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A new, simple and robust radioligand binding method used to determine kinetic off-rate constants for unlabeled ligands. Application at α_{2A}- and α_{2C}-adrenoceptors

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ABSTRACT

Kinetic on and off rate constants for many receptor ligands are difficult to determine with regular radioligand binding technique since only few of the ligands are available in labeled form.

Here we developed a new and simple radioligand binding method for determining the kinetic off-rate constant for unlabeled ligands, using whole cells expressing α_{2A}- and α_{2C}-adrenoceptors. The new method involves pre-incubation with unlabeled ligand, centrifugation of microtiter plates in order to adhere the cells to the bottom surface, and then upside-down centrifugation of the plates for few seconds to wash away the non-bound fraction of the pre-incubated ligand. The final on-reaction assay for the radioligand is then started by quick addition of a relatively fast-associating radioligand to the cells. The curve obtained is defined by a fairly simple mathematical formula that reflects the simultaneous dissociation of pre-incubated ligand and association of the radioligand. The method proved to produce highly reproducible results in determining the k_{off} constants for various unlabeled ligands.

The results show that the α_{2C}-selectivity of MK912 depends mainly on a very slow off-rate at the α_{2C}-adrenoceptor subtype. Regarding the markedly α_{2C}- over α_{2A}-selective compound spiroxatrine, its much faster on-rate at α_{2C}- than α_{2A}-adrenoceptors explains much of its exceptional α_{2C}-selectivity.

Several new techniques for determining the kinetic component of ligand-receptor interactions at molecular level are currently developing. As a reference, based on standard radioligand binding techniques, the present study describes a simple and robust experimental and mathematical procedure for determining k_{off} constants of unlabeled drugs.

1. Introduction

In a recent review, the importance of quantitative data regarding drug interaction with receptors was highlighted (Bylund and Toews, 2014). It was pointed out that such interaction almost always follows well-defined mathematical relationships that are based on receptor theory. In the same special issue, the importance of drug-receptor theory in the field of pharmacology in biomedical research was highlighted (Kenakin and Williams, 2014). To date, almost all studies involved in determining the affinity of many ligands to a receptor have been performed at equilibrium. Thereby, the kinetic component of the ligand-receptor interaction becomes undervalued (Pollard, 2010; Hoffmann et al., 2015). The shortage of kinetic studies for unlabeled ligands is probably due to the difficulty of performing and analyzing such kinetic experiments, in which, when including a radioligand, four kinetic constants plus the time factor are participating simultaneously in the process. In the present study, we present an experimental method that reduces the complexity of the experimental data and consequently eases the mathematical analysis. The new radioligand binding experimental method was used to determine the kinetic off-rate constants for several unlabeled drugs at α_{2A}- and α_{2C}-adrenoceptors.

Previously, other studies have described different experimental and mathematical strategies for determining the kinetic constants for unlabeled small ligands acting at G protein-coupled receptors. For example, Motulsky and Mahan (1984) described the mathematics for analyzing the curves obtained after the simultaneous addition of radioligand and competitor, including four rate constants in the analysis. Another study presents a laboratory manual account for the full experimental procedure (Sykes et al., 2010). Yet another study addressed the same subject as the present study, i.e. the development of a simplified method for the determination of

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the off-rate k_{off} constant of unlabeled ligands (Packeu et al., 2010). Both that study and the present study use an approach consisting of pre-incubating the receptor preparation with ligand, washing out free ligand molecules, adding radioligand and monitoring its binding after various times. However, the practical solutions and technical details differ widely (see Section 4). The methodology presented in the present study is rather robust, well suited for studies aiming at determining the off-rate constant for many unlabeled ligands at a receptor.

In a recent study, the K_d value of the radioligand [^{3}H]-RX821002, and the K_i values of seven competing drugs at the human α_{2A}-, α_{2C}-, and 4 chimeric α_{2A}-/α_{2C}-adrenoceptors were determined (Jahnsen and Uhlén, 2013). The results gave clues about what regions of the receptors are causing the subtype-selectivity for the ligands. In the present study, aiming at gathering more information about specific ligand-receptor interactions, the off-rate constants for [^{3}H]-RX821002 and 8 unlabeled drugs were determined at the human α_{2A}- and α_{2C}-adrenoceptors and at two chimeric α_{2A}-/α_{2C}-adrenoceptors. The mathematical differential equation formula used for fitting the data to the curves, described by Malany and coworkers (2009), is evaluated in some detail.

2. Materials and methods

2.1. Materials

[^{3}H]-RX821002 (NET1153; 49Ci mmol^{-1}; [^{3}H]−2-(2-methoxy-1,4-benzodioxan-2-yl)−2-imidazoline hydrochloride) was from PerkinElmer. Atipamezole (A-9611), chlorpromazine hydrochloride (C-8138), MK912 (M-7065), yohimbine (Y-3125), Dulbecco's modified Eagle's medium (DMEM) (d-6429), fetal calf serum (F-7524), penicillin-streptomycin (P-4333) and trypsin/EDTA (T-3924) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). BRL44408 (1133), RS79948–197 (0987) and RX821002 (1324) were from Tocris, Bristol, UK. Spiroxatrine (S-103) was from RBI, Natick, MA, U.S.A. PCR primers were purchased from TAG Copenhagen A/S, Denmark. Lipofectamine™ 2000, OptiMEM, and Platinum Pfx DNA polymerase were from Invitrogen. The wild type α_{2A}-adrenoceptor (RefSeq NM_000681), and wild type α_{2C}-adrenoceptor (RefSeq NM_000683) were inserted in the expression vector pcDNA3.1+. The chimeric constructs AppC and CppA have been described previously (Jahnsen and Uhlén, 2013). The HEK293 (Human Embryonic Kidney) cell line was from ATCC (Rockville, U.S.A.). All other chemicals were purchased from the appropriate commercial sources.

2.2. Chemical compounds studied in this article

Atipamezole hydrochloride (PubChem CID: 13649426); BRL44408 (PubChem CID: 121850); Chlorpromazine hydrochloride (PubChem CID: 6240); MK912 (PubChem CID: 5311298); RS79948–197 (PubChem CID:9908991); RX821002 (PubChem CID: 108094); Spiroxatrine (PubChem CID: 5268); Yohimbine hydrochloride (PubChem CID: 6169).

2.3. Selection of ligands

The radioligand [^{3}H]-RX821002 is a small, fairly hydrophilic drug, showing fast on-rate at the α_{2A}- and α_{2C}-adrenoceptors, and low non-specific binding to the HEK293 cells. [^{3}H]-RX821002 is therefore an excellent radioligand for the study. Spiroxatrine and chlorpromazine were chosen to be tested because these molecules are bulky and α_{2C}/α_{2B}- over α_{2A}-selective (Laurila et al., 2011), MK912 because it is bulky and α_{2C}- but not α_{2B}- over α_{2A}-selective (Uhlén et al., 1998), BRL44408 because it is α_{2A}-selective, and atipamezole and RX820102 because they are small and non-selective molecules (Jahnsen and Uhlén, 2013), RS79948–197 and yohimbine were included since they are non-selective (Uhlén et al., 1998), but structurally remind of MK912.

2.4. Plastic ware

The drug MK912 sticks to polystyrene (PS) surfaces, but not to polypropylene (PP) (Uhlén et al., 1994). Therefore, MK912 was dissolved, diluted, and assayed in PP tubes and PP microtiter plates. In other cases, PS microplates were used, since PS plates are transparent, enabling casual observation of the cells in a microscope. For upside down centrifugation, Greiner 96-well flat bottom microtiter plates (polystyrene # 655101; polypropylene # 655201) were fit upside down into slightly trimmed bottom parts of Finntip filter 200 µl yellow tip boxes (# 94052300) from Thermo Fisher Scientific Oy, Vantaa, Finland, and then centrifuged.

2.5. Cell culture

The HEK293 cells were grown in standard tissue culture plastic material at 37 °C, 95% air, 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 9% fetal calf serum and 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were subcultured every 3–4 days, by detachment with 0.5 mg/ml trypsin and 0.2 mg/ml EDTA.

2.6. Transient expression

The HEK293 cells were transiently transfected with the receptor constructs. 1.2 µg of plasmid DNA was diluted in 245 µl of OptiMEM, and 6 µl of Lipofectamine was diluted in 245 µl of OptiMEM. 5 min later these two solutions were mixed. After 30 min of incubation, the complex was added to HEK293 cells in 25 cm^2 culture dishes in a total volume of 2.8 ml of OptiMEM. After transfection, the cells were further cultured for 48 h.

2.7. Cell preparation

Cells transfected with receptor constructs were detached from 25 cm^2 culture dishes with 8 ml of isotonic 50 mM Tris, 1.5 mM EDTA, 100 mM NaCl, pH 7.5. Each cell suspension was centrifuged at 400 g for 3 min, and the cell pellets were re-suspended in 4 ml of binding buffer (50 mM Tris, 100 mM NaCl, pH 7.5). Cell clumps were thoroughly dispersed by pipetting up and down many times with a 1-ml pipette. The suspensions were further diluted (12 times for α_{2A}, 6 times for CppA, and 3 times for α_{2C} and AppC) in order to obtain about 0.1 nM receptor concentration in the pre-incubation assays.

2.8. [^{3}H]-RX821002 binding to whole cells, for obtaining the k_{off} and k_{on} constants for the radioligand

For the radioligand bound to a receptor, the k_{off} value is easily measured in radioligand binding experiments by blocking the on-reaction with a high concentration of an antagonist ($LR = LR_{eq} * exp^{-koff*t}$). In order to determine the k_{off} constant for [^{3}H]-RX821002 at the 4 tested receptors, 125 µl of cell suspension aliquots were mixed with 25 µl radioligand in the binding assays. After 60 min of incubation, one microplate row, with assays representing the binding equilibrium at T_0, was filtered. To the remaining assays, 150 µl of 20; µM atipamezole was added, and then, after various times the assays were filtered.

The on-rate constant (k_{on}) is somewhat more complicated to measure, since the experimental on-reaction curve also involves the k_{off} value, the formula being $LR = LR_{eq} * (1-exp^{-(kon*L+koff)*t})$.

Table 1.
The k_{on} and k_{off} rate constants for [^{3}H]-RX821002, determined at native α_{2A}- and α_{2C}-adrenoceptors and at two chimeric receptors. Shown are the rate constant values ± StdP, and number of experiments. The experiments were performed on transfected whole cells at room temp.

	α_{2A}	α_{2C}	AppC	CppA
k_{on}, association rate constant ($nM^{-1}min^{-1}$)	0.055 ± 0.026 (19)	0.189 ± 0.127 (19)	0.309 ± 0.189 (15)	0.042 ± 0.028 (15)
k_{off}, dissociation rate constant (min^{-1})	0.113 ± 0.016 (5)	0.238 ± 0.066 (4)	0.116 ± 0.018 (4)	0.206 ± 0.041 (4)

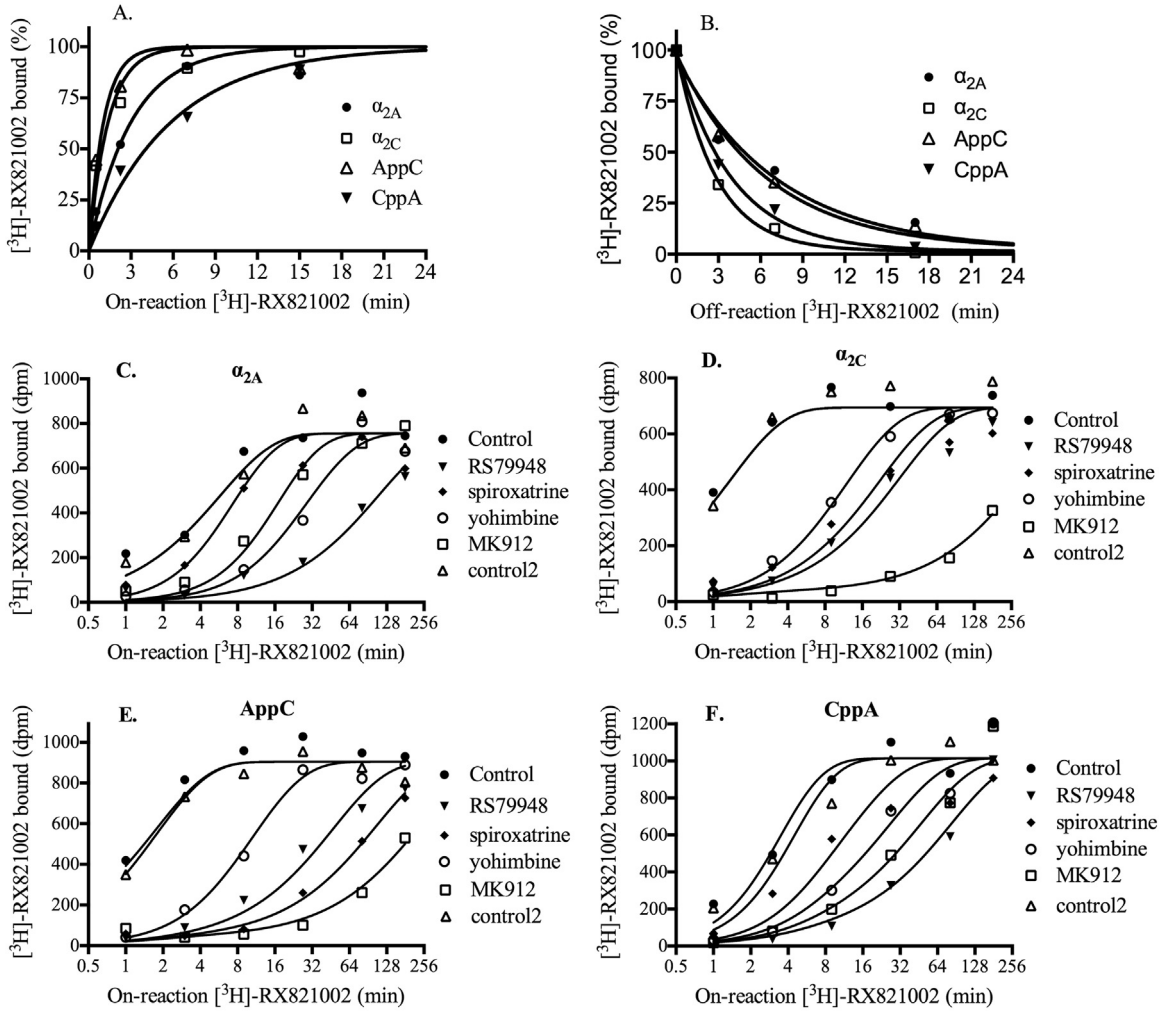

Fig. 1. A-F. On- and off-reaction curves for [^{3}H-RX821002] at α_{2A}, α_{2C}, AppC, and CppA receptors. Panel A shows on-reaction curves for [^{3}H-RX821002]. The concentration of the radioligand was 2.36 nM. Panel B shows the off-kinetic curves for 1.73 nM [^{3}H]-RX821002, obtained after diluting the assay 2-fold and at the same time blocking re-binding with 10 μM atipamezole. Panels C-F show the on-reaction curves for [^{3}H-RX821002] at α_{2A}, α_{2C}, AppC, and CppA receptors, after pre-incubation with 19 × K_i of the unlabeled competitors. The on-reaction curves enable analysis of the off-rate of the pre-incubated competitors, by validating the delay inflicted on the [^{3}H]-RX821002 on-reaction curves by the competitors. The concentration of [^{3}H]-RX821002 in the shown examples were 2.0 nM, i.e. 4.5 × K_i for α_{2A}, 4.1 × K_i for α_{2C}, 8.3 × K_i for AppC, and 2.3 × K_i for CppA.

Anyhow, knowing the k_{off} value, it is rather straightforward to establish the k_{on} value from an on-reaction curve. In principle 125 μl of cell suspension were mixed with 25 μl radioligand in the binding assays, and after various times the assays were filtered. The filtering was performed on Whatman GF/C filters, using a Brandel cell harvester. Radioactivity retained on the filters was counted in a Beckman β-scintillation counter. Curves were fitted using GraphPad Prism. The k_{off} and k_{on} constants obtained from these experiments are presented in Table 1, and the curves are shown in Fig. 1A-B.

2.9. Experimental design developed for the determination of k_{off} constants for unlabeled drugs

In order to facilitate the mathematical analysis, we developed an experimental design where the impact of the on-reaction for the competitors was almost eliminated, and therefore the on-rate could be approximated to zero. Using whole cells in suspension, the cell surface receptors were pre-incubated for 1 h in 96-wells microtiter plates (see paragraph 2.3) with a high (19xK_i) concentration of an antagonist in order to obtain 95% saturation of antagonist binding to the receptors. The assay volume was 150 μl. Then, the microtiter plates were centrifuged at 3000 rpm (1110 g) for 3 min. This attached the HEK 293 cells with antagonist-blocked receptors to the bottom surface. The microplate was then centrifuged upside-down at 600 rpm (44 g) for 10 s to remove the

liquid and unbound ligand while retaining cells, including bound ligand, attached to the bottom surface. Immediately, 300 µl of binding buffer (including about 2 nM [^{3}H]-RX821002) was added rather forcefully to the cells. Thereby, most of the HEK293 cells became re-suspended, due to that cell attachment was weak since attachment of the cells had been achieved just by the previous centrifugation in the calcium free buffer. This step also defines T_0 in the on-reaction for the radioligand as well as T_0 in the off-reaction for the antagonist. Thereafter, the assays were filtered at different time points. The first filtration was performed after 30 s, then after 2, 6, 15, 36 and 72 min. At T_0 the on-rate for the binding of [^{3}H]-RX821002 to receptors will be very small, since there are very few free receptors available. Depending on the off-reaction for the antagonist more receptors become available for [^{3}H]-RX821002 to bind to. The off-reaction of the unlabeled ligand is easily inferred from the delay imposed on the on-reaction curve of the radioligand.

In practice, from each one-day experiment we obtained the k_{off} constants of 4 ligands at 2 different receptors, with n=1. This equals the k_{off} constants of 8 ligands at 4 different receptors after 12 one-day experiments, with n=3. Some advice on the application of the experimental method is provided in supplement 1.

2.10. Formulas used for the curve fitting

The differential equation developed by Malany et al. (2009) describes the on-reaction curve for the radioligand when the receptors have been pre-incubated with a competitor as described above. In their analysis it is assumed that all receptors are blocked by the pre-incubated competing drug at the time of addition of the radioligand, and that the free concentration of competitor, left from the pre-incubation or derived from subsequently dissociated bound competitor, is negligible. Under these conditions, the binding curve is defined by Eq. (1):

$$RL=B_{max}*(k_1*L)/(k_1*L+k_2)*(1-\exp^{-(k1*L+k2)*t})+((k_1*L)/(k_1*L+k_2-k_4))*(B_{max}*(\exp^{-(k1*L+k2)*t}-\exp^{-k4*t})) \quad (1)$$

The first part of the equation above, i.e. $RL=B_{max}*(k1*L)/(k1*L+k2)*(1-\exp^{-(k1*L+k2)*t})$, describes the association binding curve for the radioligand in the absence of a competitor. Within this part, the term $k1*L/(k1*L+k_2)$ describes occupancy based on the rate constants, while $(1-\exp^{-(k1*L+k2)*t})$ describes the association rate.

The second part of the equation, i.e. $((k_1*L)/(k_1*L+k_2-k_4))*(B_{max}*(\exp^{-(k1*L+k2)*t}-\exp^{-k4*t}))$, describes the factor to be subtracted from the first part due to the pre-bound competitor. This factor always has a negative value, lowering the on-rate of the radioligand, especially at early time points of the reaction. It is this part that makes Eq. (1) a differential equation (see Supplements 3A-B for details).

The formula actually used in the present study (Eq. (2), below) is a slight modification of Eq. (1), and describes the on-reaction curve for the radioligand when initially only 95% of the receptors are blocked. This level of blockade occurs after pre-incubation with the competing drug at a concentration 19 times its K_i value. The explicit writing of Eq. (2) in the curve fitting software Graph Pad Prism is detailed in Supplement 2.

$$RL=0.05*B_{max}*(k_1*L)/(k_1*L+k_2)*(1-\exp^{-(k1*L+k2)*t})+0.95*((k_1*L)/(k_1*L+k_2))*B_{max}*(1-\exp^{-(k1*L+k2)*t})+((k1*L)/(k_1*L+k_2-k_4))*(0.95*B_{max}*(\exp^{-(k1*L+k2)*t}-\exp^{-k4*t})) \quad (2)$$

The curve fitting becomes fairly easy. The on-rate constant of the radioligand k_1 is detectable in the control curve, and is then set constant for all curves in that particular experiment (exemplified in Fig. 1(C)-F). The off-rate constant of the radioligand k_2 was obtained in earlier experiments, and is also set constant. The concentration of the radioligand L can be approximated to $[L]\approx[L]_{added}$, or can be calculated precisely as $[L]=[L]_{added}-[LR]$. Since very little competitor is present in the final radioligand binding assay almost no receptors are blocked by competitor at the final equilibrium, and LR_{eq} can be approximated as being shared among the control curve and all the other curves in that experiment. It can be noted that the competitor's k_{on} constant, k_3, is absent from the formula, since the on-reaction of the competitor is negligible due to the low concentration of the competitor after the washout of free competitor, and due to the blocking effect by the radioligand and the large 300 µl final assay volume. The remaining rate constant, i.e. the searched for k_{off} constant for the competing drugs, k_4, should be well detectable using our experimental set-up. The best applicability is for drugs where the $t_{1/2}$ of the drug is longer than the duration of time before the first measurement, which in our case was 30 s

Each experiment aimed at determining the k_{off} constant of unlabeled drugs included six curves, whereof one duplicate control curve for determining the on-rate constant of the radioligand in that particular experiment. After calculating k_1 from the control curves, k_1 and k_2 were set constant in the full 6-curve analysis (2 control curves and 4 curves obtained after preincubation with antagonists), and the B_{max} value was set shared for the 6 curves. Except the shared B_{max} value, the only constant the mathematical analysis had to define was the k_{off} constant of the pre-incubated unlabeled drugs, i.e. the k_4 constants. Eq. (2) describes the curves obtained by the herein described experimental design, and under these conditions Eq. (2) is fully sufficient, and easier to use than the full 4 kinetic constant analysis described by Motulsky and Mahan (1984). The main limitation of our method is that only drugs with half-lives in the range 1–60 min were readily analyzable, i.e. drugs with k_{off} constants between 0.693 min^{-1} and 0.012 min^{-1} ($t_{1/2}=\ln 2/k_4$). At the fast dissociation rate extreme, the dissociation rate was hard to detect for competitors having k_4 values higher than 1.386 min^{-1}, i.e. when it takes less than half a min for the pre-incubated drug to dissociate from half of the receptors.

Pre-incubating with not more than 19 fold the K_i values of the unlabeled compounds seems to avoid the problem of residual presence of competitors in the final assays (such "contamination" was seen with higher doses in pilot experiments). Using ligand pre-incubation concentrations at 19X their K_i values, all curves in the experiments approached the shared B_{eq} after 72 min, except the curves with MK912. However, even for MK912 the analysis indicated that the residual blocking of [^{3}H]-RX821002 binding by pre-incubated MK912, at the α_{2C}- and AppC-adrenoceptors, at 72 min, was due to not yet dissociated molecules, since the drug was continuously dissociating for up to 3 h in experiments with extended incubation times (not shown). In addition, pilot experiments performed at 37 °C showed that all MK912 eventually dissociated from pre-incubated α_{2C}-adrenoceptors,

In a study resembling the present study, Packeu et al. (2010) used plated cultured cells in their approach to determine the off-rate constant of unlabeled ligands. They introduced the term "two-step" procedure, involving a pre-incubation step with unlabeled ligand. The main technical differences between their study and our study are that we started with cell suspensions instead of plated cells, and we removed free ligand by upside-down centrifugation instead of aspiration. Using cell suspensions is very convenient, especially when many drugs are to be tested, since the cell solutions are homogeneous and can be diluted at will, and there is no

delay between plating of the cells and performing the kinetic experiment. Un-binding of the pre-incubated ligand, as well as binding of the radioligand, occur at cells which to a large extent had become re-suspended by the forceful addition of the diluting radioligand solution. This allows good diffusion accessibility for the ligands. In addition, our mathematics is even more reductive than that of Packeu and coworkers, since we use just one concentration of pre-incubated competitor, while they constructed dose-response curves. Our on-reaction curves represent the same type of curves that are present in their Figure 4C.

3. Results

3.1. Experimental methodology

The concept behind the present study was to practically eliminate the on-reaction of the competitors at the stage after the pre-incubation. Thereby, we arrive at a simpler mathematical formula. At the start of the present project, such an elimination of the on-reaction seemed straightforward in theory, but technically it turned out to be more difficult than anticipated. In series of pilot experiments we tried in vain an experimental setup where membrane preparations expressing the α_{2A}-adrenoceptors were pre-incubated with competitors. After the pre-incubation, excess competitor was washed away by centrifugation at high speed (38,000 g), and the supernatant discarded. However, re-suspension of the pellets took too long time, and no reproducible results were obtained. Therefore, we turned to whole cell preparations. [^{3}H]-RX821002 does not enter whole cells, and thereby it labels plasma membrane receptors from the extracellular side only (Jahnsen and Uhlén, 2012). Whole HEK293 cells (150 µl suspension) pre-incubated with competitors (19xK_i) in a microtiter-plate can be centrifuged at 3000 rpm (1110 g) for three min, attaching the cells to the bottom surface. Then, turning the microplate upside-down and centrifuging at 600 rpm (44 g) for 10 s removes essentially all medium into the attached waste container. Immediately after the centrifugation process, 300 µl of [^{3}H]-RX821002 was quickly and rather forcefully added to the cells. This action resulted in the re-suspension of most of the cells in one step. Assuming pre-bound competitor does not start to dissociate from the receptors until the addition of the diluting buffer, including the radioligand, the on-reaction for [^{3}H]-RX821002 and the off-reaction for the pre-bound competitor start simultaneously.

3.2. Experimental k_{off} and k_{on} values for [^{3}H]-RX821002 at the α_{2A}-, α_{2C}-, and two chimeric α_2-adrenoceptors

In Fig. 1(A) is shown the on-rate curves for [^{3}H]-RX821002, obtained at whole cells expressing α_{2A}, α_{2C}, and two chimeric α_2-adrenoceptors. The curves represent the binding of [^{3}H]-RX821002 at various time points after adding the radioligand. The association $t_{1/2}$ values at the various receptors were in the range 0.5–3 min Fig. 1(B) shows the off-rate curves for [^{3}H]-RX821002 from its equilibrium binding state. The procedure was to add an excess of atipamezole after a 60 min of pre-incubation with [^{3}H]-RX821002, and then filtering at various time points. The dissociation $t_{1/2}$ values at different receptors were in the range 3–6 min. Among the α_{2A}-, α_{2C}-and chimeric α_2-adrenoceptors, the on-rate constant (k_{on}) of [^{3}H]-RX821002 was highest at the α_{2C}- and AppC-receptors, while the off-rate constant (k_{off}) was highest at the α_{2C}- and CppA-receptors (Table 1). The calculated K_d values, i.e. the k_{off}/k_{on} ratios, were 2.06 nM at the α_{2A}-, 1.26 nM at the α_{2C}-adrenoceptors, 0.38 nM at the AppC receptor and 4.90 nM at the CppA receptor. The order of these values corresponds to the corresponding K_d values determined by equilibrium binding (Jahnsen and Uhlén, 2013). The kinetically determined K_d values are slightly higher than the K_d values determined at equilibrium, an effect likely induced by the high concentration of blocking competitor added in the off-rate experiments (see Vauquelin and van Liefde, 2012).

3.3. Experimental k_{off} values and calculated k_{on} values for unlabeled ligands at the α_{2A}-, α_{2C}-, and two chimeric α_2-adrenoceptors

Fig. 1(C)-(F) show the on-rate curves for [^{3}H]-RX821002 after pre-incubation with competitors. Displaying the curves in a semi-logarithmic plot, the curves are shifted to the right as compared to the antagonist-free control time-curve, though not in parallel since the blocking effect of the pre-incubated ligands is largest at early time points. The binding curves, which are influenced by the off-rates of the competitors, look quite similar among the α_{2A}- and CppA- receptors, and so are the binding curves among the α_{2C}- and AppC- receptors. This implies the C-terminal half of these receptors has the largest subtype-selective influence on the off-rate of the ligands, confirming that the C-terminal half of these receptors is the main determinant for the receptor's drug selectivity (Jahnsen and Uhlén, 2013).

Table 2 shows the experimentally obtained k_{off} values for the tested compounds. The lowest off-rate was for MK912 at the α_{2C}-adrenoceptor (k_{off}=0.00477 min^{-1}), indicating a half-life of bound MK912 of 145 min, at room temperature. Despite its high affinity, the ligand atipamezole showed a k_{off} value of 1.156 at the α_{2C}-adrenoceptor, indicating a half-life of bound atipamezole of 36 s. For ligands having half-lives shorter than 30 s accurate k_{off} values could not be obtained using our assay. Therefore, for chlorpromazine no accurate k_{off} values were obtained, and for BRL44408 the only accurate k_{off} values obtained was the k_{off} value at the α_{2A}-adrenoceptor.

Dividing the k_{off} values from Table 2 with the K_i values of the ligands yields the calculated k_{on} values (k_{on}= k_{off}/K_i). The K_i values of RS79948–197 and yohimbine were obtained in the present study, while the K_i-values of all the other tested compounds had already been determined in our previous study (Jahnsen and Uhlén, 2013). The here obtained K_i values of RS79948–197 at the four tested receptors were 0.245 ± 0.138 at the α_{2A}, 0.221 ± 0.121 at the α_{2C}, 0.236 ± 0.108 at the AppC and 0.198 ± 0.025 at the CppA receptors. The K_i values for yohimbine were 1.88 ± 0.10 at the α_{2A}, 1.06 ± 0.47 at the α_{2C}, 1.71 ± 0.79 at the AppC and 1.99 ± 0.84 at the CppA receptors. The number of experiments were n=3 at the α_{2A}- and α_{2C}-adrenoceptors, and n=4 at the AppC and CppA receptors.

The calculated k_{on} constants are shown in Table 3. As can be seen in Table 3, the ligand with the lowest calculated k_{on} value was yohimbine at the α_{2A}-adrenoceptor (k_{on}=0.0152 nM^{-1} min^{-1}). The ligand with the highest calculated k_{on} value was atipamezole at the AppC receptor (k_{on}=1.671 nM^{-1} min^{-1}).

Table 4 shows the α_{2C}/α_{2A} selectivity ratios of six ligands, regarding k_{off} constants, k_{on} constants, and affinities. The drugs with lowest k_{off} ratio between α_{2C}/α_{2A} were spiroxatrine and MK912. This contributes to their α_{2C}-selectivity. The drug with the highest k_{on} ratio between α_{2C}/α_{2A} was spiroxatrine. The high on-rate and slow off-rate for spiroxatrine at the α_{2C}-adrenoceptor makes spiroxatrine markedly α_{2C}-selective.

The results show that the α_{2C}- over α_{2A}-adrenoceptor selectivity of MK912 depends mainly on a very low off-rate for MK912 at the α_{2C}-adrenoceptor. On the other hand, the α_{2C}- over α_{2A}-selectivity of spiroxatrine depends both on its high on-rate as well as on its low off-rate at the α_{2C}-adrenoceptor. Atipamezole showed very high off-rate at the α_{2A}- and α_{2C}-adrenoceptors, but still shows high affinity thanks to its concomitant very high on-

Table 2.
Experimental k_{off} dissociation rate constants for pre-incubated ligands, determined at native α_{2A}- and α_{2C}-adrenoceptors and at two chimeric receptors. Shown are the $k_{off} \pm$ StdP values (unit min^{-1}), and number of experiments. The k_{off} values in Table 2 are also presented graphically in Fig. 2(A).

Ligand	α_{2A}	α_{2C}	AppC	CppA
MK912	0.0606 ± 0.0167 (10)	0.0048 ± 0.0034 (12)	0.0066 ± 0.0026 (9)	0.0442 ± 0.0072 (8)
RS79948–197	0.0135 ± 0.0022 (6)	0.0281 ± 0.0058 (6)	0.0202 ± 0.0032 (3)	0.0221 ± 0.0070 (3)
spiroxatrine	0.642 ± 0.455 (9)	0.0466 ± 0.0129 (12)	0.0430 ± 0.0179 (10)	0.403 ± 0.354 (8)
yohimbine	0.0285 ± 0.0054 (6)	0.0955 ± 0.0512 (6)	0.126 ± 0.021 (3)	0.153 ± 0.116 (3)
RX821002	0.0734 ± 0.0145 (9)	0.216 ± 0.134 (12)	0.0823 ± 0.0215 (10)	0.141 ± 0.034 (9)
atipamezole	0.865 ± 0.464 (8)	1.156 ± 0.484 (9)	0.665 ± 0.318 (9)	0.939 ± 0.587 (6)
clozapine	> 1.386 (6)	1.756 ± 0.602 (6)	1.498 ± 1.002 (5)	1.629 ± 0.330 (3)
BRL44408	1.308 ± 0.393 (7)	> 1.386 (8)	> 1.386 (8)	> 1.386 (8)
chlorpromazine	> 1.386 (8)	> 1.386 (4)	> 1.386 (4)	> 1.386 (4)

Table 3.
Calculated k_{on} association rate constants (nM^{-1} min^{-1}) for the pre-incubated ligands at native α_{2A}- and α_{2C}-adrenoceptors and at two chimeric receptors. The formula used is $k_{on} = k_{off}/K_i$. The k_{off} values were taken from Table 2. The K_i values were taken from Jahnsen and Uhlen, 2013, except for RS79948–197 and yohimbine where the K_i values were obtained in the present study. The calculated k_{on} values in Table 3 are also presented graphically in Fig. 2(B).

Ligand	α_{2A}	α_{2C}	AppC	CppA
MK912	0.0798	0.0467	0.106	0.0609
RS79948–197	0.0549	0.127	0.0854	0.111
spiroxatrine	0.0268	0.112	0.121	0.0320
yohimbine	0.0152	0.0901	0.0736	0.0771
RX821002	0.164	0.440	0.343	0.161
atipamezole	0.971	1.297	1.671	0.763
clozapine	–	1.112	0.462	0.087
BRL44408	0.217	–	–	–
chlorpromazine	–	–	–	–

Table 4.
Ratio of α_{2C}- over α_{2A}-selectivity for 7 ligands, regarding dissociation rates, association rates, and affinities. In the second column is shown the calculated α_{2C}/α_{2A} k_{off} values ratio. In the third column is shown the calculated α_{2C}/α_{2A} k_{on} values ratio. These ratios were calculated from data presented in the previous tables. In the fourth column are shown the α_{2C}- over α_{2A}-selectivity of the ligands, calculated from the k_{on} value ratios in column 3 divided with the k_{off} value ratios in column 2. The same α_{2C}- over α_{2A}-selectivity value is extractable from the K_i values, since the k_{on} values were derived by calculation from the ligand's k_{off} and K_i values.

Ligand	Ratio of dissociation rates (k_{off} α_{2C}/ k_{off} α_{2A})	Ratio of association rates (k_{on} α_{2C}/ k_{on} α_{2A})	α_{2C}- over α_{2A}-selectivity (k_{on} ratio / k_{off} ratio)
MK912	0.079	0.59	7.44
RS79948–197	2.09	2.31	1.11
spiroxatrine	0.073	4.18	57.6
yohimbine	3.35	5.93	1.77
RX821002	2.94	2.68	0.91
[^{3}H]-RX821002	2.11	2.42	1.14
atipamezole	1.34	1.34	1.00

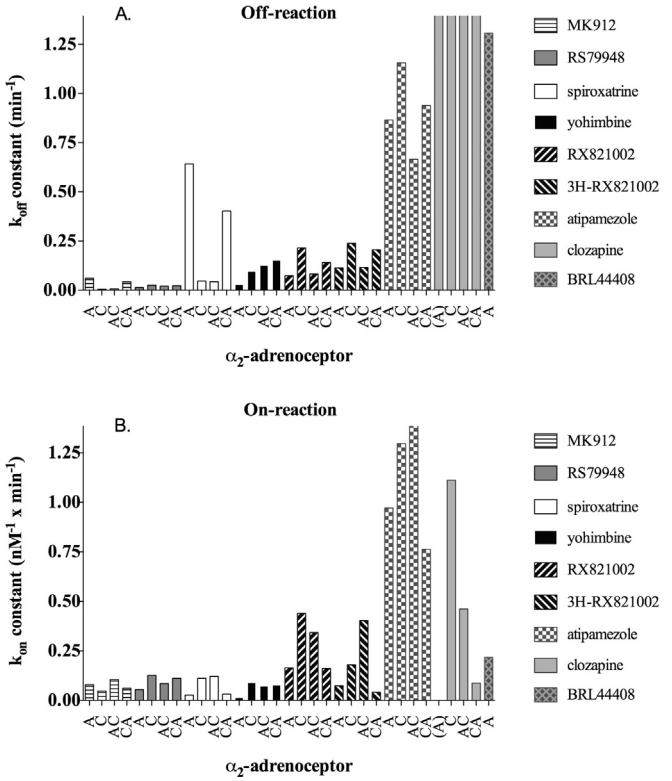

Fig. 2. A-B. Staple diagram of the k_{off} and k_{on} constants for unlabeled competitors. Section A shows experimental k_{off} constants for [^{3}H]-RX8321002 and 8 unlabeled competitors, at the α_{2A}, α_{2C}, AppC, and CppA receptors. The receptors are indicated below the x-axis by the letters A, C, AC, and CA. Section B shows the experimental k_{on} constant for [^{3}H]-RX8321002 and the calculated k_{on} constants for the 8 unlabeled competitors. Cut-offs were set at $k_{off} = 1.386\ min^{-1}$ and $k_{on} = 1.386\ nM^{-1}\ min^{-1}$. At higher k_{off} values half of the dissociation occurs in less than 30 s, and therefore determination of off-rate constants above this value is less accurate.

rate (see Fig. 2(A)-(B)).

3.4. Affinity range for applicability of the experimental method

The dissociation of MK912, RS79948–197, spiroxatrine, yohimbine, and RX821002 had $t_{1/2}$ values above one min at the receptors. These ligands produced a clear delay of [^{3}H]-RX821002 binding. Atipamezole, BRL44408, and chlorpromazine had $t_{1/2}$ values near or below one min at the receptors, and the calculated k_{off} values for these drugs are therefore less adequate. Nevertheless, it is clear that chlorpromazine dissociates quickly from all the tested receptors. As a rule of thumb, the methodology enabled clear detection of the off-rate constant for unlabeled ligands with K_i values in the range 200 pM– 10 nM. The drug with lowest affinity where k_{off} still was detectable was spiroxatrine at α_{2A}, where the K_i value was 24 nM, and the k_{off} value 0.64 min^{-1} (Table 3). This is a fairly low k_{off} value, and low off-rate, for such a low-affinity drug, so the low affinity seems to be caused by a corresponding low k_{on} value. The drug with highest affinity that still dissociated completely within 72 min was RX821002 at AppC, where the K_i value was 0.24 nM and the k_{off} value 0.082 min^{-1} (Table 3). Since, RX821002 had rather high on- and off-rates, the half-life of bound RX821002 was fairly short despite its high affinity, namely about 8 min ($t_{1/2} = \ln 2/k_{off}$).

4. Discussion

4.1. A radioligand binding experimental design for determining the

kinetic off-rate constants for unlabeled drugs at α_2-adrenoceptors

The major aim of the present study was to develop a simple radioligand binding experimental design for determining the kinetic off-rate constants for unlabeled drugs. Previous radioligand binding studies, including in-depth mathematical analysis, have been performed, characterizing the kinetics of competitive binding at β-adrenoceptors (Motulsky and Mahan, 1984), muscarinic receptors (Schreiber et al., 1985; Dowling and Charlton, 2006), D_2 dopamine and CB_1 cannabinoid receptors (Packeu et al., 2010), and adenosine A_1 receptors (Sykes et al., 2010; Guo et al., 2013; Xia et al., 2016). Most of the methods used in these studies have limitations, either due to high complexity or due to narrow applicability. In an all-at-once assay, several factors ([C], [CR], [R], [L] and [LR]) are varying simultaneously, and four different kinetic constants are involved (k_1, k_2, k_3, k_4) (see Motulsky and Mahan, 1984). Using a two-step method, as in the present study, the only unknown kinetic constant in the final on-rate curve for the radioligand is the k_{off} constant of the unlabeled competitor (k_4). Thus, the on-rate constant for the radioligand (k_1) is set constant with the value obtained from the control curve. The off-rate constant for the radioligand (k_2) is known from previous experiments (Table 1). The on-rate constant for the competitor (k_3) becomes redundant by the efficient washing-out of the competitor.

The results confirm that the experimental concept works, the wet-lab methodology essentially consisting of pre-incubation of receptors at whole cells with competitor, then quick washout by upside-down centrifugation, followed by performing the on-reaction for the radioligand. In the analysis, the k_{off} constant of the unlabeled competitor was calculated by fairly simple curve fitting mathematics (Eq. (2)), derived from the differential equation (Eq. (1)) of Malany et al. (2009). Our results show that spiroxatrine associates slowly and dissociates rapidly at the α_{2A}- and the CppA-receptors. Conversely, at the α_{2C}- and AppC-adrenoceptors, spiroxatrine associates rapidly and dissociates slowly. This pattern constitutes the α_{2C}- and AppC-selectivity of spiroxatrine. Regarding MK912, the off-kinetics experiments showed that MK912 dissociated very slowly from α_{2C} and AppC. In PET studies it has been shown that [^{11}C]MK-912 washed out problematically slow from the brain (Shiue et al., 1998). A contributing reason for this slow washout might be the low off-rate of MK912 at α_{2C}-adrenoceptors, as shown in the present study.

4.2. Kinetic parameters of ligand-receptor interactions

There are two established methodologies for studying the kinetic component of ligand-receptor interactions, namely radioligand binding and surface plasmon resonance, while several fluorescence-based methods were classified as in development [Hoffmann et al., 2015]. However, it is not until just recently that surface plasmon resonance technique (Biacore) has been used for determining the kinetic constants for small molecules interacting with G protein-coupled receptors (Rich et al., 2011). This technique still is laborious in the preparation of receptor-coated sensor chips and it is more difficult to analyze the binding kinetics for small molecules than for larger molecules. A different but technically advanced method is currently developed, namely a single molecule nanoscopic method for tip-attached peptides, enabling detection of the contact force between the ligand and receptor, i.e. measuring the required off-kinetic energy (Alsteens et al., 2015). To large extent, the off-kinetic energy determines the residence time for a ligand at a receptor. Copeland and coworkers defined the term residence time to refer to the temporal duration of the binary receptor-ligand complex, and stated that the residence time is affected by the dissociation rate (see Copeland, 2016). According to Copeland et al. (2006) the residence time (τ) is defined by $\tau = 1/k_{off}$, making residence time proportionally 1.443 times longer than dissociation half-life defined by $t_{1/2} = \ln 2/k_{off}$. Thus, in mass action kinetics, residence time and dissociation half-life are interchangeable. These two time-constants do not fully define ligand affinity or receptor occupancy, which according to the law of mass action are dependent on both the on- and off-rates. However, at the individual receptor level, the residence time of a ligand might influence its biological effect, so the quantitative assessment of this parameter has a role in the overall evaluation of receptor-ligand interactions in pharmacology (Tummino and Copeland, 2008; Guo et al., 2014; Hothersall et al., 2016; Vauquelin, 2015). Intuitively, residence time can be mistaken for the temporal presence of the ligand-receptor complex, i.e. receptor occupancy × B_{max} × time ((mol/liter)xmin), which is visualized as an area under the curve. However, residence time in the simplest model (Hothersall et al., 2016) is just a time point (min) at the dissociation curve. In binding kinetics experiments, the temporal presence of the ligand-receptor complex becomes larger with faster on-rate or slower off-rate, while residence time only is influenced by the off-rate. In our experiments, the observed k_{off} values reflect detachment and dissociation from the receptor, i.e. the freeing of the receptor from pre-bound competitor. This rate is somewhat modified by immediate re-binding of the ligand to the original receptor, i.e. by a local un-binding - re-binding process (see Vauquelin, 2016 in press). Thus, our observed k_{off} value represents dissociation of the ligand all the way out of the receptor (Fig. 3), which may resemble a true situation at individual receptors.

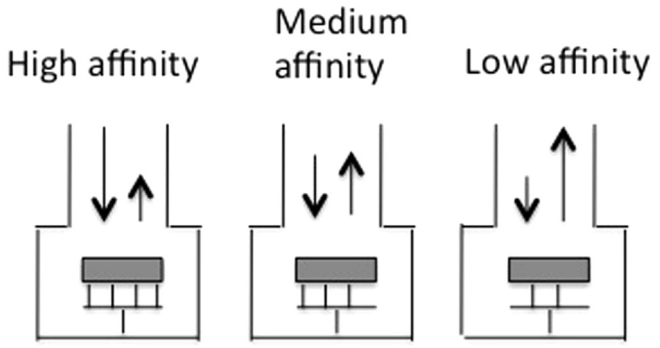

Fig. 3. Model of ligand-receptor interactions. The lower part of the figures represents the proper binding site with ligand bound to the receptor. Here the affinity of a ligand is influenced by the number of contact points and by the strength of the bonds. The upper part of the figures represents the translocation pathway for a ligand to and from the binding site of the receptor, with arrows indicating fast or slow movements. Together, the bonds and the translocation processes define the k_{on} and k_{off} values, and thereby the affinity of a ligand for a receptor.

4.3. The chimeric AppC and CppA receptors

The chimeras were designed in order to evaluate whether the first transmembrane helix of the α_{2A}-adrenoceptor is indirectly interacting with the second extracellular loop of the receptor, hypothetically inducing slow on-rate (or fast off-rate) for bulky drugs like chlorpromazine, rauwolscine and spiroxatrine at the α_{2A}-subtype, and thereby induce α_{2C}- over α_{2A}- selectivity (Laurila et al., 2011). Our results could not confirm any hypothetical interaction between TM1 and extracellular loop 2 in the α_{2A}-adrenoceptor, such that bulky drugs would associate slowly and/or dissociate rapidly at the α_{2A}- but not at all of the α_{2C}-, AppC and CppA-receptors. That complete pattern was in fact not observed for any of the four successfully tested bulky ligands MK912, RS79948–197, spiroxatrine or yohimbine. For example, the specifically slow on-rate of spiroxatrine was shared among the α_{2A}- and the CppA-receptors, even though the CppA-receptor has its first transmembrane helix from

the α_{2C}-adrenoceptor. However, a general observation was that most of the tested ligands had slower on-rates at the α_{2A}- as compared to at the α_{2C}-adrenoceptor (Fig. 2(B)).

4.4. Off-rate detection range between too fast and too slow off-rates for the pre-incubated ligands

Among the herein tested drugs the detection range corresponded to drugs with K_i values in the range 0.2 – 10 nM. Drugs with K_i values above 10 nM tended to have too high off-rate for the k_{off} constant to be detectable. The fact that low affinity drugs usually have high off-rates is obvious from the formula $K_i = k_{off}/k_{on}$. However, for analysis of drug binding to receptors there are plenty of substances with K_i values in the range 0.2 – 10 nM, and among them some may show unexpected on- or off-rates at various receptors.

4.5. Kinetic ligand-receptor interactions

In the present study we show that the on-reaction rate may vary substantially among different drugs as well as for any drug at two different receptors (Table 2). The results implies that the on-rate for a ligand is dependent on diffusion, positioning at the receptor, and attachment to the binding site, while the off-rate is dependent on detachment and diffusion away from the binding site. The physical bond forces at the binding site constitute the main factor in determining the dissociation half-life and affinity of a ligand for the receptor, but the on- and off-movements of the ligand may also play a role for its affinity. Thus, in a recent molecular dynamics simulation and experimental study it was demonstrated that even receptor features with little contribution to affinity may prove critical to the dissociation process (Guo et al., 2016). The fact that some drugs, like spiroxatrine, have a higher k_{on} constant at the α_{2C}- than at the α_{2A}-adrenoceptor seems to indicate that the α_{2A}- and α_{2C}-adrenoceptors interact differentially with spiroxatrine at the entrance process for the drug. RS79948 and yohimbine moved slowly in and out of the binding site at all tested receptors, while RX821002 and atipamezole moved fast in and out. Notably, MK912 dissociates extremely slowly from the α_{2C}-adrenoceptor subtype. Finally, at the α_{2C}-adrenoceptor subtype, spiroxatrine enters the binding site fast, but dissociates slowly, explaining its α_{2C}-selectivity. In Fig. 3 we present a model where the movements of the ligand are separated from the physical bonds between ligand and binding site. The model is meant to illustrate a theoretical separation between on one hand the movements of the ligand into and out of the binding site, and on the other hand the physical binding force keeping the ligand attached at the binding site. The model gives an idea about what is defining the affinity of a reversible drug for the receptor, and may help theorists refine *in silico* receptor models. For example, the affinity of a prospective ligand could be optimized, by creating a new molecular structure, having a higher on-rate.

Authorship contributions

Participated in research design: Uhlén. Conducted experiments: Jahnsen, Uhlén. Performed data analysis: Uhlén. Contributed to the writing of the manuscript: Jahnsen, Schiöth, Uhlén.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2016.06.021.

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