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Species-dependent binding of new synthesized bicalutamide analogues to albumin by optical biosensor analysis

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ABSTRACT

The binding of some novel bicalutamide analogues to human serum albumin (HSA) and rat serum albumin (RSA) was investigated by surface plasmon resonance (SPR) based optical biosensor technique. The serum protein binding of the bicalutamide analogues was determined and compared to that of the parent compound. Furthermore, HSA and RSA were used as target plasma proteins, in order to highlight possible differences among species when performing pharmacokinetic studies.

HSA and RSA were covalently immobilized on carboxymethyl dextran matrixes, using an amine coupling procedure. The anchoring method was validated by determining the dissociation constant (K_D) of a standard analyte to confirm that the binding properties of the proteins were maintained. The ranking of the bicalutamide analogues for their HSA and RSA bound fractions was used to compare the behaviour of the two albumins. Most of the bicalutamide analogues showed higher binding levels with respect to the lead compound, (R)-bicalutamide. Further, meaningful differences in the binding level to the two serum proteins were obtained. The dissociation constants (K_D) of the interaction between the lead compound, (R)-bicalutamide, and the two proteins were calculated. As a result, the K_D obtained with HSA was one order of magnitude higher than that obtained with RSA.

The observed differences in the HSA and RSA bonding of the bicalutamide analogues increase the knowledge on the possible low reliability in extrapolating the distribution data obtained on animals to humans. This work demonstrates that SPR based optical biosensor technique is well suited for the medium-high throughput screening of compounds' ligand binding to serum albumins.

1. Introduction

The process to generate a successful medicine is characterized by a high attrition rate. The main reason of these liabilities is related to molecular characteristics that make the ADMET (absorption, distribution, metabolism, excretion, toxicity) profile of a drug candidate unsuitable. Among all, binding to plasma protein plays an important role on the in vivo distribution of a drug. Human serum albumin (HSA) is the most abundant protein in plasma, with a concentration of about 600 μM [1]. This protein represents the major serum carrier being responsible for the transport of numerous endogenous factors and various xenobiotics, 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 [2–4]. Furthermore, the bound drug fraction acts as a reservoir for a longer drug action, increasing its half-life in the human body and thus maintaining its therapeutic level.

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 acid sequences of albumins from different animal species, the existence of significant interspecies differences has already been demonstrated. Hence this kind of investigation is very useful to evaluate the reliability of extrapolating to humans the distribution data obtained on animals in preclinical studies [5,6].

It is also 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

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elements that drive the process. The most widely used techniques for protein binding measurements are equilibrium dialysis, ultrafiltration, affinity chromatography, capillary electrophoresis, spectroscopy (fluorescence, circular dichroism, nuclear magnetic resonance) and mass spectrometry [7–14].

Surface plasmon resonance (SPR) based optical biosensor technique is one of the most successful in the biomolecular interaction analysis field. Its use to characterize target macromolecules and biopharmaceuticals is well documented in the current literatures [15–20]. The optical biosensor is based on the SPR optical phenomenon. 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 not only 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, the 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 complete view of the binding mechanisms to understand different biological and pathological phenomena [2,4,9]. Since the sensitivity of this technique is directly related to the mass of the analyte under investigation, it is obvious that it is the technique of choice to study protein–protein interactions. However, recent improvements in the SPR biosensor technology have made its use suitable also for the detection of small molecule drugs (<200 Da).

The main advantage of this technique is the lack of a labelling step for both the ligand and the analyte, thus avoiding extra time and costs as well as misinterpretation of the collected data due to the occlusion of specific binding sites [20]. However, the immobilization always remains a critical step, because the immobilized compound may show a different behaviour 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 also be 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. This technique has already been successfully used for the screening of drugs or drug candidates for their binding to HSA. The serum protein immobilization process was proved to give binding data comparable with those obtained in solution [21]. In the present work the amine-coupling procedure was used to covalently immobilize both HSA and RSA

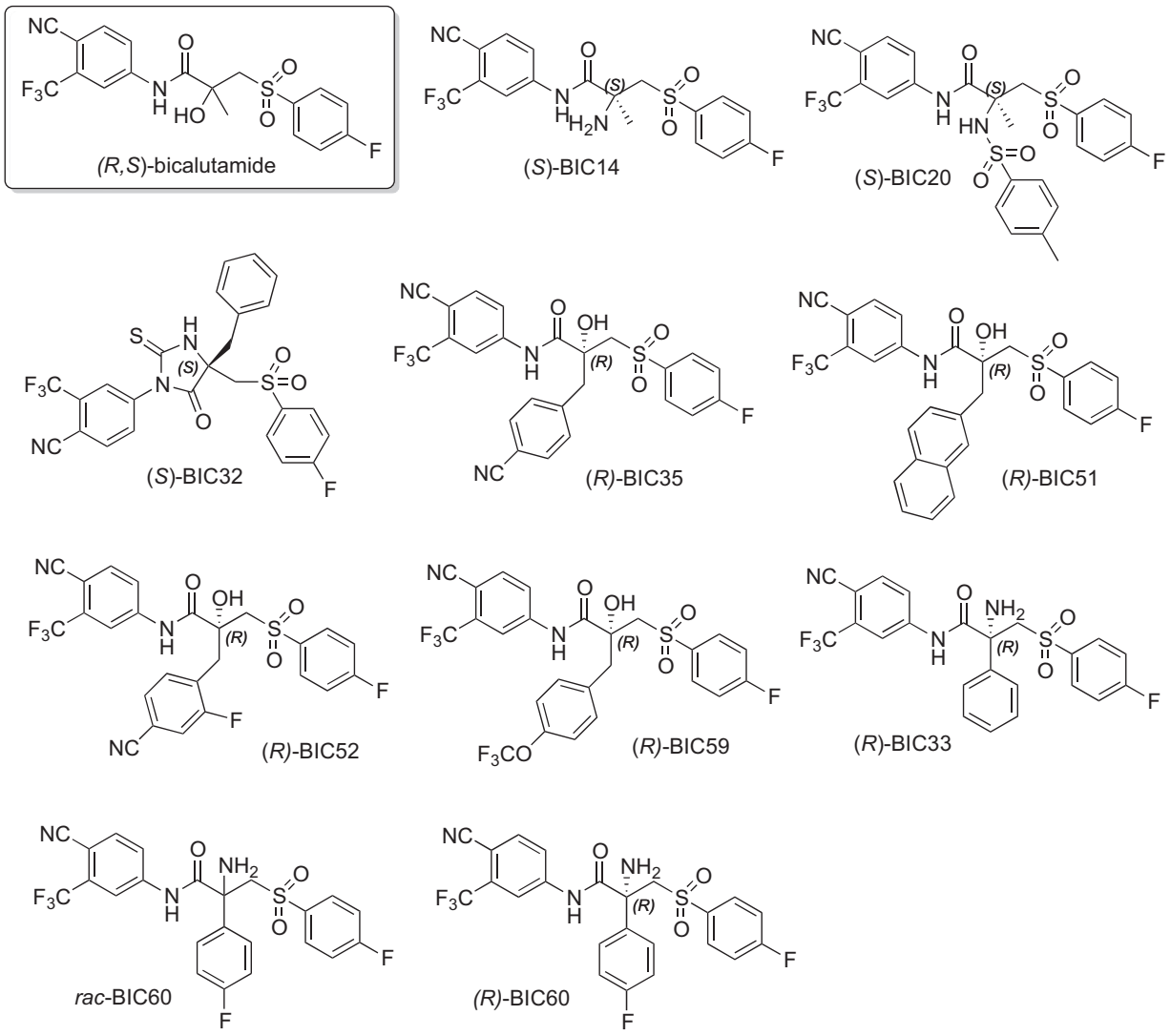

Fig. 1. Structures of (R)-bicalutamide and its analogues.

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on a carboxy methyl dextran matrix. The immobilized albumin chips were then used to obtain a ranking of sterically hindered C2-substituted bicalutamide analogues (Fig. 1).

Indeed, among the non-steroidal androgen receptor antagonists, (R,S)-bicalutamide (Casodex) is considered the most efficacious and safe, being the (R)-enantiomer the most active. Ten different compounds were selected from our non-steroidal antiandrogens library [22–26], which bear small but significant structural diversities. In fact, it has been demonstrated that small structural changes of non-steroidal antiandrogens greatly alter the nature of the androgen receptor–ligand interaction and lead to quite divergent pharmacological responses (i.e. antagonist versus agonist activity). Moreover, our previous studies confirmed that, as for bicalutamide, the (R) enantiomer is the most active [22–24]. In light of this, the interaction with albumins from different species, including enantiomers, has been evaluated with the aim to provide more insights on the pharmacokinetic behaviour of those derivatives.

2. Materials and methods

2.1. Chemicals

Human serum albumin (HSA, essentially fatty acid free), rat serum albumin (RSA, essentially fatty acid free and globulin free), dimethyl sulfoxide (DMSO) and rac-warfarin were obtained from Sigma–Aldrich (Milan, Italy). Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \times 2H_2O$), sodium chloride, and potassium chloride were purchased from Carlo Erba (Darmstadt, Germany). Sodium acetate and sodium hydroxide were obtained from Sigma–Aldrich (Milan, Italy). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine-HCl and the sensor chip with carboxymethylated matrix CM5 were supplied by Biacore (GE Healthcare, Milan, Italy).

Compounds (R)-bicalutamide, rac-bicalutamide, (S)-BIC14, (S)-BIC20, (S)-BIC32, (R)-BIC33, (R)-BIC35, (R)-BIC51, (R)-BIC52, (R)-BIC59, rac-BIC60, and (R)-BIC60 were prepared as previously reported [22–27]. The spectroscopic data of compounds (R)-BIC60 and rac-BIC60 are reported in the Supporting information. The structures of the compounds are given in Fig. 1.

The running buffer was phosphate buffered saline (PBS): 10 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride. The pH of this solution was adjusted with the addition of 1 mM NaOH. Deionized water was used for mobile phase's preparation. The buffer solutions were filtered through a 0.22 μm membrane filter and degassed by ultrasound before use.

2.2. Instrumentation

Surface plasmon resonance analyses were performed using a Biacore X100 optical biosensor with Biacore X100 Plus Package (GE Healthcare). The system was thermostated at 25 °C and equipped with research-grade CM5 sensor chips (GE Healthcare). A sensorgram describes changes in SPR angle in real time, with responses measured in resonance units (RU). One RU corresponds to 0.0001° shift in SPR angle, or to 1 pg/mm^2 of molecule on the surface.

Biosensor data were analysed using Biacore™ X100 2.0 Evaluation Software. Data processing was performed with BIAevaluation software.

2.3. Serum albumin immobilization

The human serum albumin surface was prepared by using a standard amine-coupling procedure [28]. The HSA was coupled through its surface amine groups with the carboxyl groups of the CM5 sensor chip (carboxymethylated dextran covalently attached to a gold film) of the Biacore X100 instrument. The running buffer was PBS at 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 EDC and 0.1 M 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 of 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 given in the Supplementary information (Fig. S1), where the optical biosensor response (in 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 (six injections, 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 pulses of 12 s of 50 mM NaOH were injected to wash non-covalently bound protein and stabilize the baseline. RSA 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 was 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.

2.4. Preparation of sample solutions

All the analysed compounds were stored as 1 mM stock solutions in 100% DMSO. Stock samples were diluted 1:20 with PBS buffer immediately prior to analysis. A suitable range of concentrations (1–300 μM) was then obtained by further diluting with the running buffer containing 5% of DMSO. In order to avoid disturbance of the baseline, all the dilutions were done directly into the analysis tubes. The running buffer and the sample solutions should contain the same percentage of DMSO, because of the high refractive index contribution of DMSO. Imbalanced concentrations of DMSO could determine a significant variation of the final SPR signals. The running buffer for these analyses was prepared as already described: the pH was adjusted to 6.85 by adding NaOH. The final pH value was 7.4, after adding the 5% (v/v) of DMSO [29].

2.5. Solvent refractive index correction

A DMSO calibration procedure was included in the assay protocols, in order to eliminate variations in the bulk responses between samples 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 misinterpretation of the final data. Therefore, a series of eight

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buffer solutions containing concentrations of DMSO ranging from 4.5 to 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 [21]. All the response levels obtained during the analysis were corrected with these calibration data. The DMSO calibration plot was built every day, prior starting the binding analysis.

2.6. 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 HSA 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. A buffer injection between every sample cycle was included to check the possible carry-over of analyte to the next cycle. This also allowed the stabilization of the chip surface. All samples were injected in duplicate.

2.7. 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 analysed 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, thus preventing the accumulation of the analyte on the sensor chip. All sensorgrams were processed by using double referencing method. Rac-warfarin and S-warfarin were analysed in the presence and in the absence of DMSO in the running buffer, instead (R)-bicalutamide and its analogues were always analysed in the presence of 5% (v/v) of DMSO in the running buffer. The association phase was followed for 60 s, because the kinetic of the interaction was very fast. The dissociation phase was followed for 60 s when rac-warfarin, (S)-warfarin and (R)-bicalutamide were the analytes; 10 min were necessary 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. Each equilibrium analysis was performed twice over the same sensor chip.

For (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:

$$R_{eq} = \frac{R_{max} \times [L]}{K_D + [L]} \quad (1)$$

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 obtained equilibrium responses 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:

$$R_{eq} = \frac{R_{max1} \times [L]}{K_{D1} + [L]} + \frac{R_{\max 2} \times [L]}{K_{D2} + [L]} \quad (2)$$

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:

$$R_{eq} = \frac{R_{max1} \times [L]}{K_{D1} + [L]} + \frac{R_{\max 2} \times [L]}{K_{D2} + [L]} + \frac{R_{\max 3} \times [L]}{K_{D3} + [L]} + \frac{R_{\max 4} \times [L]}{K_{D4} + [L]} \quad (3)$$

3. Results and discussion

3.1. Immobilization of serum albumin

HSA and RSA were immobilized on CM5 chips. 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. Since the covalent binding occurs through 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 protein 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 (IP) of the ligand (IP = 5.67); 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 treated the same as the active channel, without anchoring the 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 resulted in 16,114 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. The amount of protein immobilized was checked in real time, using the manual run windows of the Biacore™ X100 2.0 Control software. The final amount on the second sensor chip corresponded to 5572 RU. The lower immobilization level reached prevent 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 collected data. The binding of rac-warfarin was used to ensure that the two immobilization processes did not involve the principal binding site of the protein. As a result the experimental K_D values of the reference compound obtained with both chips were in agreement with those reported in literatures (see Section 3.3) [30,31].

Concerning RSA, the two coupling procedures resulted in 12,000 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 3.3) [32–34].

3.2. Ranking analysis

3.2.1. HSA investigation

(R)-bicalutamide and seven analogues, namely (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 for each compound was used. The analytes were injected over the HSA surface at 25 μM, at a 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 analysed compound. Since the SPR response is

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directly correlated with the specific molecular mass of the analytes, all the data collected from this analysis were divided by this value, in order to obtain comparable molar values (Fig. 2A). (R)-BIC51, (R)-BIC59, (S)-BIC20 and (R)-BIC33 showed higher HSA binding level than the reference compound, (R)-bicalutamide; whereas (S)-BIC32, (S)-BIC14 and rac-BIC60 possess lower HSA binding level. The compounds of the former group, the strongly bound ones, show a large and lipophilic substituent in their structure. The (R) configuration compounds showed to promote higher albumin affinity, thus confirming the literature data on the superior activity and pharmacokinetic profile of this enantiomer compared to the (S) one [35]. Overall, the results trend suggests that the presence of a steric hindered and lipophilic substituent at the chiral stereocentre allows a better stabilization of the molecule in the protein binding pocket, thus increasing the binding level, as well as the stability of the specific complex.

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 relevant advantages of the 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 is 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 is required for a complete characterization of a ligand–analyte interaction (60 min for the most bounded compounds in HPALC). Therefore this technique is well-suited to rapidly classify synthesized compounds in low-, medium- and high-level binders.

3.2.2. RSA investigation

All the compounds showed in Fig. 1 were tested for their RSA binding level 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 Fig. 2B. (R)-BIC51 and (S)-BIC20 showed the highest RSA binding level, whereas (S)-BIC14 represents the more weakly bound analogue. Therefore also in the case of RSA, the presence of a sterically hindered, and lipophilic substituent at the chiral stereocentre allows a better accommodation of the analogues in the protein binding pocket.

One of the major differences involves the binding ability of (R)-BIC59, which is one of the compounds showing the highest HSA binding level and a very low RSA binding level. Another difference was observed for (R)-BIC33: with the human protein it showed a higher binding ability when compared to the reference compound, while with the rat protein it showed a lower binding ability than the one displayed with reference compound. The racemate form of the reference compound, e.g. rac-bicalutamide, results in a higher RSA binding level than its (R) enantiomer [(R)-BIC (Fig. 2)]. The comparison of rac-BIC60 and (R)-BIC60 led to the same results, thus confirming a higher binding level of the (S) enantiomers of both compounds investigated with rat albumin.

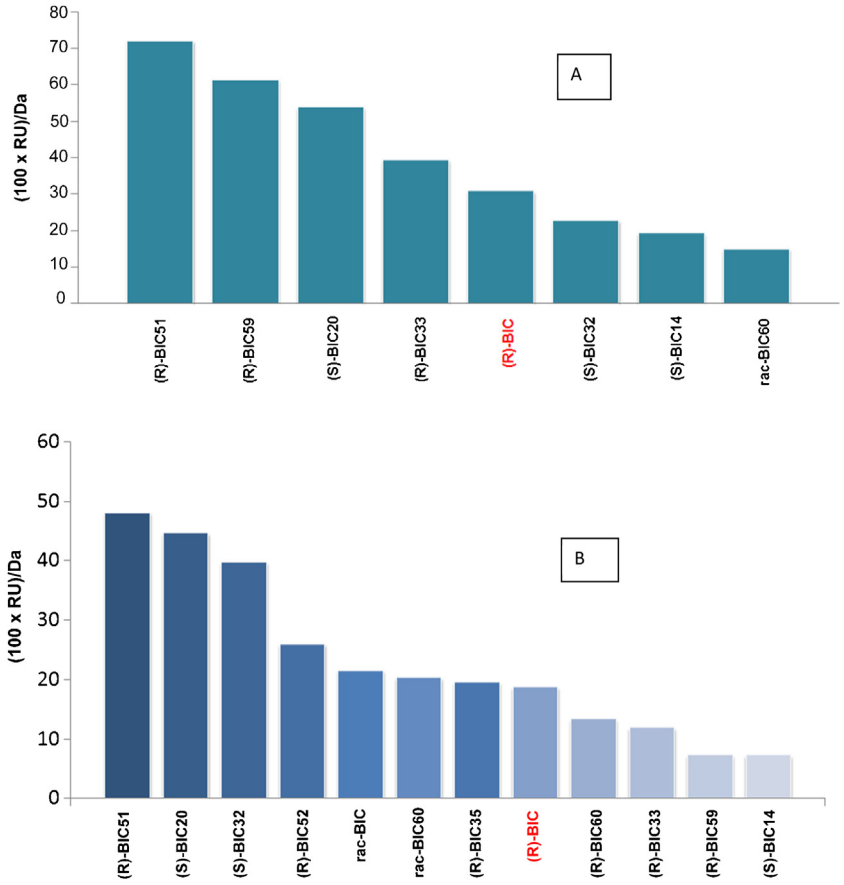

Fig. 2. (A) Ranking analyses of bicalutamide analogues performed with the HSA chip at a concentration of 25 μM in PBS 10 mM + 5% DMSO, pH 7.4. Binding data were adjusted for the molecular weight of each compound, in order to give comparable molar values. (B) Ranking analyses of bicalutamide analogues 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 compound.

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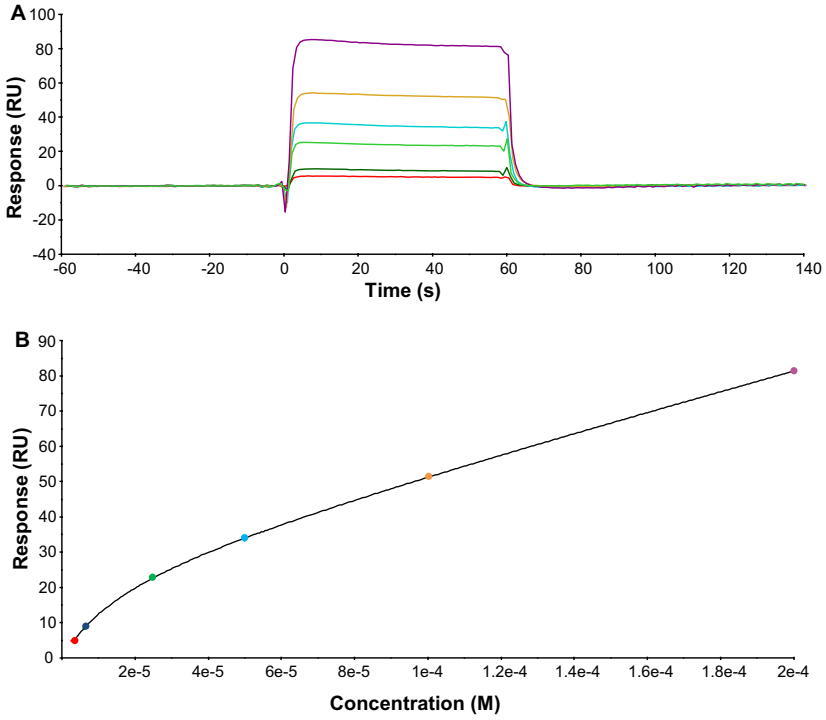

Fig. 3. (A) Corrected overlay of sensorgrams for (R)-bicalutamide binding to human serum albumin, obtained using different (R)-bicalutamide concentrations. The signal increases by increasing of the analyte concentration: [(R)-bicalutamide] = 3.13, 6.25, 25, 50, 100, and 200 μM. (B) Response data at equilibrium versus (R)-bicalutamide concentration. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

Table 1
Equilibrium dissociation constants (K_D) for Rac-warfarin/HSA interaction.

	HSA (16,113.9 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^{-6} M[a] 3.3×10^{-4} M[b]	1.0×10^{-4}[c] 1.49×10^{-4}[d]

[a] Buffer: PBS 10 mM, pH 7.4.
[b] Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.
[c] [30].
[d] [31].

3.3. 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 determined.

First of all, rac-warfarin was investigated for its binding to the human serum protein, in order to check that the immobilization step did not impact the binding results when compared to those obtained in solution. The interaction was monitored firstly in pseudo-physiological condition (PBS 10 mM, pH 7.4) and secondly in the presence of 5% (v/v) of DMSO in the running buffer. During the injection of all the set of concentrations under investigation (ranging from 1 to 300 μM) a steady state was reached in few seconds; also the dissociation occurred very rapidly when the analyte solution was replaced by the running buffer. Fig. S2 shows 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 two sites binding equation on Biacore's software (Eq. (2)) it was possible to fit equilibrium responses at different concentrations to obtain the two equilibrium dissociation constants.

The experimental K_D values (Table 1) are in good agreement with those reported in literature obtained by using the same technique [30], as well as with those obtained by different techniques [31]. 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 used for the RSA chips. The concentrations used ranged from 1 to 300 μM for the analysis in pseudo-physiological conditions (PBS 10 mM, pH 7.4), and from 4 to 600 μM for the analysis in the presence of 5% of 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 [30]. Therefore, a four-site model was used to calculate the affinity of (S)-warfarin to this protein (Eq. (3)), which yielded four different K_D values: 7.625×10^{-6} M, 9.481×10^{-5}, 5.752×10^{-4} and 2.005×10^{-4} (Fig. S3). These data are in good agreement with literature values obtained with different techniques [32–34]. The same experiment was repeated several times on the same RSA sensor chip, and the data were also confirmed by using the second RSA immobilized chip. The optimal repeatability and reproducibility of the obtained data underline how the SPR technique represents a suitable method for the accurate equilibrium dissociation constants evaluation, which deeply describes the affinity between a drug and its target protein.

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 guided over the sensor chip surfaces, ranging from 3 to 200 μM when HSA was the ligand; and from 5 to 350 μM when RSA was used. Also in this case both the association and dissociation phases were very rapid, as can be seen in Figs. 3A and 4A. A

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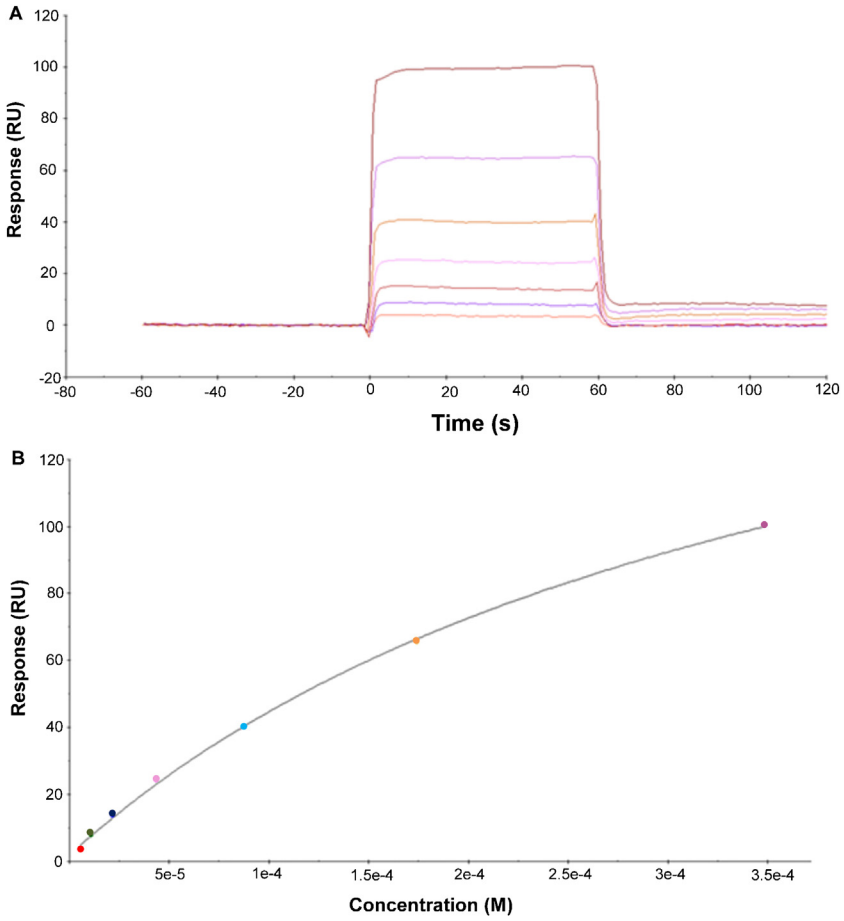

Fig. 4. (A) Corrected overlay of sensorgrams for (R)-bicalutamide binding to rat serum albumin, obtained using different (R)-bicalutamide concentrations. The signal increases by increasing of the analyte concentration: [(R)-bicalutamide] = 5.5, 10.9, 21.9, 43.8, 87.5, 175, and 350 μM. (B) Response data at equilibrium versus (R)-bicalutamide concentration. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

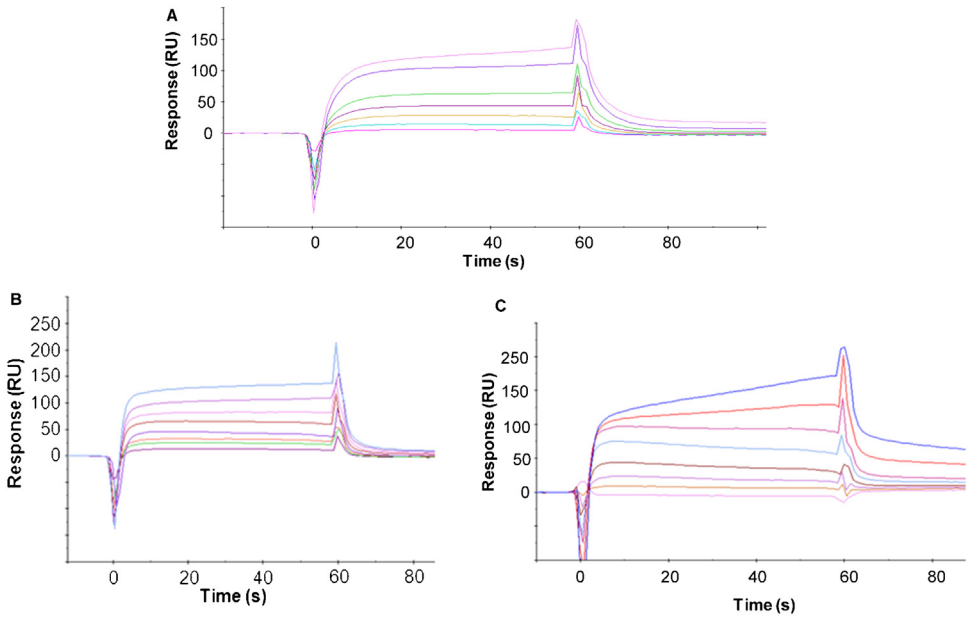

Fig. 5. Corrected overlay of sensorgrams for (A) (R)-BIC51, (B) (S)-BIC20 and (C) (S)-BIC32 binding to rat serum albumin, obtained using different analyte concentrations. The signal increases by increasing of the analyte concentration: (A) [(R)BIC-51] = 2.5, 4.2, 7.0, 11.6, 19.4, 53.9, and 89.8 μM; (B) [(S)BIC20] = 2.4, 4, 6.7, 11.1, 18.5, 30.8, 51.4, and 85.7 μM; (C) [(S)BIC32] = 2.6, 4.3, 7.1, 11.8, 19.7, 32.9, 54.8, and 91.3 μM. After reference subtraction, large spikes are recorded for each injection, because the association and dissociation curves are not corrected at the beginning (1–4 s) and end of the injection. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

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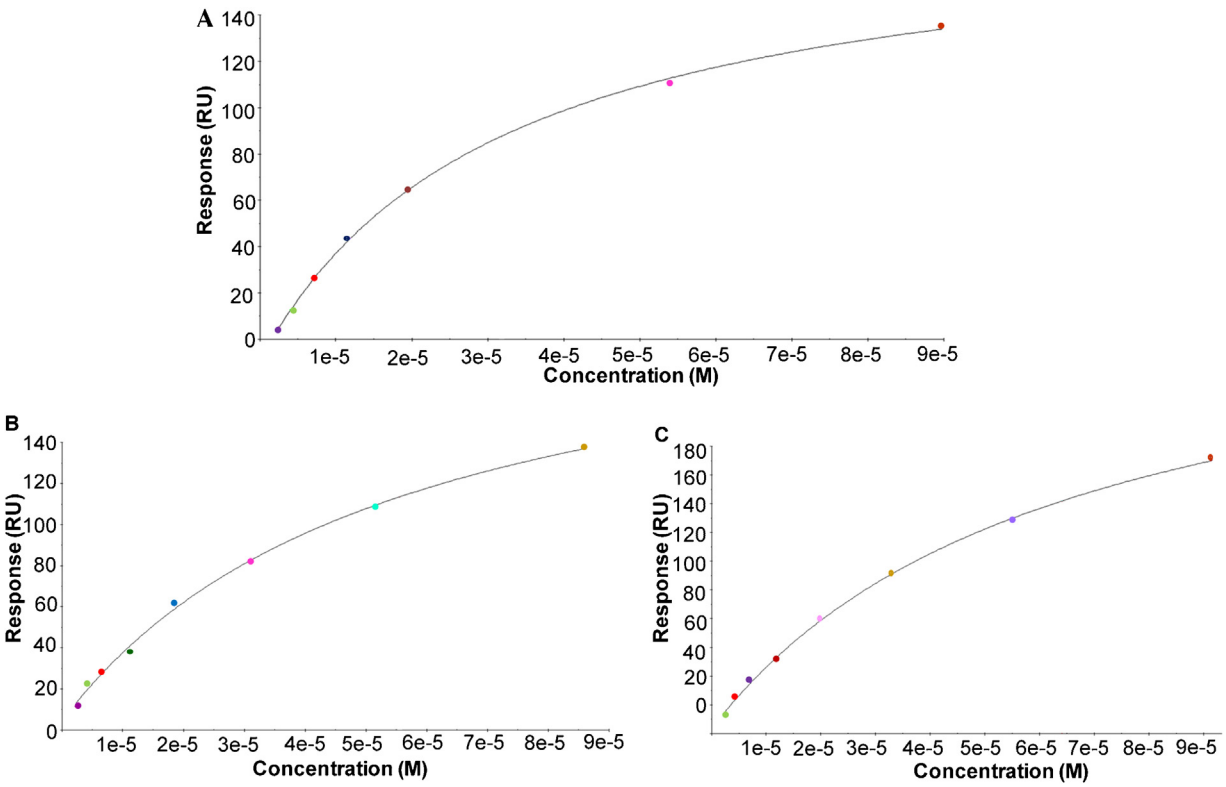

Fig. 6. Response data at equilibrium versus (A) (R)-BIC51, (B) (S)-BIC20 and (C) (S)-BIC32 concentrations. Buffer: PBS 10 mM + 5% (v/v) DMSO, pH 7.4.

single-site model (Eq. (1)) was used to fit the obtained binding data (Figs. 3B and 4B). The value of K_D calculated with the highest density HSA sensor chip was 1.8×10^{-5} M, which is in good agreement with those obtained in the same experimental conditions with the lowest density chip (1.6×10^{-5} M). The dissociation constant found with the RSA chip was 3.8×10^{-4} M, one order of magnitude lower than those observed with HSA (Table 2). The data confirm once again the importance of studying the drug affinities for albumins from different species in an early phase of drug discovery.

3.3.1. 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 RSA. A wide range of concentrations (2–90 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. 60 s after injection a steady-state plateau was reached. The dissociation phases were followed by 10 min of running buffer injection, 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. 5. A single site equation was applied to analyse the plot of the SPR equilibrium responses at different analyte concentrations (Fig. 6).

After an accurate evaluation of the collected data, K_D values of 3.18×10^{-5} M, 5.55×10^{-5} M and 6.41×10^{-5} M were found for (R)-BIC51, (S)-BIC20 and (S)-BIC32, respectively (Table 2). The experimental values of K_D are consistent with the ranking results discussed above (see Section 3.2.2). Unfortunately, it was not possible to compare the RSA K_D values obtained for these compounds with the HSA ones, because the overlaid sensorgrams obtained during the HSA binding study did not allow the extrapolation of reliable K_D values.

Table 2
Equilibrium dissociation constants (K_D) for ligand/HSA and ligand/RSA interaction.

Sample	K_D (M) HSA	K_D (M) RSA
R-bicalutamide	1.8×10^{-5} [a] 1.6×10^{-5} [b]	3.78×10^{-4} [a] 3.05×10^{-4} [b]
(R)-BIC51		3.18×10^{-5}
(S)-BIC20		5.55×10^{-5}
(S)-BIC32		6.41×10^{-5}

[a] Value obtained with the highest density sensor chip.
[b] Value obtained with the lowest density sensor chip.

4. Conclusions

Human serum albumin and rat serum albumin were immobilized on CM5 sensor chips; the reliability of the immobilization procedure was proved using warfarin as a marker for the binding. The bicalutamide analogues under investigation were ranked for their HSA and RSA binding; lipophilicity was one of the driving structural parameter in determining higher affinity for the proteins. Furthermore significant differences were observed in the binding level of the analysed compounds to the two albumins. In particular, the affinity for HSA of the lead compound, (R)-bicalutamide, was one order of magnitude higher than the affinity for RSA. All the differences in bicalutamide analogues behaviour against the two proteins highlighted in this work should be carefully taken into account for the extrapolation of the in vivo distribution data obtained in preclinical studies to humans.

SPR technique proved to be well suited for medium-high throughput screening applications, requiring small amounts of both the ligand and the ligate, and short assay time. Moreover, a clear and rapid structure–activity study in early stages of drug discovery can be achieved with this technique contributing to a rational design of the new pharmacological entities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2015.02.010.

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