

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/62615> holds various files of this Leiden University dissertation.

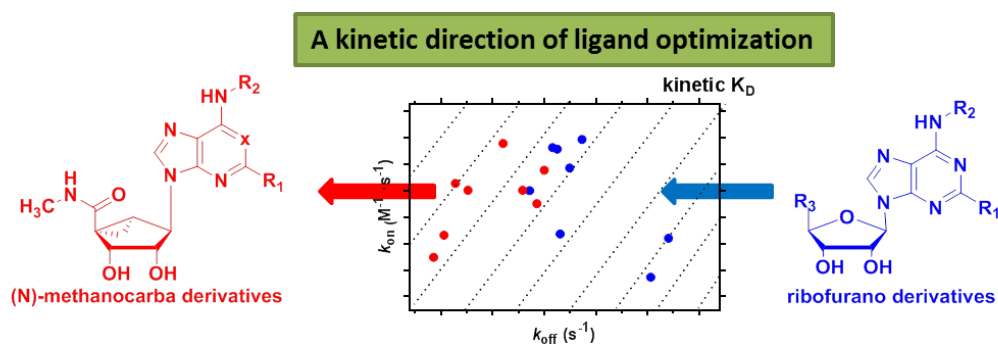
**Author:** Xia, L.

**Title:** Corpora non agunt nisi fixata : ligand receptor binding kinetics in G protein-coupled receptors

**Issue Date:** 2018-05-30

# Chapter 5

## A Binding Kinetics Study of Human Adenosine A3 Receptor Agonists



Lizi Xia, Athina Kyrizaki, Dilip K. Tosh, Tirsa T. van Duijl, Jacomina Cornelia Roorda,

Kenneth A. Jacobson, Adriaan P. IJzerman, Laura H. Heitman

Adapted from: *Biochem. Pharmacol.* **2018** doi: 10.1016/j.bcp.2017.12.026. [Epub ahead of print]

## About this chapter

The human adenosine A<sub>3</sub> (hA<sub>3</sub>) receptor has been suggested as a viable drug target in inflammatory diseases and in cancer. So far, a number of selective hA<sub>3</sub> receptor agonists (e.g. IB-MECA and 2-Cl-IB-MECA) inducing anti-inflammatory or anticancer effects are under clinical investigation. Drug-target binding kinetics is increasingly recognized as another pharmacological parameter, next to affinity, for compound triage in the early phases of drug discovery. However, such a kinetics-driven analysis has not yet been performed for the hA<sub>3</sub> receptor. In this study, we first validated a competition association assay for adenosine A<sub>3</sub> receptor agonists to determine the target interaction kinetics. Affinities and Kinetic Rate Index (KRI) values of 11 ribofurano and 10 methanocarba nucleosides were determined in radioligand binding assays. Afterwards, 15 analogues were further selected (KRI <0.70 or KRI >1.35) for full kinetics characterization. The structure-kinetics relationships (SKRs) were derived and longer residence times were associated with methanocarba and enlarged adenine N<sup>6</sup> and C2 substitutions. In addition, from a  $k_{on}$ - $k_{off}$ -K<sub>D</sub> kinetic map we divided the agonists into three subgroups. A residence time “cliff” was observed, which might be relevant to (N)-methanocarba derivatives’ rigid C2-arylalkynyl substitutions. Our findings provide substantial evidence that, next to affinity, additional knowledge of binding kinetics is useful for developing and selecting new hA<sub>3</sub>R agonists in the early phase of the drug discovery process.

## Introduction

The adenosine A<sub>3</sub> receptor is the youngest member discovered in the family of adenosine receptors (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>), all of which belong to class A G protein-coupled receptors (GPCR).<sup>1</sup> Unlike the other subtypes, the adenosine A<sub>3</sub> receptor is endogenously activated by both adenosine and inosine.<sup>2</sup> Following agonist activation, the receptor causes a decrease in cAMP levels as it primarily couples to G<sub>i</sub> protein. The adenosine A<sub>3</sub> receptor is widely distributed throughout the body, albeit at low expression levels, and its activation can affect numerous organs, tissues and systems. However, this receptor is overexpressed in inflammatory and cancer cells, reflecting its importance as a therapeutic target and biological marker in these tissues.<sup>3,4</sup> Moreover, the adenosine A<sub>3</sub> receptor is rapidly internalized within 30 min after agonist exposure.<sup>5, 6</sup> It is unresolved whether receptor desensitization terminates agonist-induced signaling or if this signaling persists after internalization.<sup>7</sup>

Since agonists cause effects of anti-inflammation, cardioprotection and neuroprotection, numerous potent and selective agonists have been extensively studied and developed.<sup>8-13</sup> Most of these agonists are derivatives of adenosine, with substitutions on the adenine nucleobase or the ribose moiety. In particular, IB-MECA and 2-Cl-IB-MECA are two potent clinical candidates with excellent bioavailability and safety profiles. Selective hA<sub>3</sub> receptor agonists are being evaluated for the treatment of chronic inflammatory diseases (e.g., rheumatoid arthritis or psoriasis), neuropathic pain and cancer (i.e. hepatocellular carcinoma).<sup>14-17</sup>

Traditional drug discovery approaches mainly focus on affinity, a parameter that is defined under equilibrium conditions. However, it is emerging that selecting ligands based on their affinity alone, does not necessarily predict *in vivo* efficacy very well. This may be due to the dynamic conditions *in vivo*, that often are in contrast to the equilibrium conditions applied in *in vitro* assays.<sup>18</sup> In fact a ligand's kinetic properties may provide a better indication of how a ligand will perform *in vivo*.<sup>19</sup> Notably, the parameter of residence time (RT) has been proposed as a more relevant selection

criterion. The RT reflects the lifetime of the ligand-receptor complex and can be calculated as the reciprocal of the ligand's dissociation constant.<sup>20, 21</sup>

Although the binding kinetics of hA<sub>3</sub> receptor agonists are occasionally reported,<sup>22, 23</sup> a systematic kinetics analysis yielding structure-kinetics relationships (SKRs) has not been conducted. Therefore, we firstly validated the binding kinetics of the prototypical hA<sub>3</sub> receptor agonists (i.e. IB-MECA and 2-Cl-IB-MECA), using radioligand displacement and competition association assays. Then, two series of ribofurano and methanocarba ([3.1.0]bicyclohexane) adenosine derivatives were evaluated for both their affinity (K<sub>i</sub>) and kinetics (kinetic rate index values, k<sub>on</sub>, k<sub>off</sub>, and RTs). This allowed a complete SKR analysis next to a more traditional SAR study. Afterwards, a retrospective evaluation linking residence times and *in vivo* efficacies was discussed. Last but not least, from a k<sub>on</sub>-k<sub>off</sub>-K<sub>D</sub> kinetic map we divided the agonists into three subgroups, providing a possible direction for the further development of hA<sub>3</sub>R agonists.

## Method

**Chemicals and reagents.** [<sup>3</sup>H]8-Ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-*i*]purin-5-one ([<sup>3</sup>H]PSB-11, specific activity 56 Ci·mmol<sup>-1</sup>) was obtained with the kind help of Prof. C.E. Müller (University of Bonn, Germany). 5'-N-methylcarboxamidoadenosine (MECA) was provided by one of the authors (KAJ). Unlabeled PSB-11, 1-deoxy-1-[6-[[[(3-Iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), and 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (2-Cl-IB-MECA) were purchased from Tocris Ltd. (Abingdon, UK). 5'-N-ethylcarboxamidoadenosine (NECA) was purchased from Sigma-Aldrich (Steinheim, Germany). LUF5501, LUF5505, LUF5500, LUF5506 and LUF5521 have been synthesized and described previously by de Zwart *et al.*<sup>24</sup>; LUF5595, LUF5586 and LUF5589 have been described by van Tilburg *et al.*<sup>25</sup>; MRS7140, MRS5980, MRS7154, MRS7549, MRS5655, MRS5679, MRS5698, MRS5644, MRS5667 and MRS7294 have been described by Tosh *et al.*<sup>26-29</sup>, and MRS3558 by Tchilibon *et al.*<sup>30</sup>. Adenosine deaminase (ADA) was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic

acid (BCA) and BCA protein assay reagents were purchased from Pierce Chemical Company (Rockford, IL, USA). Chinese hamster ovary (CHO) cells stably expressing the human adenosine A<sub>3</sub> receptor (CHOhA<sub>3</sub>) were a gift from Dr. K-N Klotz (University of Würzburg, Germany). All other chemicals were obtained from standard commercial sources and were of analytical grade.

*Cell culture and membrane preparation.* CHOhA<sub>3</sub> cells were cultured and membranes were prepared and stored as previously described.<sup>22</sup> Protein determination was done through use of the bicinchoninic acid (BCA) method.<sup>31</sup>

*Radioligand equilibrium displacement assays.* Membrane aliquots containing ~15 µg of CHOhA<sub>3</sub> protein were incubated in a total volume of 100 µL assay buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, supplemented with 0.01% CHAPS and 1 mM EDTA, pH 7.4) at 10 °C for 240 min. Displacement experiments were performed using 6 concentrations of competing agonist in the presence of a final concentration of ~10 nM [<sup>3</sup>H]PSB-11. At this concentration, total radioligand binding did not exceed 10% of that added to prevent ligand depletion. Nonspecific binding (NSB) was determined in the presence of 100 µM NECA. Incubation was terminated by rapid filtration performed on 96-well GF/B filter plates (Perkin Elmer, Groningen, the Netherlands), using a PerkinElmer Filtermate-harvester (Perkin Elmer, Groningen, the Netherlands). After drying the filter plate at 50 °C for 30 min, the filter-bound radioactivity was determined by scintillation spectrometry using the 2450 MicroBeta<sup>2</sup> Plate Counter (Perkin Elmer, Boston, MA).

*Radioligand competition association assays.* The binding kinetics of unlabeled ligands were quantified using the competition association assay based on the theoretical framework by Motulsky and Mahan.<sup>32</sup> The competition association assay was initiated by adding membrane aliquots (15 µg/well) at different time points for a total of 240 min to a total volume of 100 µl of assay buffer at 10 °C with ~10 nM [<sup>3</sup>H]PSB-11 in the absence or presence of a single concentration of competing hA<sub>3</sub> receptor agonists (i.e. at their IC<sub>50</sub> value), and, in some experiments indicated, in the simultaneous presence of 1 mM GTP. Incubations were terminated and samples were obtained as described under

*Radioligand Displacement Assay.* The “dual-point” competition association assays were designed as described previously,<sup>33</sup> where in this case the two time points were selected at 20 min ( $t_1$ ) and 240 min ( $t_2$ ).

*Data analysis.* All experimental data were analyzed using the nonlinear regression curve fitting program GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). From displacement assays, IC<sub>50</sub> values were obtained by non-linear regression analysis of the displacement curves. The obtained IC<sub>50</sub> values were converted into K<sub>i</sub> values using the Cheng-Prusoff equation to determine the affinity of the ligands,<sup>34</sup> using a K<sub>D</sub> value for the radioligand of 1.04 nM at 10 °C as determined from radioligand association and dissociation assays, as previously reported.<sup>35</sup> The residence time (RT, in min) was calculated using the equation  $RT = 1/(60 * k_{off})$ , as  $k_{off}$  is in s<sup>-1</sup>. Association and dissociation rate constants for unlabeled compounds were calculated by fitting the data into the competition association model using “kinetics of competitive binding”:

$$\begin{aligned}
 K_A &= k_1[L] \cdot 10^{-9} + k_2 \\
 K_B &= k_3[I] \cdot 10^{-9} + k_4 \\
 S &= \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot L \cdot I \cdot 10^{-18}} \\
 K_F &= 0.5(K_A + K_B + S) \\
 K_S &= 0.5(K_A + K_B - S) \\
 Q &= \frac{B_{max} \cdot k_1 \cdot L \cdot 10^{-9}}{K_F - K_S} \\
 Y &= Q \cdot \left( \frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot X)} \right)
 \end{aligned}$$

where  $k_1$  is the  $k_{on}$  of the radioligand (M<sup>-1</sup>s<sup>-1</sup>),  $k_2$  is the  $k_{off}$  of the radioligand (s<sup>-1</sup>), L is the radioligand concentration (nM), I is the concentration of the unlabeled competitor (nM), X is the time (s) and Y is the specific binding of the radioligand (DPM). The control curve (without competitor) from competition association assays generates the  $k_1$  value, and the  $k_2$  value was obtained from previous radioligand association and dissociation assays.<sup>35</sup> With that the  $k_3$ ,  $k_4$  and  $B_{max}$  were calculated,

where  $k_3$  represents the  $k_{on}$  ( $M^{-1}s^{-1}$ ) of the unlabeled ligand,  $k_4$  stands for the  $k_{off}$  ( $s^{-1}$ ) of the unlabeled ligand and  $B_{max}$  equals the total binding (DPM). All competition association data were globally fitted.

## Results

### *The effects of GTP on affinities and kinetics of the reference hA<sub>3</sub> receptor agonists IB-MECA and 2-Cl-IB-MECA*

Firstly, we determined the effects of GTP (1 mM) on both the affinities and binding kinetics of the reference hA<sub>3</sub> receptor agonists IB-MECA and 2-Cl-IB-MECA. Under the two different assay conditions (i.e. in the absence or presence of GTP) the affinities of IB-MECA and 2-Cl-IB-MECA were significantly different (**Table 1**). We observed a rightward shift of the displacement curves in the presence of GTP (**Figure 1A**, closed symbols), representing an approx. 3-fold decrease in affinity for both compounds. Of note, under both experimental conditions, monophasic displacement curves were obtained with pseudo-Hill coefficients close to unity (**Table 1**).

Furthermore, there were significant differences between the kinetics of hA<sub>3</sub> receptor agonists tested in the absence or presence of 1 mM GTP. In the competition association assays without GTP, both IB-MECA and 2-Cl-IB-MECA produced a typical “overshoot”, representative of a slower dissociation from the hA<sub>3</sub> receptor than the radioligand [<sup>3</sup>H]PSB-11 (**Figure 1B**). This overshoot, however, disappeared in the experiments with GTP (**Figure 1C**). Thus, their KRI values were decreased from well-above-unity in the absence of GTP to close-to-unity in the presence of GTP. The  $k_{off}$  values of IB-MECA and 2-Cl-IB-MECA determined by the Motulsky-Mahan model<sup>32</sup> were  $(1.8 \pm 0.2) \times 10^{-4} s^{-1}$  and  $(7.2 \pm 1.1) \times 10^{-5} s^{-1}$  in the absence of GTP, respectively (**Table 1**), which were smaller than the corresponding values determined in the presence of GTP (IB-MECA:  $(8.5 \pm 3.0) \times 10^{-4} s^{-1}$ ; 2-Cl-IB-MECA:  $(5.7 \pm 1.5) \times 10^{-4} s^{-1}$ , **Table 1**). Consequently, the calculated RTs in the absence of GTP were longer than the values derived when GTP was present (IB-MECA: 95 min vs 20 min; 2-Cl-IB-MECA 231 min vs 29 min, **Table 1**). Last but not least, the  $k_{on}$  values of these two agonists determined under the



two assay conditions were similar. Therefore, in order to define a more agonist-relevant receptor interaction, we decided to use the competition association assay in the absence of GTP for the determination of the affinities and the binding kinetics of other unlabeled hA<sub>3</sub> receptor agonists in the remainder of the study.

**Table 1.** The effects of GTP (1 mM) on the affinities and kinetics of hA<sub>3</sub> receptor agonists IB-MECA and 2-Cl-IB-MECA.

Agonists	pK <sub>i</sub> (mean K <sub>i</sub> in nM) <sup>a</sup>	Hill Slope <sup>a</sup>	KRI <sup>b</sup>	k <sub>on</sub> (M <sup>-1</sup> s <sup>-1</sup> ) <sup>c</sup>	k <sub>off</sub> (s <sup>-1</sup> ) <sup>d</sup>	RT (min) <sup>e</sup>	Kinetic K <sub>D</sub> (mean pK <sub>D</sub> ) <sup>f</sup>
<b>no GTP</b>							
IB-MECA	8.5 ± 0.07 *** (2.9)	-0.94 ± 0.05 <sup>ns</sup>	1.55 ± 0.07 **	(5.9 ± 0.9) x 10 <sup>5 ns</sup>	(1.8 ± 0.2) x 10 <sup>-4 ns</sup>	95 ± 13 *	0.30 ± 0.06 (9.5)
2-Cl-IB-MECA	8.5 ± 0.1* (3.5)	-0.85 ± 0.21 <sup>ns</sup>	2.02 ± 0.06 ***	(4.0 ± 0.5) x 10 <sup>5 ns</sup>	(7.2 ± 1.1) x 10 <sup>-5 *</sup>	231 ± 34 *	0.18 ± 0.04 (9.7)
<b>+ 1 mM GTP</b>							
IB-MECA	8.0 ± 0.02 (11)	-0.99 ± 0.07	0.99 ± 0.08	(4.5 ± 2.5) x 10 <sup>5</sup>	(8.5 ± 3.0) x 10 <sup>-4</sup>	20 ± 7	1.9 ± 1.2 (8.7)
2-Cl-IB-MECA	8.0 ± 0.08 (10)	-1.1 ± 0.1	1.07 ± 0.07	(4.0 ± 2.3) x 10 <sup>5</sup>	(5.7 ± 1.5) x 10 <sup>-4</sup>	29 ± 8	1.4 ± 0.9 (8.9)

<sup>a</sup> pK<sub>i</sub> ± SEM (n ≥ 3, mean K<sub>i</sub> value in nM), obtained from radioligand binding assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>b</sup> KRI ± SEM (n = 3) obtained from dual-point competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>c</sup> k<sub>on</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

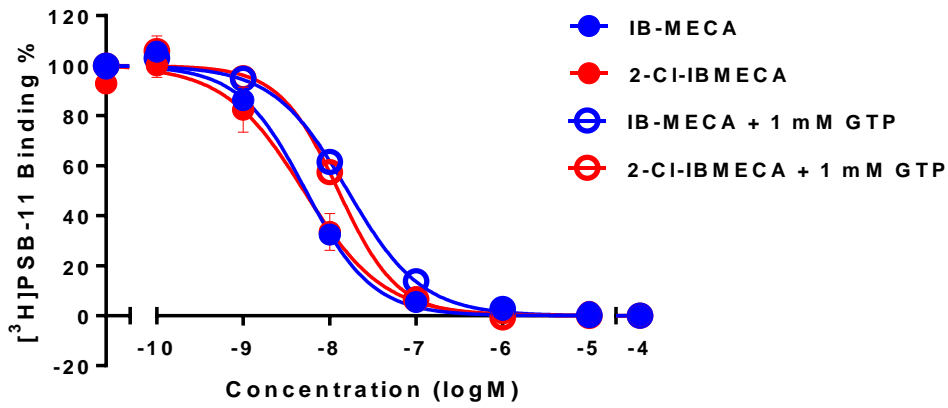
<sup>d</sup> k<sub>off</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>e</sup> RT = 1/(60\*k<sub>off</sub>); RT is expressed in min, whereas k<sub>off</sub> is expressed in s<sup>-1</sup>.

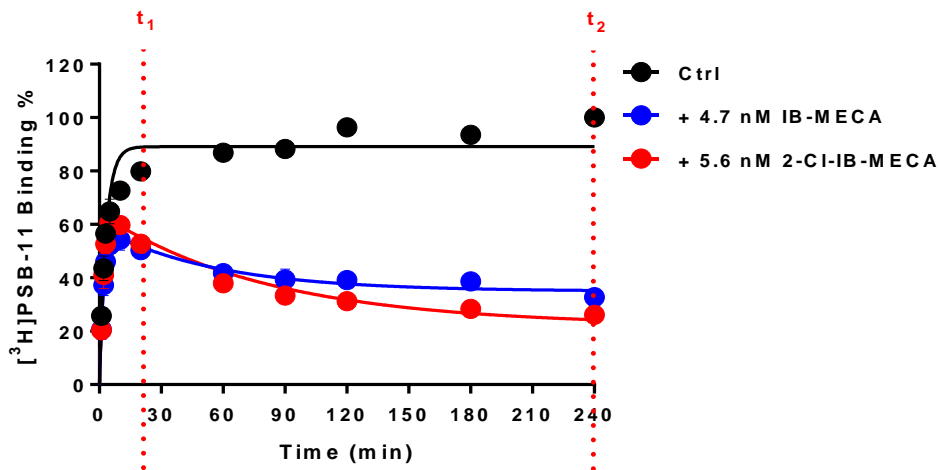
<sup>f</sup> K<sub>D</sub> = k<sub>off</sub>/k<sub>on</sub>

Student's t-test was applied for the comparison of values of  $pK_i$ ,  $KRI$ ,  $k_{on}$ ,  $k_{off}$ ,  $RT$  obtained in the absence vs presence of GTP (1 mM) in the assay conditions, \*\*\* $p < 0.0005$ , \*\* $p < 0.005$ , \*  $p < 0.05$ , ns for not significant

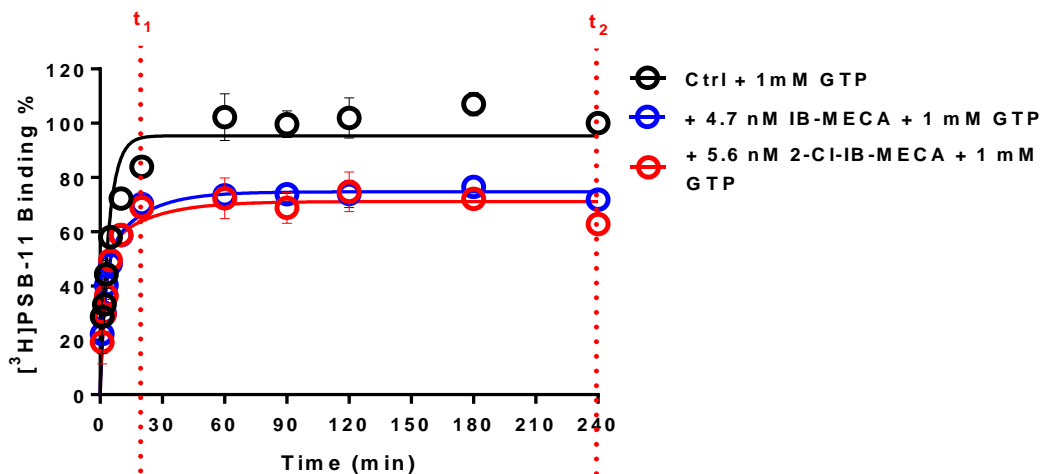
A.



B.



C.



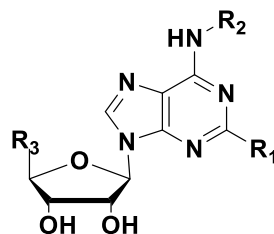
**Figure 1:** Displacement of specific [<sup>3</sup>H]PSB-11 binding from the recombinant hA<sub>3</sub> receptor stably expressed on CHO cell membranes by IB-MECA and 2-Cl-IB-MECA in the absence (closed symbols) or presence of 1 mM GTP (open symbols) (**A**); [<sup>3</sup>H]PSB-11 competition association experiments in the absence (**B**) or presence (**C**) of 1 mM GTP with IB-MECA (blue) or 2-Cl-IB-MECA (red). Combined graphs are shown from at least three experiments performed in duplicate (see **Table 1** for pK<sub>i</sub> values and kinetic parameters). Note, t<sub>1</sub>, t<sub>2</sub> are indicated, which are the two time points used in KRI determinations.

#### *Binding affinity (K<sub>i</sub>) of hA<sub>3</sub> receptor agonists*

The binding affinities of 22 human A<sub>3</sub> receptor agonists in total were determined in equilibrium radioligand displacement studies. All agonists were able to concentration-dependently inhibit specific [<sup>3</sup>H]PSB-11 binding to the human A<sub>3</sub> receptor and their affinities are listed in **Tables 2** and **3**. The agonists displayed moderate to high binding affinities, ranging from 82 nM for agonist MRS5679 to 0.72 nM for agonist MRS5980, while the reference agonists IB-MECA and 2-Cl-IB-MECA had an affinity of 2.9 and 3.5 nM, respectively.

#### *Kinetic Rate Index (KRI) values of hA<sub>3</sub> receptor agonists*

Subsequently, these hA<sub>3</sub> receptor agonists were screened in what is termed a “dual-point” competition association assay. The specific binding of [<sup>3</sup>H]PSB-11 was measured after 20 and 240 minutes in the absence and presence of a single concentration (i.e. IC<sub>50</sub>) of unlabeled hA<sub>3</sub> receptor agonists, which yielded their Kinetic Rate Index (KRI). The KRI values of the hA<sub>3</sub> receptor agonists ranged from 0.64 (LUF5501) to 5.56 (MRS5679) (**Tables 2** and **3**). Agonists with a KRI value larger than unity are considered to have a slower dissociation rate, and thus a longer RT, than the radioligand used, i.e. [<sup>3</sup>H]PSB-11, and *vice versa*. Agonists having KRI values below 0.70 (LUF5501 and LUF5505) or above 1.35 (LUF5521, IBMECA to LUF5595, LUF5589, MRS7140, MRS5980, and MRS7154 to MRS5667) were selected for further kinetic profiling.

**Table 2.** Binding Affinities and Kinetic Parameters (KRI,  $k_{on}$ ,  $k_{off}$ , RT and  $K_D$ ) for ribofurano derivatives as hA<sub>3</sub> receptor agonists.

Agonists	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	pKi (mean K <sub>i</sub> in nM) <sup>a</sup>	KRI <sup>b</sup>	$k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> ) <sup>c</sup>	$k_{off}$ (s <sup>-1</sup> ) <sup>d</sup>	RT (min) <sup>e</sup>	Kinetic K <sub>D</sub> (mean pK <sub>D</sub> ) <sup>f</sup>
LUF5501	H	-Ph-3-Cl	-CH <sub>2</sub> OH	7.5 ± 0.02 (31)	0.64 (0.53; 0.75)	(1.7 ± 0.2) × 10 <sup>5</sup>	(1.6 ± 0.2) × 10 <sup>-3</sup>	10 ± 1.3	9.2 ± 1.4 (8.0)
LUF5505	H	-Ph-3-Br	-CH <sub>2</sub> OH	7.1 ± 0.1 (78)	0.65 (0.63; 0.66)	(8.8 ± 1.4) × 10 <sup>4</sup>	(1.1 ± 0.04) × 10 <sup>-3</sup>	16 ± 0.6	12 ± 1.9 (7.9)
LUF5500	H	-Ph-4-Cl	-CH <sub>2</sub> OH	7.3 ± 0.03 (51)	0.98 (0.95; 1.01)	N.D.	N.D.	N.D.	N.D.
LUF5506	H	-Ph-4-I	-CH <sub>2</sub> OH	7.6 ± 0.08 (29)	0.99 (0.98; 1.00)	N.D.	N.D.	N.D.	N.D.
LUF5521	H	-Ph-4-I	-CONHCH <sub>2</sub> CH <sub>3</sub>	7.9 ± 0.06 (14)	2.06 ± 0.82	(1.9 ± 0.3) × 10 <sup>5</sup>	(1.4 ± 0.1) × 10 <sup>-4</sup>	117 ± 12	0.76 ± 0.13 (9.1)
MECA	H	-CH <sub>2</sub> Ph	-CONHCH <sub>3</sub>	7.4 ± 0.04 (42)	1.24 ± 0.09	N.D. <sup>h</sup>	N.D.	N.D.	N.D.

IB-MECA <sup>g</sup>	H	-CH <sub>2</sub> Ph-3-I	-CONHCH <sub>3</sub>	8.5 ± 0.07 (2.9)	1.55 ± 0.07	(5.9 ± 0.9) x 10 <sup>5</sup>	(1.8 ± 0.2) x 10 <sup>-4</sup>	95 ± 13	0.30 ± 0.06 (9.5)
2-Cl-IB-MECA <sup>g</sup>	-Cl	-CH <sub>2</sub> Ph-3-I	-CONHCH <sub>3</sub>	8.5 ± 0.1 (3.5)	2.02 ± 0.06	(4.0 ± 0.5) x 10 <sup>5</sup>	(7.2 ± 1.1) x 10 <sup>-5</sup>	231 ± 34	0.18 ± 0.04 (9.7)
LUF5595	-Cl	-CH <sub>2</sub> Ph-3-I	-CH <sub>2</sub> OC <sub>3</sub> H <sub>5</sub>	8.5 ± 0.09 (3.2)	1.37 (1.39; 1.35)	(9.6 ± 3.1) x 10 <sup>5</sup>	(2.3 ± 1.0) x 10 <sup>-4</sup>	72 ± 30	0.24 ± 0.13 (9.6)
LUF5586	H	-CH <sub>2</sub> Ph-3-I	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>3</sub>	8.6 ± 0.04 (2.8)	1.30 (1.30; 1.29)	N.D.	N.D.	N.D.	N.D.
LUF5589	-Cl	-CH <sub>2</sub> Ph-3-I	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>3</sub>	8.7 ± 0.08 (2.2)	1.66 ± 0.21	(8.4 ± 0.7) x 10 <sup>5</sup>	(1.2 ± 0.3) x 10 <sup>-4</sup>	138 ± 37	0.14 ± 0.04 (9.8)
MRS7294	-C≡C-C <sub>4</sub> H <sub>2</sub> S-5-Cl	-CH <sub>3</sub>	-CH <sub>2</sub> OH	8.4 ± 0.05 (3.7)	1.63 (1.50; 1.75)	(8.2 ± 3.8) x 10 <sup>5</sup>	(1.3 ± 0.2) x 10 <sup>-4</sup>	125 ± 14	0.16 ± 0.02 (9.8)

<sup>a</sup> pK<sub>i</sub> ± SEM (n ≥ 3, mean K<sub>i</sub> value in nM), obtained from radioligand binding assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>b</sup> KRI ± SEM (n = 3) or KRI (n1,n2) (n = 2), obtained from dual-point competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>c</sup> k<sub>on</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>d</sup> k<sub>off</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

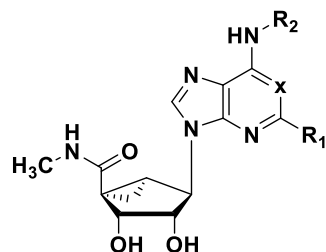
<sup>e</sup> RT = 1/(60 \* k<sub>off</sub>); RT is expressed in min, whereas k<sub>off</sub> is expressed in s<sup>-1</sup>.

<sup>f</sup> K<sub>D</sub> = k<sub>off</sub>/k<sub>on</sub>, expressed in nM

<sup>g</sup> values taken from **Table 1**

<sup>h</sup> N.D. = not determined

**Table 3.** Binding Affinities and Kinetic Parameters (KRI,  $k_{on}$ ,  $k_{off}$ , RT and  $K_D$ ) for (N)-methanocarpa derivatives as  $hA_3$  receptor agonists.



Agonists	X	R <sub>1</sub>	R <sub>2</sub>	pK <sub>i</sub> (mean K <sub>i</sub> value in nM) <sup>a</sup>	KRI <sup>b</sup>	$k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> ) <sup>c</sup>	$k_{off}$ (s <sup>-1</sup> ) <sup>d</sup>	RT (min) <sup>e</sup>	Kinetic K <sub>D</sub> (mean pK <sub>D</sub> ) <sup>f</sup>
MRS7294 <sup>g</sup>	N	-C≡C-C <sub>4</sub> H <sub>2</sub> S-5-Cl	-CH <sub>3</sub>	8.4 ± 0.05 (3.7)	1.63 (1.50; 1.75)	(8.2 ± 3.8) x 10 <sup>5</sup>	(1.3 ± 0.2) x 10 <sup>-4</sup>	125 ± 14	0.16 ± 0.02 (9.8)
MRS5980	N	-C≡C-C <sub>4</sub> H <sub>2</sub> S-5-Cl	-CH <sub>3</sub>	9.2 ± 0.09 (0.72)	1.97 (1.87; 2.06)	(9.0 ± 2.3) x 10 <sup>5</sup>	(4.0 ± 1.0) x 10 <sup>-5</sup>	417 ± 104	0.044 ± 0.016 (10.4)
MRS7140	C	-C≡C-C <sub>4</sub> H <sub>2</sub> S-5-Cl	-CH <sub>3</sub>	8.3 ± 0.08 (5.2)	1.94 (1.82; 2.07)	(3.2 ± 1.2) x 10 <sup>5</sup>	(8.5 ± 0.3) x 10 <sup>-5</sup>	196 ± 8	0.27 ± 0.10 (9.6)
MRS7154	N	-C≡C-C <sub>4</sub> H <sub>2</sub> S-5-Cl	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	8.7 ± 0.06 (2.2)	1.67 (1.75; 1.59)	(4.0 ± 0.7) x 10 <sup>5</sup>	(6.2 ± 1.2) x 10 <sup>-5</sup>	270 ± 51	0.15 ± 0.04 (9.8)
MRS3558	N	-Cl	-CH <sub>2</sub> Ph-3-Cl	9.1 ± 0.2 (1.0)	1.49 (1.33; 1.64)	(5.7 ± 0.8) x 10 <sup>5</sup>	(1.0 ± 0.2) x 10 <sup>-4</sup>	167 ± 39	0.18 ± 0.05 (9.8)

MRS5655	N	-C≡C-Ph	-CH <sub>2</sub> Ph-3-Cl	8.5 ± 0.09 (3.6)	2.58 (2.05; 3.12)	(4.0 ± 0.8) x 10 <sup>5</sup>	(1.8 ± 0.3) x 10 <sup>-5</sup>	909 ± 165	0.046 ± 0.013 (10.3)
MRS5644	N	-C≡C-Ph	-CH <sub>3</sub>	8.6 ± 0.01 (2.4)	2.42 (2.28; 2.56)	(4.5 ± 1.8) x 10 <sup>5</sup>	(1.4 ± 0.2) x 10 <sup>-5</sup>	1205 ± 174	0.031 ± 0.013 (10.5)
MRS5698	N	-C≡C-(3,4-di-F-Ph)	-CH <sub>2</sub> Ph-3-Cl	8.1 ± 0.2 (9.4)	4.21 (3.75; 4.67)	(1.3 ± 0.4) x 10 <sup>5</sup>	(8.5 ± 0.7) x 10 <sup>-6</sup>	1961 ± 154	0.068 ± 0.020 (10.2)
MRS5679	N	-C≡C-(4-biphenyl)	-CH <sub>2</sub> Ph-3-Cl	7.1 ± 0.05 (82)	5.56 (6.00; 5.11)	undefined	undefined	undefined	undefined
MRS5667	N	-C≡C-(4-biphenyl)	-CH <sub>3</sub>	8.3 ± 0.05 (4.6)	3.34 (3.14; 3.55)	(1.8 ± 0.3) x 10 <sup>5</sup>	(1.1 ± 0.3) x 10 <sup>-5</sup>	1563 ± 464	0.058 ± 0.020 (10.2)

<sup>a</sup> pK<sub>i</sub> ± SEM (n ≥ 3, mean K<sub>i</sub> value in nM), obtained from radioligand binding assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>b</sup> KRI ± SEM (n = 3) or KRI (n<sub>1</sub>,n<sub>2</sub>) (n = 2), obtained from dual-point competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>c</sup> k<sub>on</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>d</sup> k<sub>off</sub> ± SEM (n ≥ 3), obtained from competition association assays with [<sup>3</sup>H]PSB-11 on the hA<sub>3</sub> receptor stably expressed on CHO cell membranes.

<sup>e</sup> RT = 1/(60 \* k<sub>off</sub>); RT is expressed in min, whereas k<sub>off</sub> is expressed in s<sup>-1</sup>.

<sup>f</sup> K<sub>D</sub> = k<sub>off</sub>/k<sub>on</sub>, expressed in nM

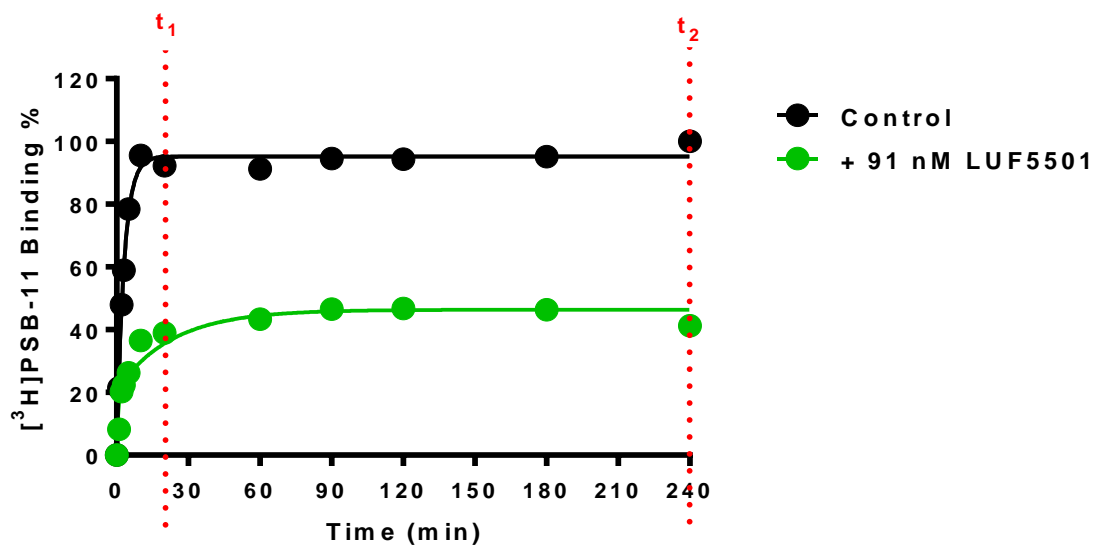
<sup>g</sup> MRS7294 has a ribofurano ring (see also **Table 2**).

### *Binding kinetics of selected hA<sub>3</sub> receptor agonists using the competition association assay*

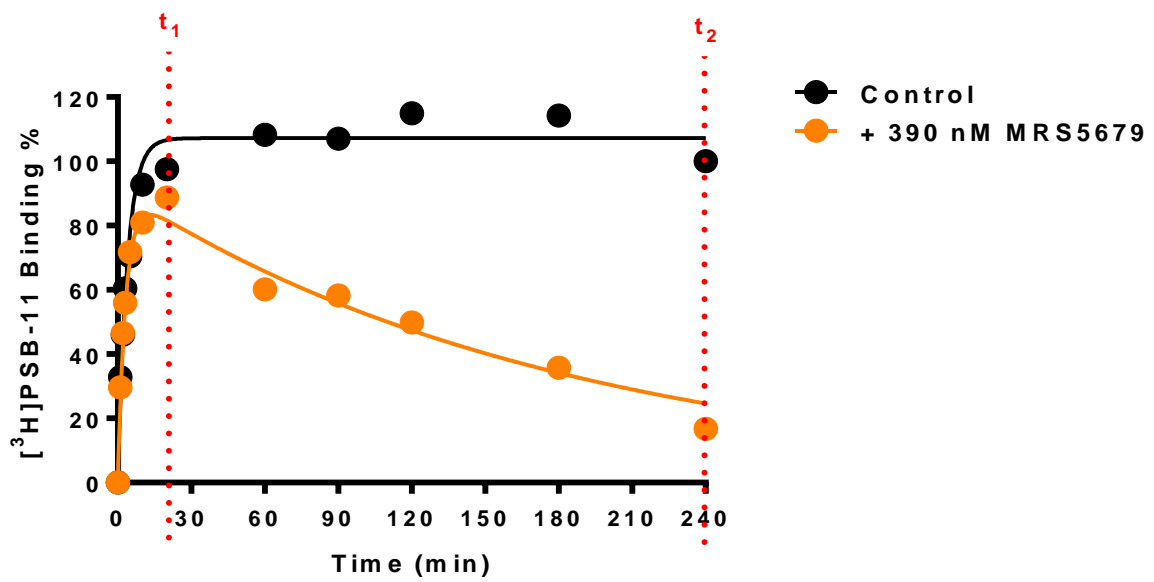
Next, the kinetic binding parameters of selected agonists that had either low or high KRI values were determined using the competition association assay with [<sup>3</sup>H]PSB-11. Association rate constants varied by only 11-fold, ranging from  $(8.8 \pm 1.4) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$  for LUF5505 to  $(9.6 \pm 3.1) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  for LUF5595 (**Tables 2 and 3**). There was a more pronounced 188-fold difference in dissociation rate constants, in line with the divergent KRI values (LUF5501:  $(1.6 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$  vs MRS5698:  $(8.5 \pm 0.7) \times 10^{-6} \text{ s}^{-1}$ ). The shortest RT agonist, LUF5501, presented a gradually ascending curve in the competition association assays (**Figure 2A**). Of note, the dissociation of MRS5679, with the largest KRI value (5.56), was determined as very slow and not readily quantitated under the current method. The very prominent “overshoot” in the competition association curve was already indicative of a much slower dissociation rate than [<sup>3</sup>H]PSB-11 (**Figure 2B**). Notably, a significant correlation between the negative logarithm of the dissociation rate constants for the selected agonists and their KRI values derived from the kinetic assays was obtained (**Figure 3A**), which confirmed that a compound’s KRI value is a good predictor for its dissociation rate constant. Besides, a significant correlation was also observed between the agonist affinities (pK<sub>i</sub> values) determined in equilibrium displacement experiments and their pK<sub>D</sub> values (“kinetic K<sub>D</sub> values”) derived from competition association experiments, although the pK<sub>D</sub> values were on average one log unit higher than the pK<sub>i</sub> values (**Figure 3B**). As to the kinetic rate constants (k<sub>on</sub> or k<sub>off</sub>) of the hA<sub>3</sub> receptor agonists, their association rate constants showed a better correlation with affinity than their dissociation rate constants (**Figure 3C and 3D**). Furthermore, a k<sub>on</sub>-k<sub>off</sub>-K<sub>D</sub> “kinetic map” (**Figure 4**) was constructed based on the compounds’ divergent affinities (expressed as kinetic K<sub>D</sub> values) and rate constants, yielding a division of these agonists into three different sub-categories: agonists that show k<sub>off</sub> values in a lower range but due to divergent k<sub>on</sub> values have various K<sub>D</sub> values (Group A); agonists that show k<sub>off</sub> values in a median range but due to divergent k<sub>on</sub> values (although smaller than in Group A) have different K<sub>D</sub> values (Group B); and two agonists that have relatively poor affinity due to larger k<sub>off</sub> values and smaller k<sub>on</sub> values (Group C).



A.

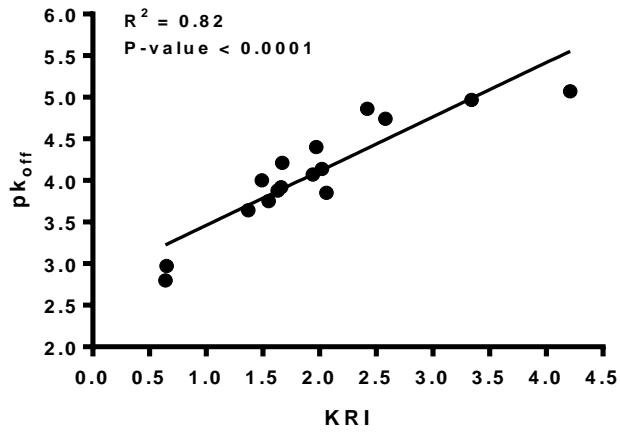


B.

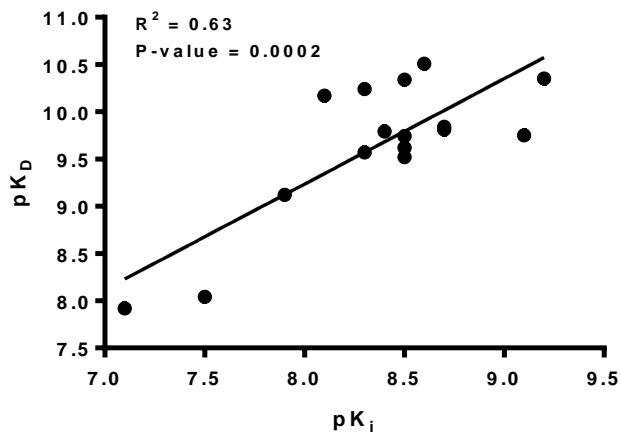


**Figure 2:** Competition association experiments with  $[^3\text{H}]$ PSB-11 binding to the recombinant  $\text{hA}_3$  receptor stably expressed on CHO cell membranes (10 °C) in the absence or presence of unlabeled short-residence-time agonist LUF5501 (A), or long-residence-time agonist MRS5679 (B). Representative graphs are shown from one experiment performed in duplicate. Note,  $t_1$ ,  $t_2$  are indicated, which are the two time points used in KRI determinations.

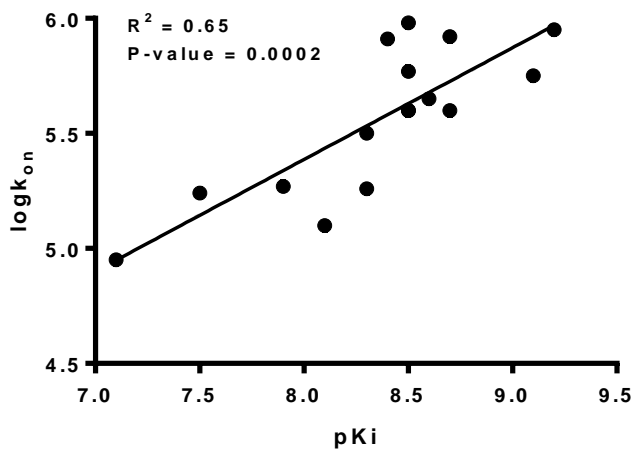
A.



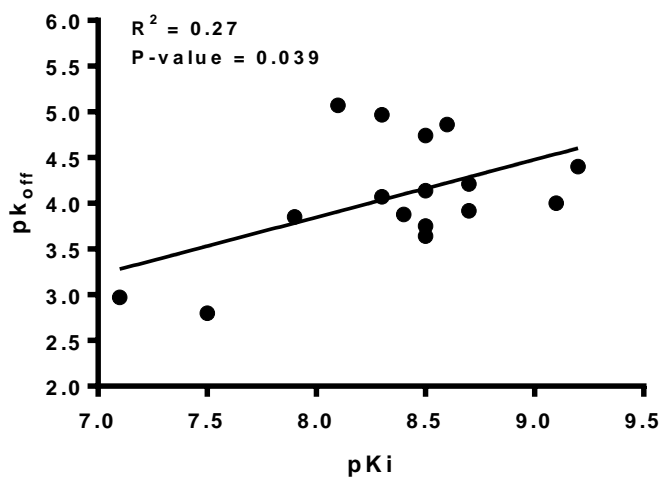
B.



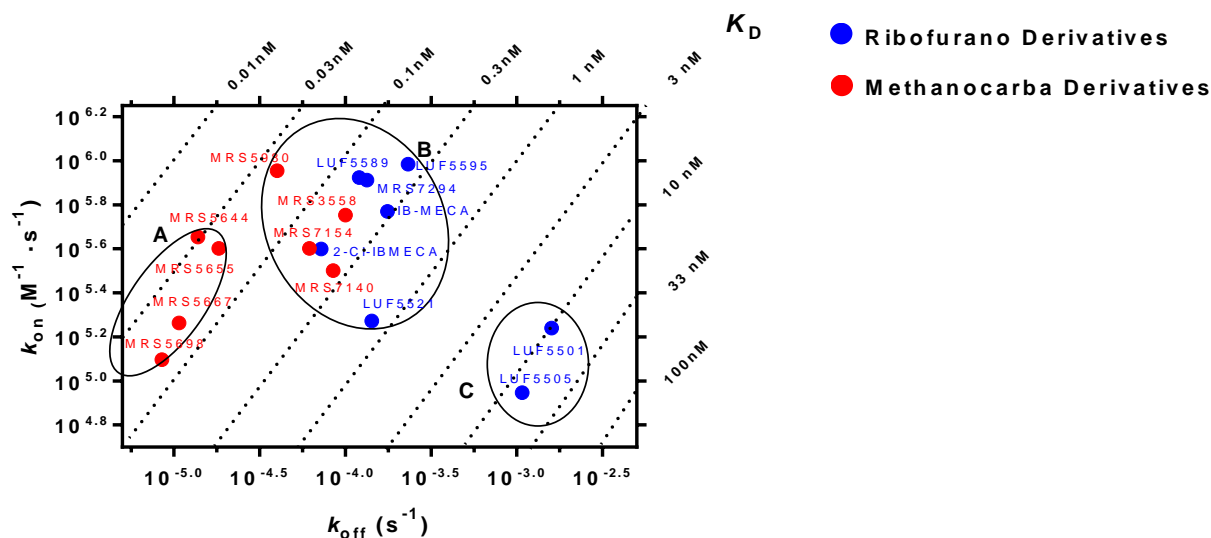
C.



D.



**Figure 3:** The correlations between the negative logarithm of each  $hA_3$  receptor agonist dissociation rate constants ( $pK_{off}$ ) and the kinetic rate index (KRI) (A), the  $A_3$  receptor agonist affinity ( $pK_i$ ) and the “kinetic  $K_D$ ” ( $pK_D$ ) (B), logarithm of association rate constants ( $\log k_{on}$ ) (C) and negative logarithm of dissociation rate constants ( $pK_{off}$ ) (D). Data used in these plots are detailed in **Tables 2 and 3**.



**Figure 4:** Kinetics map (y axis:  $k_{on}$  in  $M^{-1} \cdot s^{-1}$ , x axis:  $k_{off}$  in  $s^{-1}$ ) of all compounds that were kinetically characterized in this study.  $k_{on}$  and  $k_{off}$  values were obtained through competition association assays. The kinetically derived affinity ( $K_D = k_{off}/k_{on}$ ) is represented through diagonal parallel lines. The ribofurano derivatives are colored in blue, and the methanocarba derivatives are colored in red.

*Structure–Affinity Relationships (SAR) and Structure–Kinetics Relationships (SKR) of hA<sub>3</sub> receptor agonists*

First, a series of hA<sub>3</sub>R agonists with either a natural or 5'-modified ribose moiety was investigated (**Table 2**). LUF5501-LUF5506 have an intact ribofurano moiety and a halogen substitution at the N<sup>6</sup> phenyl group of the core scaffold. 3-Chlorophenyl substitution (LUF5501) provided somewhat better affinity than 3-bromophenyl (LUF5505) or 4-chlorophenyl (LUF5500) substitution (31 nM vs 78 nM; 31 nM vs 51 nM, respectively). The affinity of the 4-iodophenyl substituted derivative (LUF5506) was similar to LUF5501 (31 nM vs 29 nM). Meanwhile, their KRI values showed that halogen substitution at *meta*-position led to lower KRI values than those at *para*-position of the phenyl ring. The KRI values of LUF5501 and LUF5505 were at the lower end (< 0.7) with 0.64 and 0.65, respectively. The full-curve competition association experiments confirmed their similar fast dissociation rates (LUF5501:  $(1.6 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$ ; LUF5505:  $(1.1 \pm 0.04) \times 10^{-3} \text{ s}^{-1}$ ). Worthy of note, LUF5501 proved to have the shortest RT (10 min) among the entire selection of compounds.

When the 5'-hydroxymethylene group (LUF5506) was substituted by an ethylcarboxamide (-CONHCH<sub>2</sub>CH<sub>3</sub>, LUF5521), affinity (14 nM vs 29 nM) and particularly the KRI increased (2.06 vs 0.99). The association and dissociation rates of LUF5521 were  $(1.9 \pm 0.3) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  and  $(1.4 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$ , respectively, and its RT was calculated as 117 min.

We then examined benzyl substitutions at the N<sup>6</sup> position of the core scaffold, with a methylcarboxamide (-CONHCH<sub>3</sub>) modification of the 5'-position on the ribofuran moiety (MECA, IB-MECA and 2-Cl-IBMECA). MECA had an affinity of 42 nM with a KRI value of 1.24. When iodine was introduced at the 3-position of the benzyl ring, yielding IB-MECA, affinity was strongly increased (2.9 nM vs 42 nM) as well as the KRI value (1.55 vs 1.24); with a further chlorine atom substitution at the C-2 position on the core scaffold (2-Cl-IBMECA) there was no further increase in affinity (3.5 nM vs 2.9 nM), but its KRI value increased significantly (2.02 vs 1.55), which is the largest value in this series (**Table 2**). For IB-MECA and 2-Cl-IBMECA, the association rate constants were similar  $((5.9 \pm 0.9) \times$

$10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(4.0 \pm 0.5) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ), however 2-Cl-IBMECA had a slower dissociation rate ( $(7.2 \pm 1.1) \times 10^{-5} \text{ s}^{-1}$  vs  $(1.8 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$ ) as had been immanent already from their KRI values. The RTs of IBMECA and 2-Cl-IBMECA were calculated from their dissociation rate constants as 95 min and 231 min, respectively.

Interestingly, when the amide at the 5'-position on the ribofurano ring of 2-Cl-IBMECA was changed to an ether function ( $-\text{CH}_2\text{OC}_3\text{H}_5$ , LUF5595), both association ( $(9.6 \pm 3.1) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(4.0 \pm 0.5) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ) and dissociation ( $(2.3 \pm 1.0) \times 10^{-4} \text{ s}^{-1}$  vs  $(7.2 \pm 1.1) \times 10^{-5} \text{ s}^{-1}$ ) became faster, even though the compound was still a slowly dissociating agonist compared with the radioligand (KRI: 1.37).

Finally, the effect of the 2-Cl substitution was further confirmed in the comparison between LUF5586 and LUF5589. Their affinities were similar (2.8 nM vs 2.2 nM), but with 2-Cl, LUF5589 had a higher KRI than LUF5586 (1.66 vs 1.30), and the RT of LUF5589 was determined as 138 min. In addition, a larger substituent at the C-2 position (MRS7294) was tolerated with respect to both affinity and kinetics, at least in combination with a small N<sup>6</sup>-methyl substituent.

**Table 3** summarizes our findings on the series of ring-rigidified methanocarba derivatives that maintain a receptor-preferred North (N) conformation, with the flexible ribose-substituted MRS7294 included once more to allow a comparison. MRS7294, MRS5980, MRS7140, MRS7158 and MRS7154 share a common fragment ((5-chlorothiophen-2-yl)ethynyl) at the C-2-position (R<sub>1</sub> group). MRS7294, the agonist with unmodified ribofuran moiety, served as the starting of the analysis, which had both high affinity (3.7 nM) and a large KRI value (1.63). By competition association experiments its association and dissociation rates were determined as  $(8.2 \pm 3.8) \times 10^5 \text{ (M}^{-1}\text{s}^{-1})$  and  $(1.3 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$ , respectively; its RT was calculated as 125 min. When the ribofurano ring was changed by the methanocarba moiety (MRS5980), the affinity increased to the sub-nanomolar range (0.72 nM vs 3.7 nM) and its KRI value also increased largely (1.97 vs 1.63). The full-curve competition association experiments showed that MRS5980's association rate was slightly faster ( $(9.0 \pm 2.3) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(8.2 \pm 3.8) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  for the respective rate constants), but its dissociation rate was much slower

$(4.0 \pm 1.0) \times 10^{-5} \text{ s}^{-1}$  vs  $(1.3 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$ ) than its ribose-equivalent. The RT of MRS5980 was 417 min. When the nitrogen atom at the *N*-1-position was replaced by a carbon atom (MRS7140), the affinity remained high (5.2 nM), and its kinetics profile was not much altered compared with MRS5980 ( $k_{\text{on}}$ :  $(3.2 \pm 1.2) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(9.0 \pm 2.3) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ;  $k_{\text{off}}$ :  $(8.5 \pm 0.3) \times 10^{-5} \text{ s}^{-1}$  vs  $(4.0 \pm 1.0) \times 10^{-5} \text{ s}^{-1}$ ). Changing the mono-methyl substitution at the  $\text{N}^6$  position (MRS5980) for di-methyl substitution (MRS7158), severely compromised both affinity and KRI value (618 nM vs 0.72 nM; 0.93 vs 1.97). When mono-substitution at the  $\text{N}^6$  position (MRS7140) was extended to n-propyl (MRS7154), affinity remained high (2.2 nM), like the KRI value (1.67); both association and dissociation rates of MRS7154 were quite similar to MRS5980 ( $k_{\text{on}}$ :  $(4.0 \pm 0.7) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(9.0 \pm 2.3) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ;  $k_{\text{off}}$ :  $(6.2 \pm 1.2) \times 10^{-5} \text{ s}^{-1}$  vs  $(4.0 \pm 1.0) \times 10^{-5} \text{ s}^{-1}$ ), with RTs of 270 min and 417 min, respectively.

The methanocarba derivatives MRS3558, MRS5655, MRS5679 and MRS5698 share their  $\text{R}_2$  groups (3-chlorobenzyl substitution). When the 2-Cl substituent of MRS3558 was replaced by the bulky and rigid phenylethynyl (MRS5655), the affinity decreased from 1.0 nM (MRS3558) to 3.6 nM (MRS5655), while the KRI value increased, however (1.49 vs 2.58). In the competition association assays the association rate constants of MRS3558 and MRS5655 were similar ( $(5.7 \pm 0.8) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(4.0 \pm 0.8) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ), while the dissociation rate constants were 5.6-fold different ( $(1.0 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$  vs  $(1.8 \pm 0.3) \times 10^{-5} \text{ s}^{-1}$ ), leading to a RT for MRS5655 of 909 min. When the phenylethynyl of MRS5655 was 3,4-di-fluorinated as in MRS5698, the affinity was reduced from 3.6 nM (MRS5655) to 9.4 nM (MRS5698), whilst the KRI value increased significantly from 2.58 (MRS5655) to 4.21 (MRS5698), with both slower association and dissociation rate constants ( $k_{\text{on}}$ :  $(4.0 \pm 0.8) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  vs  $(1.3 \pm 0.4) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ;  $k_{\text{off}}$ :  $(1.8 \pm 0.3) \times 10^{-5} \text{ s}^{-1}$  vs  $(8.5 \pm 0.7) \times 10^{-6} \text{ s}^{-1}$ ), thus extending RT from 909 min to 1961 min. Furthermore, when the phenylethynyl fragment of MRS5655 was expanded to a 4-biphenylethynyl substitution on the  $\text{R}_1$  group (MRS5679), the affinity suffered from 3.6 nM (MRS5655) to 82 nM (MRS5679); however, its KRI value increased dramatically from 2.58 to 5.56. This high value rendered the determination of the kinetics profile of MRS5679 impossible. Obviously,

from all these compounds we learned there can be a clear dichotomy between affinity and residence time.

When the benzyl substitutions at the  $N^6$  position ( $R_2$  in **Table 3**) were replaced by a simple monomethyl (i.e. MRS5655 to MRS5644; MRS5679 to MRS5667), their binding affinities to  $hA_3$  receptors were not compromised (MRS5655/MRS5644: 3.6 nM vs 2.4 nM) or even increased (MRS5679/MRS5667: 82 nM vs 4.6 nM), and so were the kinetics profiles of MRS5655 and MRS5644. The KRI values of MRS5679 and MRS5667 were both at the higher end (5.56 and 3.34).

## Discussion

### *Effects of GTP on agonist binding to the human adenosine $A_3$ receptor*

In the current study, the binding interactions of unlabeled  $hA_3$  receptor agonists were determined in radioligand binding experiments with the reference tritiated antagonist [ $^3H$ ]PSB-11. The favorable binding characteristics of this radioligand served us particularly well in the competition association experiments where robust binding over time could still be measured in the presence of unlabeled ligands, essential for the calculation of the association and dissociation rate constants. It is well known that antagonists occupy a GPCR irrespective of whether it is coupled to a G protein, whereas agonists prefer the G protein-coupled state of the receptor <sup>36</sup>. Addition of GTP to the incubation mixture induces an uncoupling between receptor and available G protein, which leads to a lower apparent receptor affinity for agonists. We confirmed this behavior in our equilibrium displacement experiments, in which the two reference agonists IB-MECA and 2-Cl-IB-MECA had a 3-4-fold lower affinity in the presence of GTP (**Figure 1A, Table 1**). Interestingly, all displacement curves had pseudo-Hill coefficients of approx. unity (**Table 1**), suggesting that the agonists recognized one receptor state in both the presence and absence of GTP, most likely a G protein-coupled state (no GTP) or an uncoupled state (with GTP). We then determined the agonist binding kinetics for the two

states in subsequent competition association assays by measuring these in the absence or presence of GTP (**Figure 1B**). Interestingly, the association rate constants were identical for the two states (**Table 1**). Apparently, the ligand, when approaching the receptor from the extracellular side, is insensitive to the coupling status at the intracellular G protein interface. However, the dissociation kinetics proved to be (significantly) different between the states, up to 8-fold for 2-Cl-IB-MECA (**Table 1**), suggesting that dissociating from the receptor is influenced by the absence or presence of the G protein. We then performed further experiments in the absence of GTP, as we felt that for the case of the hA<sub>3</sub> receptor this experimental condition provides us information on the more relevant high-affinity agonist binding state of the receptor. The mathematical modeling provided by Motulsky and Mahan<sup>32</sup> does not allow the calculation of two receptor states with corresponding kinetic parameters. Hence, in a number of cases similar to ours it was decided to include GTP in the assays. For the adenosine A<sub>1</sub> receptor, we included GTP in our assays, forcing the receptor to be in one lower affinity, G protein-uncoupled state only; with this restriction, the kinetic parameters of agonists were determined as conveniently as antagonists.<sup>37</sup> Likewise, the team of Charlton included GTP in kinetic assays on the muscarinic M<sub>3</sub> receptor.<sup>38</sup> On the other hand, GTP was excluded in assays on the adenosine A<sub>2A</sub> receptor, as agonist affinity at this protein is insensitive to GTP in both equilibrium and kinetics assays.<sup>39</sup>

#### *Methodological aspects of the radioligand binding assays*

To ensure an accurate and robust kinetic quantification, experiments were performed at 10 °C, as reported and documented previously.<sup>35</sup> Initially, a so-called dual-point competition association assay yielding KRI values for the hA<sub>3</sub> receptor was performed as a “kinetic screening campaign” to increase throughput in comparison to the traditional, more elaborate, competition association experiments.<sup>35</sup> Although fast kinetics of agonist binding (e.g. LUF5501, **Figure 2A**) was determined accurately at a relatively low temperature, the kinetics of one agonist with obviously very slow dissociation



characteristics (MRS5679, **Figure 2B**) could not be reliably determined with the Motulsky-Mahan equations. Meanwhile, its large KRI value (5.56) undoubtedly reflects its slow dissociation.

Besides the significant correlation between the agonists' KRI values and dissociation rate constants (**Figure 3A**), the equilibrium  $K_i$  and kinetic  $K_D$  values were also well correlated (**Figure 3B**). Despite this correlation there are large discrepancies in values between the equilibrium affinities ( $K_i$ ) and kinetic  $K_D$  values. In general, the kinetic  $pK_D$  values are higher than  $pK_i$  values, with differences from 0.5 to 2.1 log-unit (LUF5501 and MRS5698), whilst the short RT agonists have smaller differences than long RT agonists. This phenomenon suggests that the affinities determined in the radioligand displacement studies may not reflect the true affinity of the compounds as many of them have residence times much longer than the (already long) incubation time of 240 min. In fact, this points to a general caveat in end-point assays in which the equilibrium characteristics of the probe, e.g., a radiolabeled or fluorescently tagged compound, determine the assay protocol without further consideration of the same characteristics of the unlabeled ligands to be tested.

Furthermore, there was a significant correlation between  $k_{on}$  ( $k_3$ ) and affinity values ( $K_i$ ) of the agonists, but no relationship was found between  $k_{off}$  ( $k_4$ ) and  $K_i$  (**Figure 3C and 3D**). Such correlation between affinity and on-rates has been reported in the case of other GPCR<sup>40</sup> and ion channels.<sup>41,42</sup> However, in the current study, variations in association rate constants were approximately 10-fold ( $\log k_{on}$ : 5.0 to 6.0), much smaller than at the other targets mentioned above. In addition, all the association rates were well below the diffusion limit of around  $10^7 \text{ M}^{-1}\text{s}^{-1}$ , which had been observed previously for membrane-bound proteins.<sup>43</sup>

#### *Ligand optimization based on Structure–Kinetics Relationships (SKR)*

All the agonists examined in the current study have been reported, and the riboside MRS7294 was most recently disclosed as a  $hA_3$  receptor agonist.<sup>28</sup> Most ribofurano derivatives (**Table 2**) had been synthesized and tested as early as 1994.<sup>44</sup> The (N)-methanocarba derivatives (**Table 3**) were explored

more recently, showing generally improved affinities over the earlier compounds.<sup>26-29</sup> Although most of these compounds have been described in the context of structure-affinity relationships (SARs), we reasoned that an extensive structure-kinetics relationships (SKRs) analysis is warranted since kinetic profiles are emerging indicators of *in vivo* functional efficacy.<sup>18, 20, 21, 45</sup>

In the series of ribofurano derivatives (**Table 2**), the most outstanding agonist with respect to residence time was 2-Cl-IB-MECA (231 min). The underlying chemical features that prolonged the residence time in the ribofurano series are: i) a chlorine atom at the C2 position ( $R_1$  in **Table 2**, IB-MECA vs 2-Cl-IB-MECA, 95 min vs 231 min; LUF5586 vs LUF5589 in KRI: 1.30 vs 1.66), ii) a bulky *meta*-iodobenzyl substitution at the  $N^6$  position of the adenine nucleobase ( $R_2$  in **Table 2**, MECA vs IB-MECA in KRI: 1.24 vs 1.55), and iii) an amide modification at the 5' position ( $R_3$  in **Table 2**, 2-Cl-IB-MECA vs LUF5595: 231 min vs 72 min; LUF5506 vs LUF5521 in KRI: 0.99 vs 2.06). Interestingly, slow dissociation kinetics of agonists can be maintained by introducing an extended linear side chain at the C2 position and removing the bulky substituent at the  $N^6$  position (i.e.  $N^6$ -Me analogue MRS7294 in RT: 125 min). The kinetic behavior of another known reference agonist, 2-(1-hexynyl)-N-methyladenosine (HEMADO), also confirmed this observation from comparison with other research.<sup>45, 46</sup>

More impressive kinetics were observed in the methanocarba series. From a previous study it was concluded that a possible H-bond interaction between the amide modification at the original 5' position (-CONHCH<sub>3</sub>) and residues in the binding pocket of the hA<sub>3</sub> receptor was a key feature of agonist kinetics.<sup>47</sup> Comparing the kinetics of ribofurano MRS7294 and its methanocarba equivalent MRS5980 provides evidence the conformational-constrained (N)-methanocarba moiety as a ribofurano ring substitute adds to the slow dissociation of MRS5980, suggesting this rigid ring system fits even better in the ligand binding pocket. Additionally, the N1 atom on the adenine core is contributing to slow dissociation (1-deza MRS7140 vs MRS5980 in RT: 196 min vs 417 min). The  $N^6$  position in the methanocarba series ( $R_2$  substituent) accepted a small methyl group as avidly as the

larger *n*-propyl (MRS5980 vs MRS7154) and 3-chlorobenzyl substituent (MRS5644 vs MRS5655), with respect to both affinity and kinetics. Apparently, differences in lipophilicity between the  $N^6$ -substituents did not seem to matter either. Data for the  $R_1$  (C2 position) substituent were already commented on in the Results section. It is noteworthy that the 3,4-difluorophenyl fragment contributed to a long residence time of MRS5698, quite similar to the 2,4-difluorophenyl fragment we encountered in  $A_{2A}$  receptor antagonists displaying long residence time.<sup>48</sup>

So far the human  $A_3$  receptor has not been crystallized yet, although its most homologous relative, the adenosine  $A_1$  receptor, has.<sup>49</sup> Homology models of the  $A_3$  receptor have been constructed, and their relevance has been recently reviewed and discussed.<sup>50</sup> It appeared that the interaction of the extended and rigid 2-arylethynyl groups at the C-2 position (as in e.g., MRS5679 and MRS5644) with the  $A_3$  receptor required an outward movement of TM2. Although the agonists' path of binding to or dissociation from the  $A_3$  receptor is unknown, one could speculate that such reorganization of TM2 "locks" the C2-substituted agonist. This reasoning stems from a molecular dynamics simulation and site-directed mutation study of the ligand dissociation pathway from the human adenosine  $A_{2A}$  receptor,<sup>51</sup> for which we used a high resolution crystal structure.<sup>52</sup> It taught us there may be a lid adjacent to the ligand binding pocket retaining the ligand for some time before the compound leaves the receptor. A follow-up study revealed that ligand dissociation correlated with the strength of the salt-bridge between His264 in EL3 and Glu169 in EL2.<sup>53</sup> It would be interesting to learn whether a similar mechanism exists for the  $A_3$  receptor.

#### *Retrospective evaluation of selected agonists with complete kinetic profiles*

IB-MECA (RT = 95 min) and 2-Cl-IB-MECA (RT = 231 min) are two reference full agonists for the  $hA_3$  receptor. Despite numerous reports about their controversial cardioprotection,<sup>54, 55</sup> they have been taken into a series of clinical trials: for IB-MECA, in total 12 completed or planned trials have been reported related to inflammatory conditions (e.g., keratoconjunctivitis sicca, rheumatoid arthritis, psoriasis, uveitis); for 2-Cl-IB-MECA, four trials have been registered for liver diseases (e.g.,

hepatocellular carcinoma, hepatitis C).<sup>56,57</sup> Intuitively one would expect that long residence time hA<sub>3</sub> receptor agonists could be beneficial in chronic diseases, for instance to allow once daily dosing.

More recently, the neuroprotective effect provided by hA<sub>3</sub> receptor agonists has been receiving attention.<sup>11,58</sup> A number of agonists have been designed and evaluated both in *in vitro* and *in vivo* functional experiments, especially for the treatment of chronic neuropathic pain.<sup>26,27,29</sup> MRS5698 in particular, having the longest residence time (1961 min) from our research, has been studied extensively in various pre-clinical animal models of neuropathic pain.<sup>59-61</sup> We, however, hesitate to link the binding kinetics profile of MRS5698 with its *in vivo* effects, also because issues such as pharmacokinetics and species differences (human/rodent) may play an important but yet unknown role.

The adenosine A<sub>3</sub> receptor has a peculiar and rapid desensitization/internalization profile in cultured cells.<sup>6</sup> However, the fast desensitization after A<sub>3</sub> agonist exposure under *in vitro* conditions does not generalize to *in vivo* pain models. MRS5698 maintained full efficacy over a five-day period, with the drug administered either by daily injection or by an implanted mini-pump to provide a steady state plasma concentration.<sup>55</sup> Prolonged MRS5698 exposure in the rat did not reduce its efficacy, as would be expected from agonist-induced desensitization alone. Thus, additional studies of agonist occupancy of the receptor and subsequent receptor processing are warranted to further explore this interesting target for combatting neuropathic pain.

#### *Kinetic Map*

Using the association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rate constants obtained from competition association experiments (**Tables 2 and 3**), a kinetic map (**Figure 4**) was constructed by plotting these values on the y-axis and x-axis, respectively. The dashed diagonal parallel lines represent the kinetically derived  $K_D$  values ( $K_D = k_{off}/k_{on}$ ). Out of this map three subgroups emerged, which were divided according to  $k_{off}$  values: Group A < Group B < Group C. Obviously, Group A and C are solely

composed of methanocarba derivatives (**Figure 4**, red) and ribofurano derivatives (**Figure 4**, blue), respectively. Group B is a mixture of these two compound classes, with the methanocarba derivatives mainly displaying smaller  $k_{off}$  values. Thus, rigidifying the ribose ring with the bicyclic group consistently prolonged the dissociation time. This general division indicates a different mode of receptor-ligand interaction during the binding and unbinding process of the two ligand groups. It also seems there is a clear residence-time “cliff” between Groups A (having one rigid C2-phenylethynyl or 4-biphenylethynyl group with or without one enlarged  $N^6$ -benzyl group) and B (having multiple enlarged adenine substituents) in the kinetic map.

In each subgroup, agonists exhibit  $k_{off}$  values in a similar range, but have different  $k_{on}$  values. As a consequence, variation in  $K_D$  values in each group was observed (Group A: ~10-fold; Group B: ~100-fold; Group C: ~3-fold). Previous SKR studies have primarily focused on optimizing dissociation rates and residence times for designing a kinetically favorable ligand. Yet recently, there has been increasing acknowledgement of the important role association rate constants may play in determining the efficacy of a drug as the result of increased rebinding or increased drug-target selectivity.<sup>21, 62</sup> A kinetic map would thus allow for the selection of compounds with appropriate residence times whilst exploring the role of association rate constants.

In summary, an agonist-related competition association assay at the  $hA_3$  receptor was validated, and a series of ribofurano and methanocarba derivatives were kinetically profiled for the first time. A  $k_{on}$ - $k_{off}$ - $K_D$  kinetic map was constructed and subsequently the agonists with complete kinetic profile were divided into three sub-groups based on their residence times. Longer residence times were associated with methanocarba (vs. ribose) and enlarged adenine  $N^6$  and C-2 (rigid arylalkynyl) substitutions. We identified agonists with very long residence times, but these may not be the most therapeutically favorable for treating various conditions in view of the fast desensitization of the receptor. Although far from definitive, this study suggests that proper residence times, not

necessarily longer than target turnover time, may be vital parameters in the development of hA<sub>3</sub>R agonists for therapeutic use.

## References

1. Meyerhof, W.; Müller-Brechlin, R.; Richter, D. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **1991**, *284*, 155-160.
2. Cortés, D.; Guinzberg, R.; Villalobos-Molina, R.; Piña, E. Evidence that endogenous inosine and adenosine-mediated hyperglycaemia during ischaemia–reperfusion through A<sub>3</sub> adenosine receptors. *Auton. Autacoid Pharmacol.* **2009**, *29*, 157-164.
3. Madi, L.; Ochaion, A.; Rath-Wolfson, L.; Bar-Yehuda, S.; Erlanger, A.; Ohana, G.; Harish, A.; Merimski, O.; Barer, F.; Fishman, P. The A<sub>3</sub> adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. *Clin. Cancer Res.* **2004**, *10*, 4472-4479.
4. Ochaion, A.; Bar-Yehuda, S.; Cohen, S.; Barer, F.; Patoka, R.; Amital, H.; Reitblat, T.; Reitblat, A.; Ophir, J.; Konfino, I.; Chowers, Y.; Ben-Horin, S.; Fishman, P. The anti-inflammatory target A<sub>3</sub> adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn's disease. *Cell. Immunol.* **2009**, *258*, 115-122.
5. Palmer, T. M.; Stiles, G. L. Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A<sub>3</sub> adenosine receptor. *Mol. Pharmacol.* **2000**, *57*, 539-545.
6. Klaase, E. C.; IJzerman, A. P.; de Grip, W. J.; Beukers, M. W. Internalization and desensitization of adenosine receptors. *Purinergic Signalling* **2008**, *4*, 21-37.
7. Gessi, S.; Merighi, S.; Varani, K.; Leung, E.; Mac Lennan, S.; Borea, P. A. The A<sub>3</sub> adenosine receptor: an enigmatic player in cell biology. *Pharmacol. Ther.* **2008**, *117*, 123-140.
8. Shamama, N.; Luqman, A. K.; Zafar, M. A.; Seemi, F. B. Adenosine A<sub>3</sub> receptor: a promising therapeutic target in cardiovascular disease. *Curr. Cardiol. Rev.* **2016**, *12*, 18-26.
9. Borea, P. A.; Varani, K.; Vincenzi, F.; Baraldi, P. G.; Tabrizi, M. A.; Merighi, S.; Gessi, S. The A<sub>3</sub> adenosine receptor: history and perspectives. *Pharmacol. Rev.* **2015**, *67*, 74-102.
10. Antonioli, L.; Csóka, B.; Fornai, M.; Colucci, R.; Kókai, E.; Blandizzi, C.; Haskó, G. Adenosine and inflammation: what's new on the horizon? *Drug Discovery Today* **2014**, *19*, 1051-1068.
11. Fishman, P.; Bar-Yehuda, S.; Liang, B. T.; Jacobson, K. A. Pharmacological and therapeutic effects of A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) agonists. *Drug Discovery Today* **2012**, *17*, 359-366.
12. Müller, C. E.; Jacobson, K. A. Recent developments in adenosine receptor ligands and their potential as novel drugs. *Biochim. Biophys. Acta, Biomembr.* **2011**, *1808*, 1290-1308.
13. Koscsó, B.; Csóka, B.; Pacher, P.; Haskó, G. Investigational A<sub>3</sub> adenosine receptor targeting agents. *Expert Opin. Invest. Drugs* **2011**, *20*, 757-768.
14. Salvemini, D.; Jacobson, K. A. Highly selective A<sub>3</sub> adenosine receptor agonists relieve chronic neuropathic pain. *Expert Opin. Ther. Pat.* **2017**, *27*, 967.
15. Fishman, P.; Cohen, S. The A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR): therapeutic target and predictive biological marker in rheumatoid arthritis. *Clin. Rheumatol.* **2016**, *35*, 2359-2362.
16. Cohen, S.; Stemmer, S. M.; Zozulya, G.; Ochaion, A.; Patoka, R.; Barer, F.; Bar-Yehuda, S.; Rath-Wolfson, L.; Jacobson, K. A.; Fishman, P. CF102 an A<sub>3</sub> adenosine receptor agonist mediates anti-tumor and anti-inflammatory effects in the liver. *J. Cell. Physiol.* **2011**, *226*, 2438-2447.
17. Jacobson, K. A.; Merighi, S.; Varani, K.; Borea, P. A.; Baraldi, S.; Aghazadeh Tabrizi, M.; Romagnoli, R.; Baraldi, P. G.; Ciancetta, A.; Tosh, D. K.; Gao, Z.-G.; Gessi, S. A<sub>3</sub> adenosine receptors as

modulators of inflammation: from medicinal chemistry to therapy. *Med. Res. Rev.*, 10.1002/med.21456.

18. Guo, D.; Hillger, J. M.; IJzerman, A. P.; Heitman, L. H. Drug-target residence time—a case for G protein-coupled receptors. *Med. Res. Rev.* **2014**, *34*, 856-892.

19. Cusack, K. P.; Wang, Y.; Hoemann, M. Z.; Marjanovic, J.; Heym, R. G.; Vasudevan, A. Design strategies to address kinetics of drug binding and residence time. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2019-2027.

20. Copeland, R. A.; Pompliano, D. L.; Meek, T. D. Drug-target residence time and its implications for lead optimization. *Nat. Rev. Drug Discovery* **2006**, *5*, 730-739.

21. Copeland, R. A. The drug-target residence time model: a 10-year retrospective. *Nat. Rev. Drug Discovery* **2016**, *15*, 87-95.

22. Heitman, L. H.; Göblyös, A.; Zweemer, A. M.; Bakker, R.; Mulder-Krieger, T.; van Veldhoven, J. P. D.; de Vries, H.; Brussee, J.; IJzerman, A. P. A series of 2,4-disubstituted quinolines as a new class of allosteric enhancers of the adenosine A<sub>3</sub> receptor. *J. Med. Chem.* **2009**, *52*, 926-931.

23. Gessi, S.; Varani, K.; Merighi, S.; Morelli, A.; Ferrari, D.; Leung, E.; Baraldi, P. G.; Spalluto, G.; Borea, P. A. Pharmacological and biochemical characterization of A<sub>3</sub> adenosine receptors in Jurkat T cells. *Br. J. Pharmacol.* **2001**, *134*, 116-126.

24. de Zwart, M.; de Groote, M.; van der Klein, P. A. M.; van Dun, S.; Bronsing, R.; von Frijtag Drabbe Künzel, J. K.; IJzerman, A. P. Phenyl-substituted N<sup>6</sup>-phenyladenosines and N<sup>6</sup>-phenyl-5' -N-ethylcarboxamidoadenosines with high activity at human adenosine A<sub>2B</sub> receptors. *Drug Dev. Res.* **2000**, *49*, 85-93.

25. van Tilburg, E. W.; van der Klein, P. A. M.; von Frijtag Drabbe Künzel, J.; de Groote, M.; Stannek, C.; Lorenzen, A.; IJzerman, A. P. 5'-O-alkyl ethers of N,2-substituted adenosine derivatives: partial agonists for the adenosine A<sub>1</sub> and A<sub>3</sub> receptors. *J. Med. Chem.* **2001**, *44*, 2966-2975.

26. Tosh, D. K.; Finley, A.; Paoletta, S.; Moss, S. M.; Gao, Z.-G.; Gizewski, E. T.; Auchampach, J. A.; Salvemini, D.; Jacobson, K. A. In vivo phenotypic screening for treating chronic neuropathic pain: modification of C2-arylethynyl group of conformationally constrained A<sub>3</sub> adenosine receptor agonists. *J. Med. Chem.* **2014**, *57*, 9901-9914.

27. Tosh, D. K.; Crane, S.; Chen, Z.; Paoletta, S.; Gao, Z.-G.; Gizewski, E.; Auchampach, J. A.; Salvemini, D.; Jacobson, K. A. Rigidified A<sub>3</sub> adenosine receptor agonists: 1-deazaadenine modification maintains high in vivo efficacy. *ACS Med. Chem. Lett.* **2015**, *6*, 804-808.

28. Tosh, D. K.; Janowsky, A.; Eshleman, A. J.; Warnick, E.; Gao, Z.-G.; Chen, Z.; Gizewski, E.; Auchampach, J. A.; Salvemini, D.; Jacobson, K. A. Scaffold repurposing of nucleosides (adenosine receptor agonists): enhanced activity at the human dopamine and norepinephrine sodium symporters. *J. Med. Chem.* **2017**, *60*, 3109-3123.

29. Tosh, D. K.; Deflorian, F.; Phan, K.; Gao, Z.-G.; Wan, T. C.; Gizewski, E.; Auchampach, J. A.; Jacobson, K. A. Structure-guided design of A<sub>3</sub> adenosine receptor-selective nucleosides: combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *J. Med. Chem.* **2012**, *55*, 4847-4860.

30. Tchilibon, S.; Joshi, B. V.; Kim, S.-K.; Duong, H. T.; Gao, Z.-G.; Jacobson, K. A. (N)-methanocarpa 2,N<sup>6</sup>-disubstituted adenine nucleosides as highly potent and selective A<sub>3</sub> adenosine receptor agonists. *J. Med. Chem.* **2005**, *48*, 1745-1758.

31. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76-85.

32. Motulsky, H. J.; Mahan, L. C. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* **1984**, *25*, 1-9.

33. Guo, D.; van Dorp, E. J. H.; Mulder-Krieger, T.; van Veldhoven, J. P. D.; Brussee, J.; IJzerman, A. P.; Heitman, L. H. Dual-point competition association assay: a fast and high-throughput kinetic screening method for assessing ligand-receptor binding kinetics. *J. Biomol. Screening* **2013**, *18*, 309-320.

34. Cheng, Y.-C.; Prusoff, W. H. Relationship between the inhibition constant ( $K_i$ ) and the concentration of inhibitor which causes 50 per cent inhibition ( $IC_{50}$ ) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.
35. Xia, L.; Burger, W. A. C.; van Veldhoven, J. P. D.; Kuiper, B. J.; van Duijl, T. T.; Lenselink, E. B.; Paasman, E.; Heitman, L. H.; IJzerman, A. P. Structure–affinity relationships and structure–kinetics relationships of pyrido[2,1-f]purine-2,4-dione derivatives as human adenosine  $A_3$  receptor antagonists. *J. Med. Chem.* **2017**, *60*, 7555-7568.
36. Sprang, S. R. Cell signalling: binding the receptor at both ends. *Nature* **2011**, *469*, 172-173.
37. Xia, L.; de Vries, H.; IJzerman, A. P.; Heitman, L. H. Scintillation proximity assay (SPA) as a new approach to determine a ligand's kinetic profile. A case in point for the adenosine  $A_1$  receptor. *Purinergic Signalling* **2016**, *12*, 115-126.
38. Sykes, D. A.; Dowling, M. R.; Charlton, S. J. Exploring the mechanism of agonist efficacy: a relationship between efficacy and agonist dissociation rate at the muscarinic  $M_3$  receptor. *Mol. Pharmacol.* **2009**, *76*, 543-551.
39. Guo, D.; Mulder-Krieger, T.; IJzerman, A. P.; Heitman, L. H. Functional efficacy of adenosine  $A_{2A}$  receptor agonists is positively correlated to their receptor residence time. *Br. J. Pharmacol.* **2012**, *166*, 1846-1859.
40. Doornbos, M. L. J.; Cid, J. M.; Haubrich, J.; Nunes, A.; van de Sande, J. W.; Vermond, S. C.; Mulder-Krieger, T.; Trabanco, A. A.; Ahnaou, A.; Drinkenburg, W. H.; Lavreysen, H.; Heitman, L. H.; IJzerman, A. P.; Tresadern, G. Discovery and kinetic profiling of 7-aryl-1,2,4-triazolo[4,3-a]pyridines: positive allosteric modulators of the metabotropic glutamate receptor 2. *J. Med. Chem.* **2017**, *60*, 6704–6720.
41. Yu, Z.; van Veldhoven, J. P. D.; Louvel, J.; 't Hart, I. M. E.; Rook, M. B.; van der Heyden, M. A. G.; Heitman, L. H.; IJzerman, A. P. Structure–affinity relationships (SARs) and structure–kinetics relationships (SKRs) of Kv11.1 blockers. *J. Med. Chem.* **2015**, *58*, 5916-5929.
42. Yu, Z.; IJzerman, A. P.; Heitman, L. H. K(v)11.1 (hERG)-induced cardiotoxicity: a molecular insight from a binding kinetics study of prototypical K(v)11.1 (hERG) inhibitors. *Br. J. Pharmacol.* **2015**, *172*, 940-955.
43. Smith, G. F. Medicinal chemistry by the numbers: the physicochemistry, thermodynamics and kinetics of modern drug design. In *Progress in Medicinal Chemistry*, Lawton, G.; Witty, D. R., Eds. Elsevier B.V.: Burlington, **2009**; Vol. 48, p 1.
44. Gallo-Rodriguez, C.; Ji, X.-d.; Melman, N.; Siegman, B. D.; Sanders, L. H.; Orlina, J.; Fischer, B.; Pu, Q.; Olah, M. E.; van Galen, P. J. M.; Stiles, G. L.; Jacobson, K. A. Structure – activity relationships of  $N^6$ -benzyladenosine-5' -uronamides as  $A_3$ -selective adenosine agonists. *J. Med. Chem.* **1994**, *37*, 636-646.
45. Guo, D.; Heitman, L. H.; IJzerman, A. P. Kinetic aspects of the interaction between ligand and G protein-coupled receptor: the case of the adenosine receptors. *Chem. Rev.* **2017**, *117*, 38-66.
46. Klotz, K.-N.; Falgner, N.; Kachler, S.; Lambertucci, C.; Vittori, S.; Volpini, R.; Cristalli, G. [ $^3H$ ]HEMADO— a novel tritiated agonist selective for the human adenosine  $A_3$  receptor. *Eur. J. Pharmacol.* **2007**, *556*, 14-18.
47. Auchampach, J. A.; Gizewski, E. T.; Wan, T. C.; de Castro, S.; Brown, G. G.; Jacobson, K. A. Synthesis and pharmacological characterization of [ $^{125}I$ ]MRS5127, a high affinity, selective agonist radioligand for the  $A_3$  adenosine receptor. *Biochem. Pharmacol.* **2010**, *79*, 967-973.
48. Guo, D.; Xia, L.; van Veldhoven, J. P. D.; Hazeu, M.; Mocking, T.; Brussee, J.; IJzerman, A. P.; Heitman, L. H. Binding kinetics of ZM241385 derivatives at the human adenosine  $A_{2A}$  receptor. *ChemMedChem* **2014**, *9*, 752-761.
49. Glukhova, A.; Thal, D. M.; Nguyen, A. T.; Vecchio, E. A.; Jörg, M.; Scammells, P. J.; May, L. T.; Sexton, P. M.; Christopoulos, A. Structure of the adenosine  $A_1$  receptor reveals the basis for subtype selectivity. *Cell* **2017**, *168*, 867-877.
50. Ciancetta, A.; Jacobson, K. Structural Probing and Molecular Modeling of the  $A_3$  Adenosine Receptor: A Focus on Agonist Binding. *Molecules* **2017**, *22*, 449.



51. Guo, D.; Pan, A. C.; Dror, R. O.; Mocking, T.; Liu, R.; Heitman, L. H.; Shaw, D. E.; IJzerman, A. P. Molecular basis of ligand dissociation from the adenosine A<sub>2A</sub> receptor. *Mol. Pharmacol.* **2016**, *89*, 485-491.
52. Liu, W.; Chun, E.; Thompson, A. A.; Chubukov, P.; Xu, F.; Katritch, V.; Han, G. W.; Roth, C. B.; Heitman, L. H.; IJzerman, A. P.; Cherezov, V.; Stevens, R. C. Structural basis for allosteric regulation of GPCRs by sodium ions. *Science* **2012**, *337*, 232-236.
53. Segala, E.; Guo, D.; Cheng, R. K. Y.; Bortolato, A.; Deflorian, F.; Doré, A. S.; Errey, J. C.; Heitman, L. H.; IJzerman, A. P.; Marshall, F. H.; Cooke, R. M. Controlling the Dissociation of Ligands from the Adenosine A<sub>2A</sub> Receptor through Modulation of Salt Bridge Strength. *J. Med. Chem.* **2016**, *59*, 6470-6479.
54. Xu, Z.; Jang, Y.; Mueller, R. A.; Norfleet, E. A. IB-MECA and cardioprotection. *Cardiovasc. Drug Rev.* **2006**, *24*, 227-238.
55. Ge, Z.-D.; Peart, J. N.; Kreckler, L. M.; Wan, T. C.; Jacobson, M. A.; Gross, G. J.; Auchampach, J. A. Cl-IB-MECA [2-chloro-N<sup>6</sup>-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide] reduces ischemia/reperfusion injury in mice by activating the A<sub>3</sub> adenosine receptor. *J. Pharmacol. Exp. Ther.* **2006**, *319*, 1200-1210.
56. National center for biotechnology information. Pubchem compound database; CID=123683, <https://pubchem.ncbi.nlm.nih.gov/compound/123683> (accessed June 30, 2017).
57. National center for biotechnology information. PubChem compound database; CID=3035850, <https://pubchem.ncbi.nlm.nih.gov/compound/3035850> (accessed June 30, 2017).
58. Chen, Z.; Janes, K.; Chen, C.; Doyle, T.; Bryant, L.; Tosh, D. K.; Jacobson, K. A.; Salvemini, D. Controlling murine and rat chronic pain through A<sub>3</sub> adenosine receptor activation. *FASEB J.* **2012**, *26*, 1855-1865.
59. Carlin, J. L.; Tosh, D. K.; Xiao, C.; Piñol, R. A.; Chen, Z.; Salvemini, D.; Gavrilova, O.; Jacobson, K. A.; Reitman, M. L. Peripheral adenosine A<sub>3</sub> receptor activation causes regulated hypothermia in mice that is dependent on central histamine H<sub>1</sub> receptors. *J. Pharmacol. Exp. Ther.* **2016**, *356*, 474-482.
60. Little, J. W.; Ford, A.; Symons-Liguori, A. M.; Chen, Z.; Janes, K.; Doyle, T.; Xie, J.; Luongo, L.; Tosh, D. K.; Maione, S.; Bannister, K.; Dickenson, A. H.; Vanderah, T. W.; Porreca, F.; Jacobson, K. A.; Salvemini, D. Endogenous adenosine A<sub>3</sub> receptor activation selectively alleviates persistent pain states. *Brain* **2015**, *138*, 28-35.
61. Ford, A.; Castonguay, A.; Cottet, M.; Little, J. W.; Chen, Z.; Symons-Liguori, A. M.; Doyle, T.; Egan, T. M.; Vanderah, T. W.; De Koninck, Y.; Tosh, D. K.; Jacobson, K. A.; Salvemini, D. Engagement of the GABA to KCC2 signaling pathway contributes to the analgesic effects of A<sub>3</sub>AR agonists in neuropathic pain. *J. Neurosci.* **2015**, *35*, 6057-6067.
62. de Witte, W. E. A.; Danhof, M.; van der Graaf, P. H.; de Lange, E. C. M. In vivo target residence time and kinetic selectivity: the association rate constant as determinant. *Trends Pharmacol. Sci.* **2016**, *37*, 831-842.