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

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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 analogs 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 analogs. 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.

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 toward

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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 active enantiomer of the commercial drug) and of the novel synthesized derivatives (Fig. 1) [22–25]. The androgen receptor binding level of all the analogs under investigation was ranked, allowing the identification of the higher bounded compounds, compared to the reference, e.g. (R)-bicalutamide [26]. Kinetic binding studies of (R)-bicalutamide and some of the analogs 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.

2. Materials and methods

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 ^{1}H NMR (400 MHz, C_6D_6) δ: 9.31 (s, 1H, NH), 7.56–7.50 (m, 3H, ArH), 6.97 (d, J = 2.0 Hz, 1H, ArH), 6.88–6.85 (m, 2H, ArH), 6.78 (d, J = 8.4 Hz, 1H,

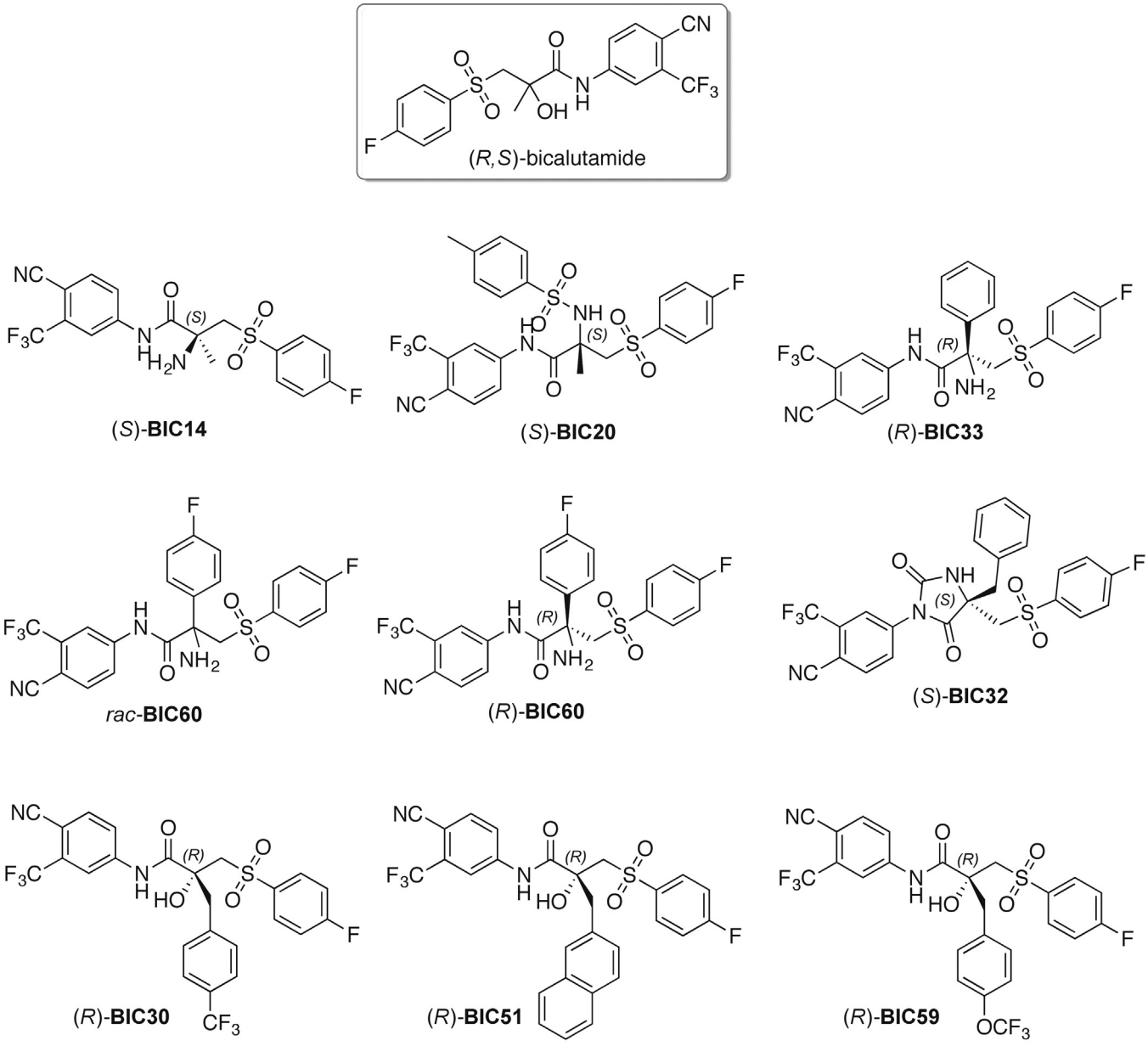

Fig. 1. Structures of (R)-bicalutamide and its analogs.

ArH), 6.60 (t, J = 8.4 Hz, 2H, ArH), 6.43 (t, J = 8.4 Hz, 2H, ArH), 4.18 (d, J = 14 Hz, 1H, CH_2), 3.12 (d, J = 14.4 Hz, 1H, CH_2), 2.27 (bs, 2H, NH_2); ^{13}C NMR (100 MHz, C_6D_6) δ: 170.6, 165.5 (d, J = 305.7), 163.0 (d, J = 298 Hz), 141.1, 136.8, 135.5, 130.8, 130.7, 127.1, 127.0, 121.3, 116.8 (q, J = 4.7 Hz), 116.4, 116.2, 115.9, 115.7, 115.4, 104.9, 64.3, 62.0.

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 ($NaH_2PO_4 \cdot 2H_2O$) 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.

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 BIAevaluation software.

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. 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

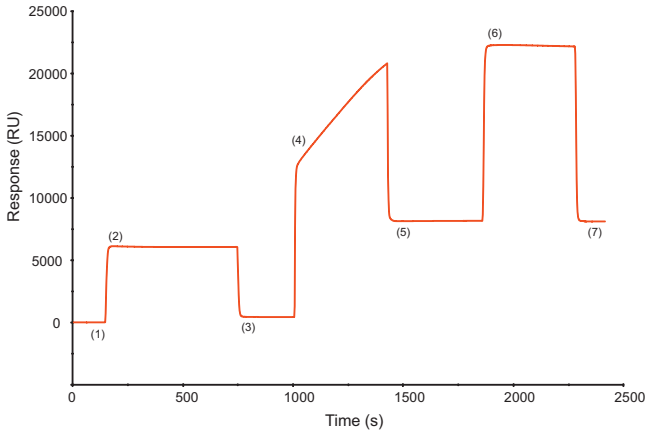

Fig. 2. Sensorgram of the androgen receptor immobilization to the carboxymethyl dextran matrix: (1) buffer baseline stabilization; (2) EDC/NHS mixture add; (3) buffer re-equilibration; (4) Androgen receptor in acetate buffer add (7 min of reaction); (5) buffer washes; (6) block of non-coupled activated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

in a refrigerator, in a 50 mL tube containing running buffer without DMSO (see Section 2.5) so that the support was completely covered.

2.4. Preparation of sample solutions

All the set of analyzed compounds were stored as 1 mg/mL stock solutions in 100% 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.

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 few minutes 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.

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 was 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 (χ^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.

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.

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.

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

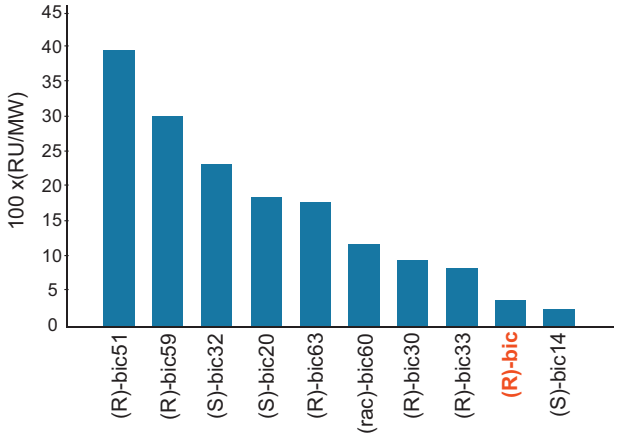

Fig. 3. Ranking analyses of bicalutamide analogs performed at a concentration of 10 μM in HBS 10 mM + NaCl 150 mM + 2% DMSO, pH 7. Binding data were adjusted for the molecular weights of each compound, in order to give comparable molar values.

of the system, represent critical aspects of the chosen strategy [16]. It has been already demonstrated that bicalutamide and its analogs 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 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. 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 4 weeks as demonstrated by the consistent ranking obtained from analyses carried out at different times for the same set of ligate concentrations.

3.2. Ranking analyses

Bicalutamide and its analogs 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 analogs 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. 3). The increasing of lipophilicity of the substituent at the chiral stereocenter also increases the affinity of

Table 1
Predicted log *P* values of the compounds under investigation. Compounds are tabulated according to the ascending degree of binding for the AR.

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

[a] Calculated using online www.molinspiration.com/cgi-bin/properties site (Molinspiration Property Engine v2009.01).

these compounds to AR. This behavior matches with the calculated log *P* of the compounds under investigation (see Table 1). In fact, (R)-bicalutamide and (S)-bic14 show a lower predicted log *P* than the other compounds. On the other hand, (R)-bic51, (R)-bic59 and (S)-bic32 hold the highest predicted log *P* values and produce the highest receptor binding affinity. Moreover, comparing the results of Fig. 3 and Table 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.

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 Fig. 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 the analyzed interaction was well described by a 1:1 interaction model (Fig. 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. 4a and b); whereas the (R)-bic33 kinetic analyses were performed using multi-cycle approach (see Fig. 4c). In both cases several analyte concentrations were injected over the surface to determine the binding data. Multi-cycle 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. 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 $2.63 \times 10^{-5}\,s^{-1}$, resulting in a K_D of 70.7 nM. This value is in good agreement with the K_D 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 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

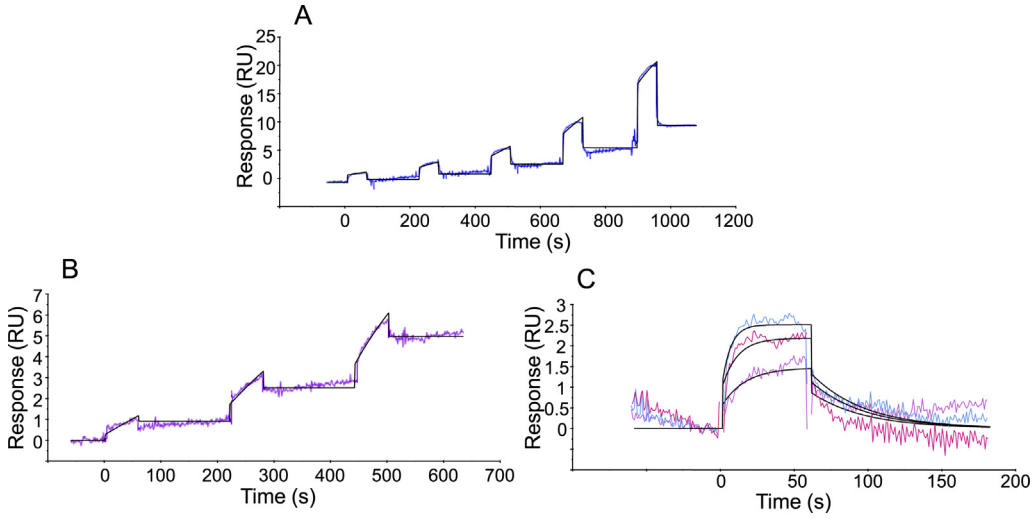

Fig. 4. Kinetic profiles for (R)-bicalutamide (A), (R)-bic32 (B) and (R)-bic33 (C) binding to AR determined using single cycle (A, B) and multi-cycle (C) kinetics. Sensorgrams show blank and reference subtracted data with kinetic fit for 1:1 interaction model overlaid in black.

Table 2
Rate constants and affinities of AR/ligand interactions. Parameters were estimated by global fit of an equation describing 1:1 binding.

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]

[a] Value in good agreement with those reported in literature obtained by using radiolabeled target [26].
[b] Not determined, because outside of instrument range.

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 (K_D) showed that (S)-bic32 had a higher affinity for the receptor than the lead compound (173 pM). This datum was in accordance with the ranking study presented before.

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 realtime 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 a 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.

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