16113.9 RU)	**HSA (557.4 RU)**	**Literature**
K_{D1}	1.6×10^{-6} M [a] 2.4×10^{-6} M [b]	7.2×10^{-6} M [a] 1.8×10^{-6} M [b]	6.8×10^{-6} [c] 4×10^{-6} [d]
K_{D2}	3.88×10^{-4} M [a] 1.8×10^{-4} M [b]	9.9×10^{-4} M [a] 3.3×10^{-4} M [b]	1.0×10^{-4} [c] 1.49×10^{-4} [d]

Table 6.1 Rac-warfarin K_D values.

[a] Buffer: PBS 10 mM, pH 7.4
[b] Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4
[c] [9]
[d] [10]

DMSO. According to literature data, (S)-warfarin binds at four different binding pockets in the rat albumin structure; in particular RSA has two sets of binding sites: one high-affinity site and three of much lower affinity [11]. Therefore, a four-site model was used to calculate the affinity of (S)-warfarin to this protein (eq. 6.3), which yielded four different K_D values: 7.625 x 10^{-6} M, 9.481 x 10^{-5}, 5.752 x 10^{-4} and 2.005 x 10^{-4} (Fig. 6.8). These data are in good agreement with the values found in literature [11-13]. The same experiments was repeated different times on the same RSA sensor chip and confirmed with the second one.

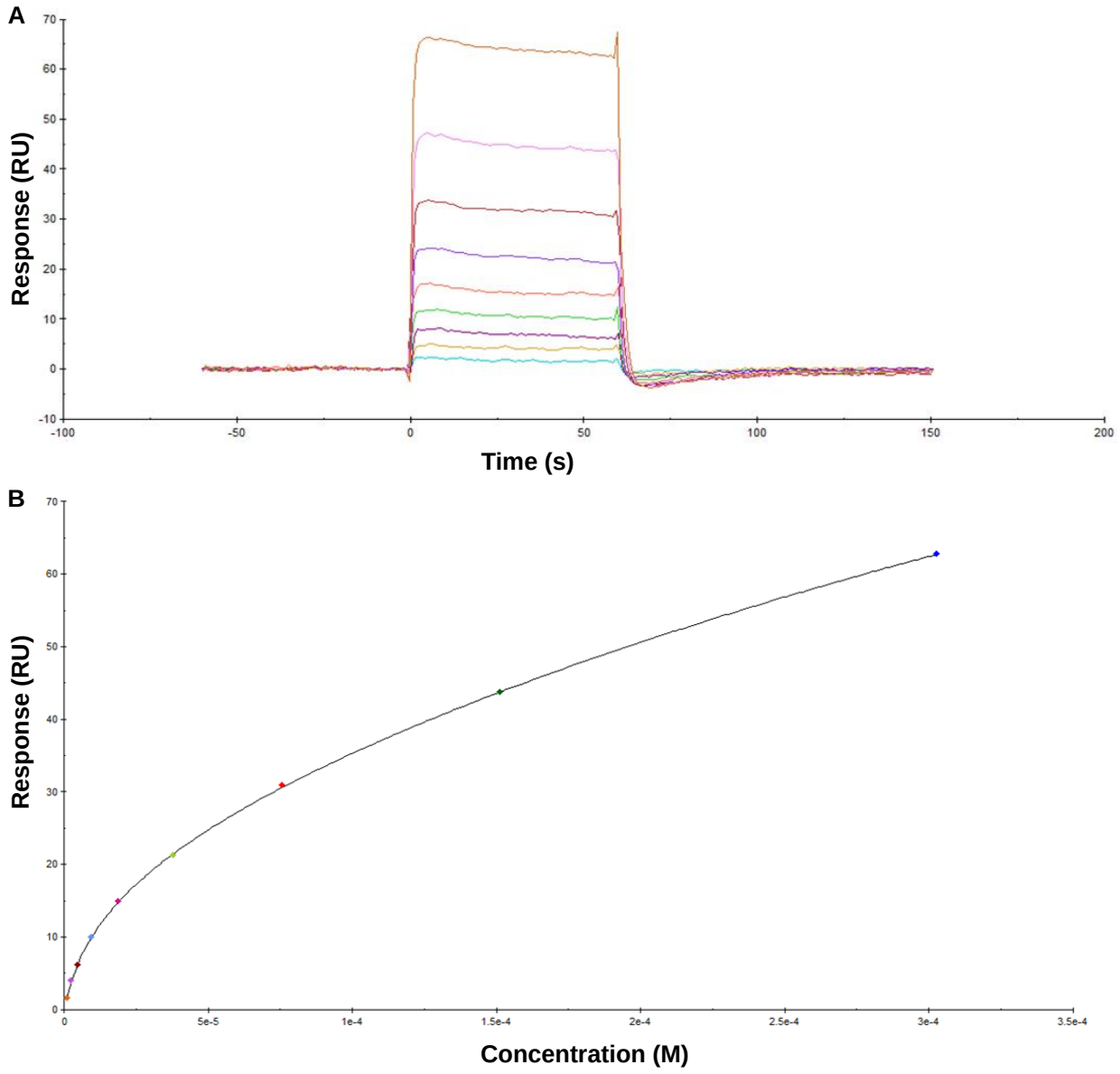

Fig.6.8 (A) Corrected overlays sensorgrams for (S)-warfarin binding to rat serum albumin, obtained using different (S)-warfarin concentrations (about 1 µM - 300 µM) in pseudo-physiological condition (PBS 10 mM, pH 7.4). (B) Response data at equilibrium versus (S)-warfarin concentration.

Since (R)-bicalutamide showed very low solubility, its K_D determination was conducted in the presence of 5% (v/v) of DMSO in the running buffer, as well as in the sample solutions, with both HSA and RSA sensor chips. A wide range of (R)-bicalutamide concentrations were floated over the sensor chip surfaces, a series of concentrations ranging from 3 µM to 200 µM when HSA was the ligand; and from 5 µM to 350 µM when RSA was used as the ligand. Also in this case both the association and dissociation phases were very rapid, as we can see in the Figure 6.9 A and 6.10 A. A single-site model (eq. 6.1) was used to fit the binding data obtained (Fig. 6.9 B, 6.10 B). The value of K_D calculated with the highest density HSA sensor chip was 1.8 x 10^{-5} M, value in good agreement with those obtained in the same experimental conditions with the lowest density chip (1.6 x 10^{-5} M). The dissociation constant found with the RSA chip was 3.8 x 10^{-4} M, one order of magnitude lower than those observed with HSA. The data confirm once again the importance of studying the drug distribution with albumins from different species in an early phase of drug discovery.

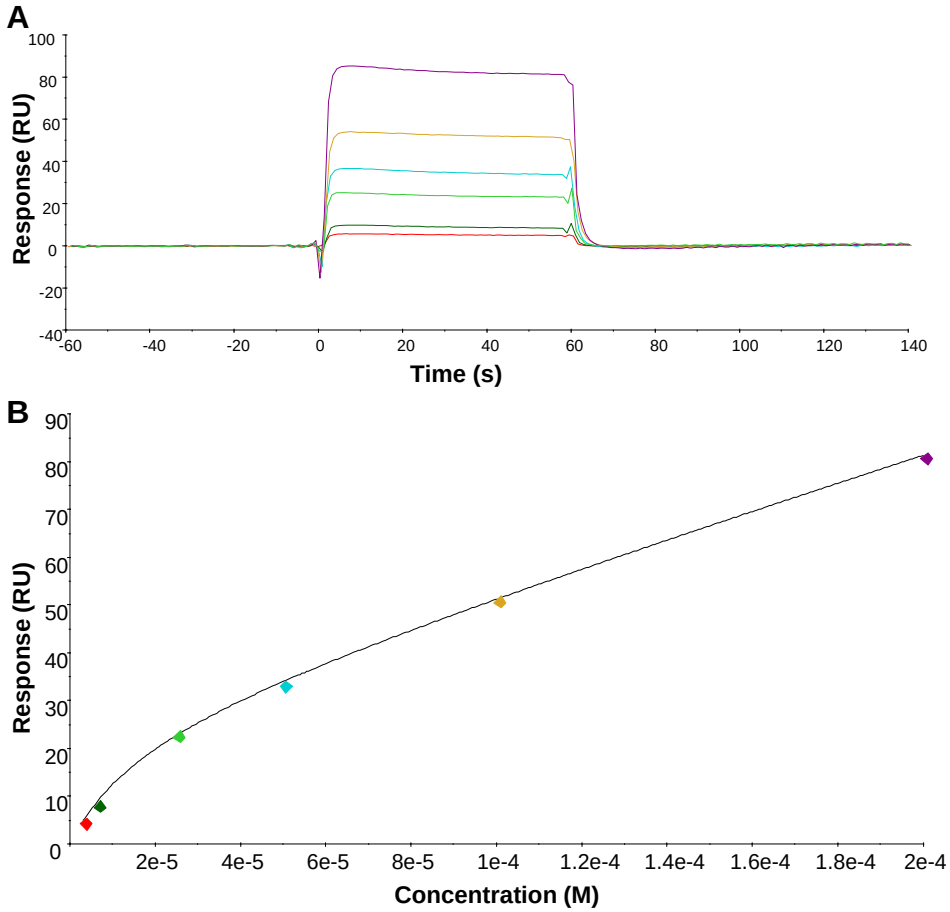

Fig.6.9 (A) Corrected overlays sensorgrams for (R)-bicalutamide binding to human serum albumin, obtained using different (R)-bicalutamide concentrations (about 3 µM - 200 µM). (B) Response data at equilibrium versus (R)-bicalutamide concentration. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

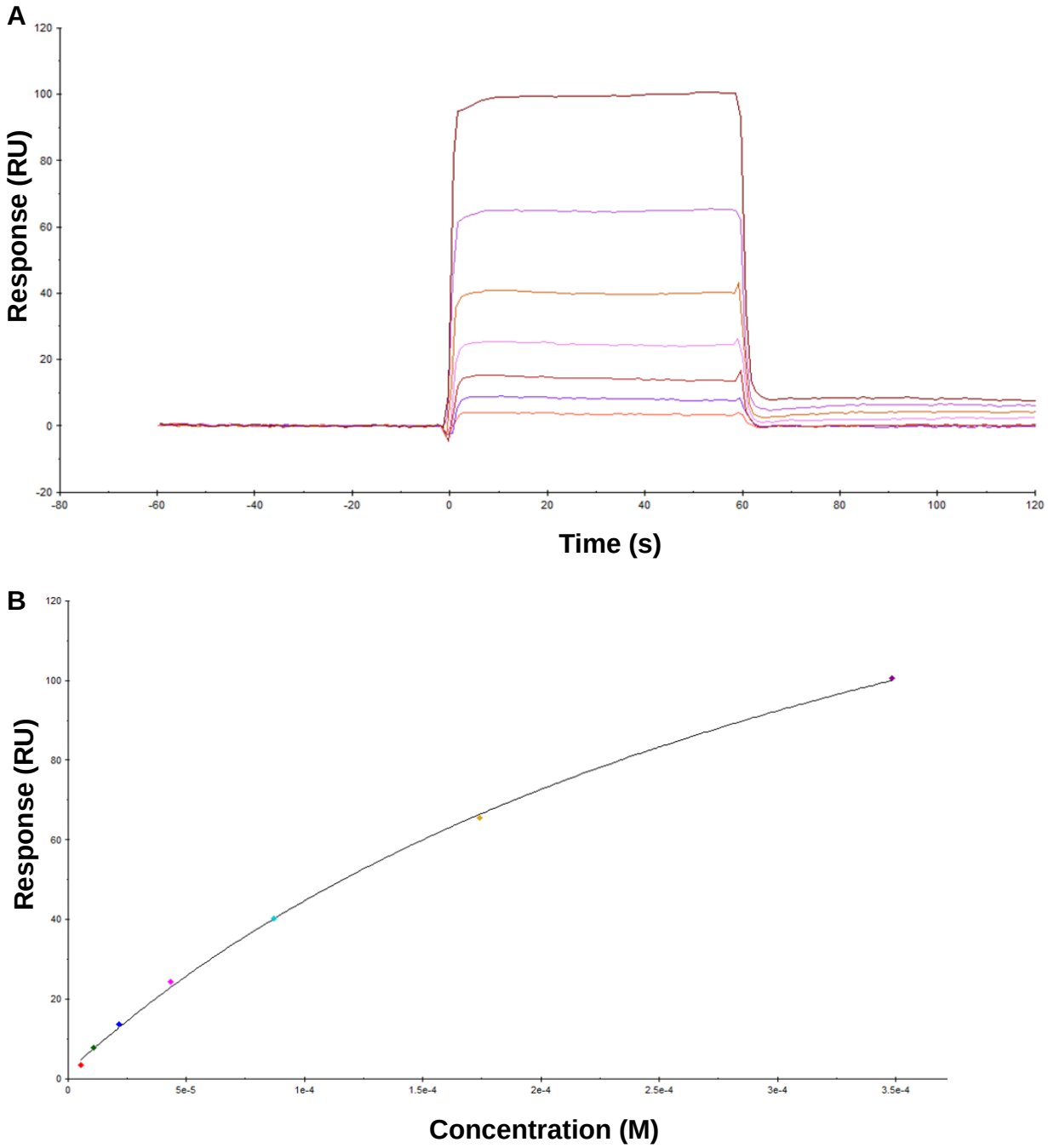

Fig.6.10 (A) Corrected overlays sensorgrams for (R)-bicalutamide binding to rat serum albumin, obtained using different (R)-bicalutamide concentrations (about 5 µM - 350 µM). (B) Response data at equilibrium versus (R)-bicalutamide concentration. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

RSA-bicalutamide analogues study

The RSA sensor chip was used to evaluate the dissociation constants of the complexes formed between the highly bound compounds and the RSA. A wide range of

concentrations (2 µM – 90 µM) of (R)-BIC51, (S)-BIC20 and (S)-BIC32 were injected over the RSA surface. Because of the poor solubility of the tested compounds all the analysis were conducted in the presence of 5% (v/v) of DMSO. After 60 s injection a steady-state plateau was reached. The dissociation phases were followed for 10 min, since a complete regeneration of the sensor chip was difficult to achieve. The binding responses of each compound plotted as a function of time are reported in Fig. 6.11. A single site equation was applied to analyze the plot of the SPR equilibrium responses at different analyte concentrations (Fig. 6.12).

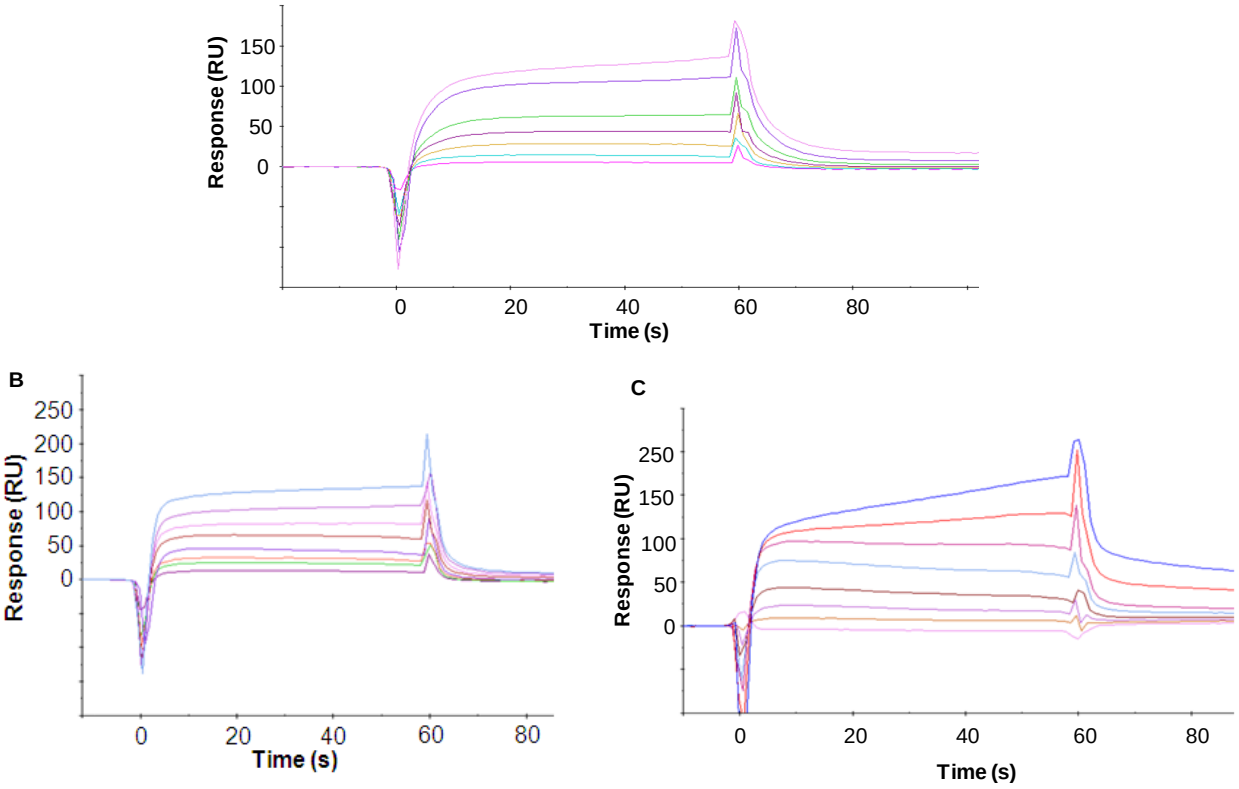

Fig.6.11 Corrected overlays sensorgrams for (A) (R)-BIC51, (B) (S)-BIC20 and (C) (S)-BIC32 binding to rat serum albumin, obtained using different analyte concentrations (about 2 µM - 90 µM). Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

After an accurate evaluation of the data collected, K_D values of 3.18 x 10^{-5} M, 5.55 x 10^{-5} M and 6.41 x 10^{-5} M were calculated for (R)-BIC51, (S)-BIC20 and (S)-BIC32, respectively. The experimental K_D obtained are consistent with the ranking results discussed above (see section 6.2.2).

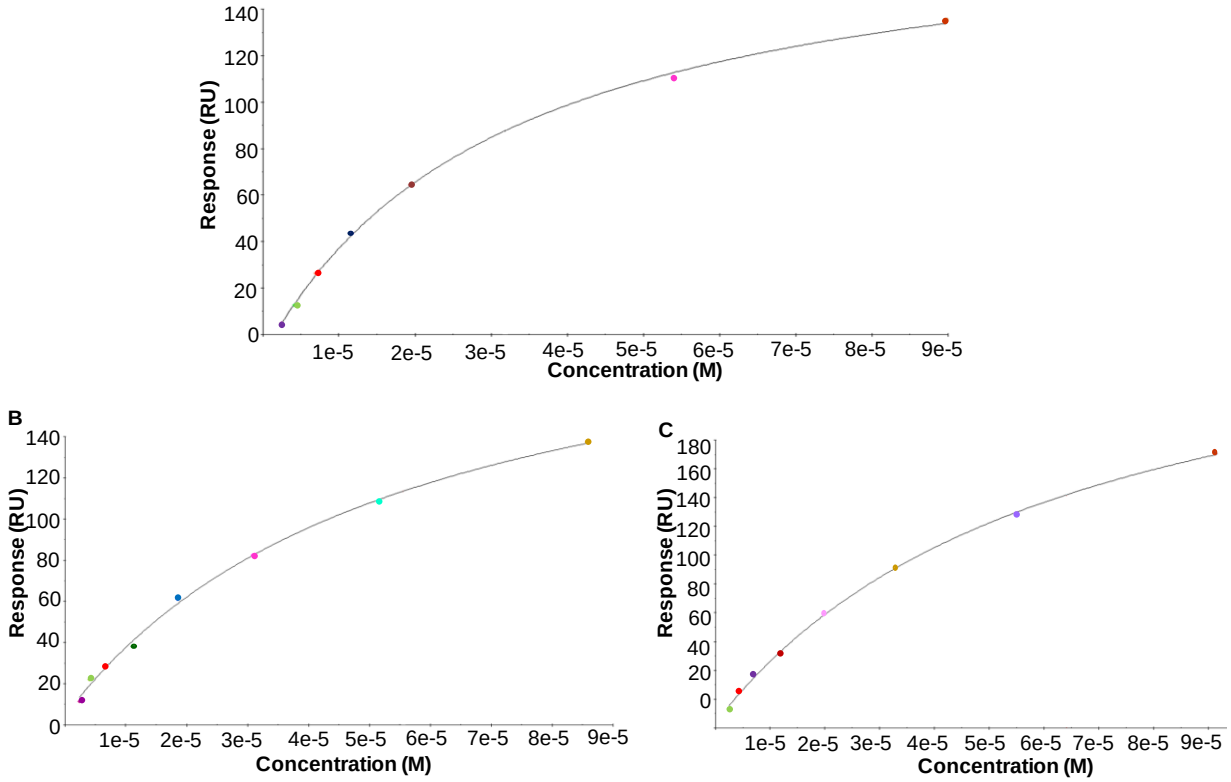

Fig. 6.12 Response data at equilibrium versus (A) (R)-BIC51, (B) (S)-BIC20 and (C) (S)-BIC32 concentrations. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

6.3 Conclusions

Human serum albumin and rat serum albumin were immobilized on CM5 sensor chips via amine coupling to investigate the binding parameters of some novel bicalutamide analogues with these two proteins. Two different immobilization processes were carried out for each protein, leading to different protein immobilization levels. Rac-warfarin and (S)-warfarin were used to evaluate the reliability of the immobilization steps, for both HSA and RSA. Good correlation of the binding data obtained with these sensor chips and those already published was found.

The bicalutamide analogues under investigation were ranked for their HSA and RSA binding level and significant differences were observed with the two different proteins. The RSA ranking results were also compared with the HSA data obtained with the HPALC study (see Chapter 5).

The K_D evaluation of the lead of this class of compounds, (R)-bicalutamide, was conducted with both HSA and RSA, using a single site equation to fit the SPR responses observed. Also in this case a huge difference was found, being the affinity for HSA one order of magnitude higher than those for RSA.

Some of the bicalutamide analogues were analyzed for their RSA K_D binding constants, by using the RSA surface. The values extrapolated are consistent with the ranking data obtained before with the first RSA sensor chip.

All the differences in the bicalutamide analogues behavior against the two proteins highlighted in this work should be carefully taken into account during the extrapolation of the in vivo distribution data obtained in preclinical studies to humans.

This work makes evident the ability of the SPR technique for screening libraries of newly synthesized compounds for their affinity to the serum carrier albumins. A clear and rapid structure-activity study in early stages of drug discovery can be achieved with this technique allowing a rational design of the new pharmacological entities.

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Conclusions

The current work demonstrates the usefulness of a multi-methodological approach to provide a better elucidation of biorecognition processes. In particular our attention was focused on the albumin binding of novel synthesized class of pharmaceutical compounds and plasma proteins from different species, HSA and RSA. Moreover the interaction between new chemical entities and their biological target have been investigated. The use of orthogonal analytical techniques was proved to be the key strategy to obtain a deeply overview of the analysed processes.

Three different classes of compounds, which possess different pharmaceutical functions have been studied: 5-Imino-1,2,4-thiadiazoles and quinazolines derivatives as glycogen synthase kinase 3b (GSK-3b) and phosphodiesterase 7 (PDE7) inhibitors; tocainide analogues as sodium channel blockers useful in the treatment of myotonic syndromes and bicalutamide analogues as anti-androgen drug used in the treatment of prostate cancer. The distribution behavior of each class of compounds was studied through three different analytical techniques: affinity chromatography (HPALC), circular dichroism (CD) and optical biosensor. Furthermore the optical biosensor technique was also applied to characterize the binding of the bicalutamide analogues with their biological target, the androgen receptor.

The affinity chromatography (HPALC) studies were conducted to rank the HSA binding level of each class of compounds, allowing the identification of the higher and lower bounded compounds to the protein. These data are very helpful to optimize the synthesis of novel pharmaceutical compounds, when a better distribution in the human body is required. In fact, albumin binding of drugs has an important role for the drug activity in vivo; furthermore, the binding of poor soluble compounds with plasma proteins improves also their solubility and their transport throughout the body. Since interactions with other drugs or endogenous factor for the same binding area can lead to significant alterations in the free drug concentration, the HSA based column was also utilized to evaluate the principal binding sites of the molecules under investigation on the protein structure. The displacement information obtained with this technique was then confirmed through CD, an in solution analytical technique which loses the drawback of the immobilization step, needed in the HPALC analysis.

Concerning the tocainide and bicalutamide analogues the same study was conducted using RSA as target. An interesting evaluation of differences among species has been highlighted. The extrapolated data suggests the relevance to assess the reliability of the operation of extrapolation of pharmacokinetic data obtained with animals to humans in early stages of drug discovery.

The bicalutamide analogues were also screened for their HSA and RSA binding levels through the optical biosensor technique. All the compounds under investigation were rapidly tested over the HSA and RSA surfaces providing not only qualitative information on weather a molecule binds or not to the proteins but also quantitative evaluation of the bond strength. In fact, the highly bounded compounds have been also analysed in terms of K_D values of the complex created with the RSA. The comparison of these data with those obtained through the chromatographic technique showed the reliability of this technique in characterizing strong drug-protein interactions in a high throughput screening mode: short time for the analysis and the miniaturization of the system represent two of the major advantages of this analytical technique.

Finally a detailed description of the bicalutamide analogues-androgen receptor recognition process was obtained by means of an androgen receptor sensor chip. A rapid screening of these new compounds for their androgen receptor binding levels was obtained. This analysis allows the selection of newly analogues with higher affinity for the target than the reference compound. Moreover, a careful kinetic analysis has been carried out with some of the compounds under investigation, providing a more detailed picture of the bio-recognition process mechanism.

The development of a reliable multi-methodological method, as the one discussed in this thesis, represent the best way to approach the study of the recognition processes that govern the biological phenomena, providing important structure-activity relationship details in early phases of drug discovery.

Addendum

Summary in Italian

Il lavoro di ricerca svolto dalla Dott.sa Cecilia Fortugno durante il triennio di Dottorato in Chimica è focalizzato sullo studio dei fenomeni di bio-riconoscimento esistenti nei processi biologici. Nelle prime fasi dello studio del farmaco questo tipo di studio è di fondamentale importanza, per poter caratterizzare nuovi target biologici e sviluppare nuove molecole 'lead'. Lo studio di questi fenomeni permette inoltre la caratterizzazione del profilo farmacocinetico dei nuovi farmaci in via di sviluppo in fasi precoci dello studio del farmaco (parametri ADMET: Assorbimento, Distribuzione, Metabolismo, Eliminazione e Tossicità), permettendo di selezionare sin da subito i composti più promettenti e di tralasciare invece quelli con caratteristiche negative, con un conseguente risparmio notevole di tempo e denaro.

Tutto il lavoro è centrato sulla caratterizzazione del legame tra molecole di nuova sintesi, interessanti dal punto di vista farmacologico, e le loro proteine bersaglio. Lo studio è stato condotto utilizzando un approccio multi-metodologico, che ha previsto l'uso di tre tecniche analitiche: cromatografia di affinità ad alta pressione (HPALC), dicroismo circolare (CD) e Biosensore Ottico. L'utilizzo di queste tecniche in sinergia ha permesso di ottenere una chiara e completa visione dei processi di bio-riconoscimento in studio.

In particolare, è stato analizzato il legame di molecole di nuova sintesi alla proteina più abbondante nel plasma, la sieroalbumina umana (HSA). Questa proteina possiede un ruolo fondamentale nel trasporto di farmaci, metaboliti e fattori endogeni; per questo lo studio del meccanismo di legame dei nuovi farmaci all'HSA è di fondamentale importanza per caratterizzare la distribuzione del farmaco. Attraverso le tecniche sopra citate è stato possibile ottenere un ranking delle diverse classi di composti in analisi per il loro legame alla proteina, permettendo di individuare facilmente i composti con caratteristiche farmacocinetiche più vantaggiose; inoltre, con l'utilizzo di specifici marker sono stati effettuati studi di spiazzamento con lo scopo di individuare i siti a più alta affinità di questi composti sulla proteina.

Parte essenziale del lavoro è stata lo studio del legame di farmaci in via di sviluppo a sieroalbumine di diverse specie; in particolare HSA e RSA (sieroalbumina di ratto). Infatti, l'utilizzo di modelli animali negli esperimenti di distribuzione effettuati in vivo rappresenta un punto critico degli studi farmacocinetici. Con questo lavoro è stato possibile evidenziare come le piccole differenze presenti nella sequenza amminoacidica delle due proteine possano portare a significative differenze nella loro struttura tridimensionale che si rifletteranno quindi sulla loro capacità di legame e sulla loro stereoselettività. Ottenere queste informazioni in fasi precoci dello studio del farmaco permetterebbe un migliore confronto tra i dati ottenuti tramite esperimenti in vitro e i dati ottenuti con esperimenti sull'animale, facilitando così la successiva estrapolazione di questi dati all'uomo.

In ultimo, per mezzo del biosensore ottico è stato analizzato il legame di una serie di potenziali antitumorali di nuova sintesi con il loro target biologico (recettore

androgeno). Questa tecnica permette la determinazione delle costanti di affinità (K_D) dei complessi in analisi e delle costanti cinetiche che guidano questi processi (k_{on} e k_{off}).

All'interno dei tre anni di dottorato la Dott.sa Fortugno ha svolto un periodo di ricerca di circa un anno presso l'Università di Ginevra, School of Pharmaceutical Sciences (con la quale è stato stretto un accordo di co-tutela, tutors Profs. Pierre Alain Carrupt e Jean Luc Veuthey), usufruendo di una borsa Marco Polo per un periodo di sei mesi; ha partecipato a diversi convegni nazionali e internazionali presentando due comunicazioni orali e due comunicazioni poster; è inoltre coautrice di due pubblicazioni su quotate riviste internazionali (European Journal of Pharmaceutical Sciences, Journal of Chromatography B), e di altri due articoli accettati per la pubblicazione.

Summary in French

Le travail de recherche developpé pendant le Doctorat en Chimie se focalise sur l'étude des phénomènes de bioreconnaissance existante dans les processus biologiques. Dans les premieres phases d'étude du médicament, ce type d'étude est très important pour pouvoir caractériser de nouveaux target biologiques et developer de nouvelles molécules "lead". De plus, l'étude de ces phénomènes permet la caractérisation du profil pharmacocinétique de nouveaux médicaments dans les phases précoces d'étude du médicament (paramètres ADMET: absorption, Distribution, Métabolisme, Élimination et Toxicité), en permettant de sélectionner dès le début les composants les plus prometteurs et de passer, au contraire, sur ceux avec des caractéristiques négatives, en conséquence en gagnant du temps et d'argent.

Tout le travail se fonde sur la caractérisation de la liaison entre les nouvelles entités chimiques, qui sont intéressant du point de vue pharmacologique et leurs protéines-target. L'étude a été réalisée en utilisant une approche multiméthodologique, qui a impliqué l'utilisation de trois techniques d'analyse: chromatographie d'affinité à haute pression (HPALC), dichroïsme circulaire (CD) et biosenseur optique. L'utilisation de ces techniques en synergie a permis d'obtenir une vue claire et complète des processus de bioreconnaissance étudiés.

En particulier, nous avons analysé la liaison des nouvelles entités chimiques à une des protéines les plus abondantes du sérum, la sérum albumine humaine (HSA). Cette protéine joue un rôle fondamental dans le transport des médicaments, des metabolites et des facteurs endogènes, permettant de expliquer la distribution des médicaments dans l'organisme humaine. À travers ces techniques il a été possible d'obtenir un ranking de tous les composés analysés selon leur liaison avec la protéine, en permettant d'identifier facilement les composants avec les caractéristiques pharmacocinétiques les plus favorables; de plus, avec l'utilisation de markers spécifiques nous avons effectué des études de déplacement, pour identifier les sites avec haute affinité sur la protéine.

La liaison de nouvelles entités chimiques aux protéins plasmatiques des différentes espèces animales a été étudié, en particulier HSA et RSA (sérum albumine de rat). En effet, l'utilisation de modèles animaux dans les expériences de distribution réalisées in vivo représente un point critique des études pharmacocinétiques. Avec ce travail, il a été possible de montrer comment les petites différences dans la chaîne d'acides aminés de ces deux protéines peuvent conduire à des différences significatives dans leur structure tridimensionnelle qui se repércuteront, donc, sur leur capacité de liaison et sur leur stéréosélectivité. Obtenir ces informations dans les phases précoces d'étude du médicament permettrait une meilleure comparaison entre les données obtenues par des expériences in vitro et les données obtenues avec les expériences sur l'animale, en facilitant ainsi la suivante extrapolation de ces données pour les humains.

Enfin, la liaison d'une série de potentiels anticancéreux nouvellement synthétisé avec leur target biologique (récepteur des androgènes) a été analysé en utilisant une biosenseur optique. Cette technique permet la détermination des constantes d'affinité (K_D) des complexes analysés et des constantes cinétiques qui guident ces processus (k_{on} et k_{off}), en obtenant ainsi une caractérisation complète et détaillée du processus de bioreconnaissance étudié.

Scientific Communications

List of Publications

1. Fortugno C., G. Varchi, A. Guerrini, P.A. Carrupt, C.Bertucci *Optical biosensor analysis in studying new synthesized bicalutamide analogues binding to androgen.* J. Pharm. Biomed. Anal. [accepted for publication]

2. Muraglia M., De Bellis M., Catalano A., Carocci A., Franchini C., Carrieri A., Fortugno C., Bertucci C., Desaphy J.F., De Luca A., Conte D., Corbo F. *N-Aryl-2,6-dimethylbenzamides, a New Generation of Tocainide Analogues as Blockers of Skeletal Muscle Voltage-Gated Sodium Channels.* J. Med. Chem., 2014 Feb. 25, doi: 10.1021/jm401864b [accepted for publication]

3. Pistolozzi M., Fortugno C., Franchini C., Corbo F., Muraglia M., Roy M., Félix G., Bertucci C. *Species-dependent binding of tocainide analogues to albumin: Affinity chromatography and circular dichroism study.* J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2014 Jan 10. doi: 10.1016/j.jchromb.2014.01.007. [Epub ahead of print]

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Conference contributions

- C. Fortugno, G. Varchi, A. Guerrini, P.A.Carrupt, C.Bertucci (2013) *Optical biosensor analyses in studying new synthesized bicalutamide analogues binding to androgen receptor. 24th international Symposium on Pharmaceutical and Biomedical Analysis – Recent Developments in Pharmaceutical Analysis – RDPA 2013. Poster*

- C. Fortugno *Multimethodological study of molecular recognition phenomena.* Advanced Analytical Methodologies in Biopharmaceutical Quality Control – SSPA 2013. Oral communication

- C.Fortugno, M.Muraglia, F.Corbo, C.Franchini, C.Bertucci *Drug binding to albumins by high performance affinity chromatography and circular dichroism.* VII Meeting "Nuove Prospettive in Chimica Farmaceutica" - NPCF7. Oral communication

- F. Corbo, M. Muraglia, C. Bertucci, C.Fortugno, A. Carrieri, M. De Bellis, A. De Luca, R. Carbonara, J.-F. Desaphy, D. Conte Camerino and C. Franchini *New molecular requirements of Toc derivatives bearing a novel class of Nav1.4 blockers*. 21st National Meeting of Medicinal Chemistry. Poster