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SHORT COMMUNICATION

Aliskiren displays long-lasting interactions with human renin

Thomas Gossas · Lotta Vrang · Ian Henderson · Susanne Sedig · Christer Sahlberg · Erik Lindström · U. Helena Danielson

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Abstract Aliskiren is a selective renin inhibitor recently approved for use in hypertension. Efficacy duration appears longer than what would be expected based on its circulating half-life. The aim was therefore to characterize the kinetics of the interaction between aliskiren and renin. The interaction was evaluated in three assays and compared with two other renin inhibitors including remikiren. First, the inhibition of recombinant human renin was assessed by monitoring the cleavage of fluorescent substrate. Second, human plasma renin activity (PRA) was monitored by measuring generated angiotensin I over 1 h in the presence or absence of inhibitor. Finally, the affinity, association and dissociation rate constants were determined by using a surface plasmon resonance (SPR) biosensor assay. Aliskiren and remikiren were found to be equipotent inhibitors of recombinant renin activity ($K_i \leq$ 0.04 nM) while compound 1 displayed a K_i value of 1 nM. PRA was efficiently inhibited by both aliskiren and remikiren with IC_{50} values of 0.2–0.3 nM. Remikiren and aliskiren also displayed long-lasting interactions with immobilized renin having k_{off} values of 0.18 and 0.11×10^{-3} s^{-1} respectively. These dissociation rate constants corresponded to residence times of 1.5 and 2.5 h, respectively, while compound 1 had a residence time lasting only 3 min. It is therefore concluded that the long-lasting interaction between aliskiren and human renin may contribute to the 24 h anti-hypertensive effect seen in clinical trials and possibly also to target-mediated drug disposition.

Key words Renin · Aliskiren · Surface plasmon resonance (SPR) · Interaction kinetics · Inhibitors · Optical biosensor

Abbreviations

PRA	Human plasma renin activity
SPR	Surface plasmon resonance
RAS	Renin–angiotensin system
ACE	Angiotensin-converting enzyme
ARB	Angiotensin AT_1 receptor antagonist
FRET	Fluorescence resonance energy transfer

Introduction

The renin–angiotensin system (RAS) plays an important role in blood pressure control and chronic over-activation of this system is implicated in hypertension and various cardiac and renal diseases. Interventions with this signaling cascade have resulted in two classes of anti-hypertensive drugs; (1) angiotensin-converting enzyme (ACE) inhibitors, which inhibit the proteolytic conversion of angiotensin I to the vasoconstrictor angiotensin II, and (2) angiotensin AT_1 receptor antagonists (or blockers, ARBs). Interestingly, both ACE inhibitors and ARBs confer so-called end-organ protection in for instance cardiac failure or kidney disease, which appears to be, at least to some extent, independent of their blood pressure lowering effects (Weir 2007).

The first and rate-limiting step in the RAS pathway involves the proteolytic conversion of angiotensinogen to the decapeptide angiotensin I. This hydrolysis is catalyzed by renin, an aspartyl protease released from juxtaglomerular cells in the kidney. It was suggested

T. Gossas · U. H. Danielson (✉)
Department of Biochemistry and Organic Chemistry, BMC,
Uppsala University,
Box 576, 751 23 Uppsala, Sweden
e-mail: helena.danielson@biorg.uu.se

T. Gossas
e-mail: thomas.gossas@beactica.com

L. Vrang · I. Henderson · S. Sedig · C. Sahlberg · E. Lindström
Medivir AB,
Huddinge, Sweden

already 50 years ago that interfering with renin activity would be expected to produce the most pronounced inhibition of the RAS signaling cascade (Skeggs et al. 1957). However, despite the attractiveness of renin as a target, the development of renin inhibitors has been a challenge. The first renin inhibitors, although being potent and selective in vitro, suffered from poor oral bioavailability and insufficient pharmacodynamics in the clinical setting (Fisher and Hollenberg 2001). Recently, the potent and selective renin inhibitor aliskiren has been approved for the treatment of hypertension, thereby representing the first approved renin inhibitor for clinical use.

Blood pressure fluctuates according to a circadian pattern that reaches a peak in the mid-morning (the so-called early morning surge) at approximately 10.00 a.m. (Weber 2002). The early morning surge is associated with an increase in the incidence of cardiovascular events such as stroke and myocardial infarction (White 2007). Thus, optimal antihypertensive therapy should control blood pressure for 24 h, including the early morning surge period which typically starts around 4.00 a.m. Interestingly, increased renin secretion occurs concomitantly with the early morning surge and may thus play a regulatory role (Portaluppi et al. 1990). Aliskiren treatment results in sustained 24-h blood pressure reduction which may in part be due to its long metabolic half-life which range between 23 and 36 h (Oh et al. 2007; Waldmeier et al. 2007). However, the effects of aliskiren appear to last longer than expected on the basis of its circulating half-life. The mechanistic explanation for this long-lasting effect is not known, although a slow dissociation rate from the enzyme could possibly contribute (i.e., extended residence time, see Copeland et al. 2006). Indeed, the ARB candesartan, an insurmountable receptor antagonist, is suggested to have an advantage over other ARBs mainly due to an extended residence time at the AT_1 receptor (Vauquelin and Van Liefde 2006).

Although aliskiren has been described as a tight binding ligand to the renin enzyme (Wood et al. 2003), to our knowledge there are no published data regarding the dissociation rate of aliskiren or other renin inhibitors from the human renin enzyme. In a recent study, the interactions of aliskiren with renin and prorenin, which had been bound to immobilized renin receptor, was studied using an SPR-based assay (Biswas et al. 2010). However, the response to aliskiren (50 response units (RU)) was unrealistic with respect to the immobilized renin (20 RU) and the association rate constant was outside the limits of the instrument. The experiments were clearly flawed, making any conclusions on renin interactions meaningless.

Thus, the aim of the current study was for the first time to determine the association and dissociation rate constants for the interaction between aliskiren and human renin and to compare these parameters with those for other renin inhibitors such as remikiren, and to investigate if there was a correlation with a long-lasting effect in a plasma renin assay.

Materials and methods

Compounds

Aliskiren and compound 1 were synthesized according to published procedures (Dong et al. 2005; Baldwin et al. 2006). Remikiren (Fischli et al. 1991) was a kind gift from Dr. Jan Danser. The structures are shown in Fig. 1.

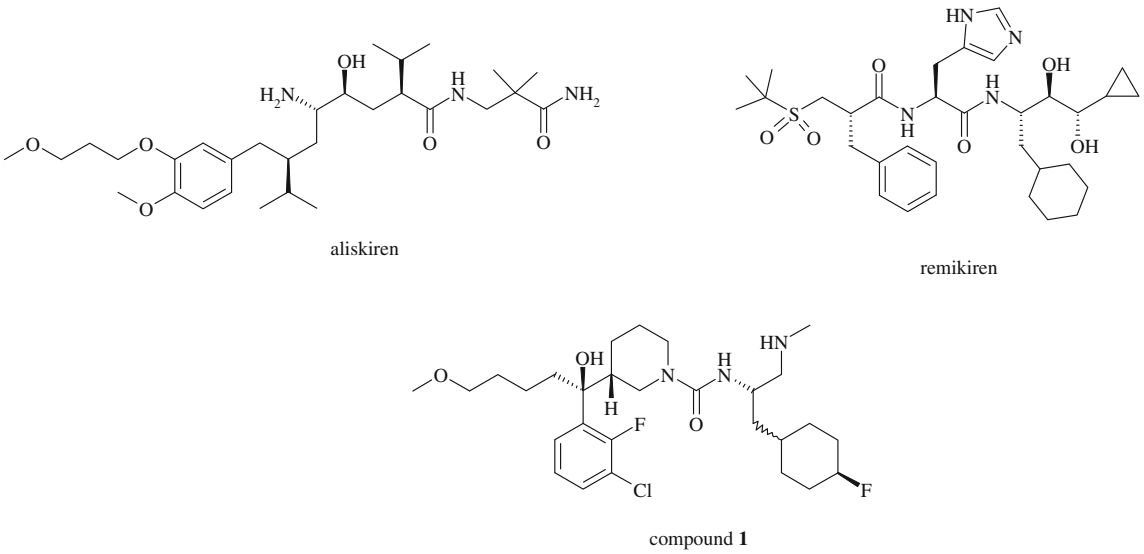

Fig. 1 Inhibitor structures

Enzyme activity studies

Renin activity measurements were carried out in white 96-well microtiter plates (Cliniplate, Thermo Fisher, Göteborg, Sweden) in an assay buffer consisting of 100 mM Tris/HCl, 50 mM NaCl, 0.5 mM EDTA, 0.05% CHAPS, pH 7.4 and a maximal DMSO concentration of 1.2%. A 1 mg/ml stock solution of renin expressed in HEK cells (Proteos Inc., Kalamazoo, MI, USA) was made up in assay buffer and kept in small aliquots at −0°C. A series of inhibitor concentrations were prepared in 100% DMSO. The fluorescence resonance energy transfer (FRET) substrate, Arg-Glu (EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys (DABCYL)-Arg (Sigma, St. Louis, MO, USA), was dissolved in DMSO at a concentration of 1 mM. A pre-incubation of 1 μl of the inhibitor stock added to 90 μl of 4 nM renin in the plate for 30 min at 37°C was followed by the addition of 10 μl of 20 μM substrate in assay buffer, which started the reaction. The fluorescence (excitation 340 nm, emission 500 nm) was monitored every minute for 20 min in a fluorescence plate reader (Fluoroskan Ascent, Thermo Labsystems, Helsinki, Finland).

Rates were determined by linear regression of the fluorescence/time data using Microsoft Excel. K_i values were obtained by non-linear regression analysis (Prism v 5, GraphPad Software Inc., La Jolla, CA, USA) of the rates as a function of the logarithm of inhibitor concentration using an equation for tight binding inhibitors (Morrison 1969), modified as below to allow fitting to logarithmic values of inhibitor concentration.

$$I_0 = 10^{(X+9)}$$
$$K_i^{\mathrm{App}} = 10^{(\log(K_i)+9+\log(1+S/K_M))}$$
$$v_0 = \frac{Q\left(\left(E_0-I_0-K_i^{\mathrm{App}}\right)+\sqrt{\left(E_0-I_0-K_i^{\mathrm{App}}\right)^2+4E_0I_0}\right)}{2}$$

X is the logarithm of the inhibitor concentration, S is the substrate concentration, E_0 is the enzyme concentration, ν_0 is the rate of fluorescence change, and Q is a conversion factor defined as the uninhibited rate of fluorescence change divided by the enzyme concentration. The Michaelis–Menten constant, K_M, was determined to be 0.7 μM in separate experiments where the substrate concentration was varied between 0.1 and 5 μM.

Plasma renin activity

For plasma renin activity (PRA) measurements, blood samples from healthy volunteers were collected in EDTA-containing tubes (Vacutainer, Swevet Piab AB, Sjöbo, Sweden). Plasma was separated from cells by centrifugation at 3,000×g for 10 min at room temperature and was immediately frozen and stored at −20°C until use.

The collected plasma had readily measureable levels of endogenous renin activity at pH 6. However, renin activity at pH 7.2 was very low and human recombinant renin was therefore added to achieve robust levels of angiotensin I. Plasma samples (220 μl) were mixed with 25 μl Tris/acetate pH 7.2 (0.05 M final concentration) containing the angiotensinase inhibitor 2,3-dimercaptopropanol (5 mM final concentration) and recombinant renin (final concentration 0.015 nM). The samples were incubated at 37°C for 1 h with or without renin inhibitor dissolved in DMSO (1% final DMSO concentration). The generated angiotensin I was determined by a commercially available radioimmunoassay kit (CA-1533, DiaSorin, Stillwater, Minnesota, USA) according to the suppliers description. The concentration of renin inhibitor that inhibited PRA by 50% (IC_{50}) was determined by non-linear regression analysis using GraphPad Prism Software version 5.01 (San Diego, CA, USA).

SPR biosensor studies

A Biacore S51 instrument (GE Healthcare, Uppsala, Sweden) was used for the interaction studies. Renin (Proteos Inc., Kalamazoo, MI, USA) was immobilized by amine- and aldehyde coupling as described in the Biacore sensor surface handbook. For aldehyde coupling, renin was oxidized with sodium (meta)periodate (Sigma) and thereafter buffer-exchanged using protein de-salting spin columns (Pierce Biotech. Inc., Rockford, IL, USA) and stored as aliquots in coupling buffer (10 mM sodium acetate, pH 5) at −80°C prior to immobilization on a CM5-chip. HBS-P (10 mM HEPES, 0.15 M NaCl, 0.005% Tween 20), pH 7.4 was used as running buffer for immobilization. The same buffer, supplemented with 3% DMSO, was also used for the interaction studies. Inhibitors were injected for 90 s at a flow rate of 30 μl/min and the dissociation was thereafter followed for up to 900 s. The sensor surface was regenerated after each cycle by 2×30 s injections of 1 M LiCl in 50% ethanediol. Solvent correction was included as described in the Biacore S51 methodology handbook. Three blank injections were made for every inhibitor concentration series. All sensorgrams were corrected by subtracting the signal from a non-modified reference surface and an average of the blank injections. Biacore S51 evaluation software 1.2.1 was used for data evaluation and Biaevaluation 4.1 for global non-linear regression analysis of the data.

Results

Inhibition of recombinant human renin and human plasma renin

The inhibitory effect of the compounds was determined in two types of activity-based assays. One was a steady-state

assay using recombinant human renin while the other was based on the inhibition of renin in human plasma. Of the renin inhibitors tested, aliskiren and remikiren displayed the highest inhibition for recombinant human renin with K_i values of 0.04 nM or below (Table 1). The inhibition constant for compound 1 was 1.2 nM. The IC_{50} values for the inhibition of PRA in human plasma ranged from 0.2 (remikiren) to 14 nM (compound 1), Table 1. These values are similar to those reported by others (van den Meiracker et al. 1990; Wood et al. 2003; Baldwin et al. 2006).

SPR biosensor assay

An SPR biosensor assay for the study of the interaction between renin and small molecules was developed. Although amine coupling of renin was possible in terms of achieving adequate immobilization levels, inhibitors failed to interact specifically with this type of sensor surface. Instead, immobilization by aldehyde coupling was found to produce a sensor surface to which inhibitors interacted specifically. The HIV protease inhibitor indinavir was used as a negative control and was found not to interact with immobilized renin. The analyte binding capacity of aldehyde-coupled renin was typically 60–80% of the theoretical maximum. This sensor surface was stable and could be used for several days without significant loss of binding capacity. The surface could also withstand a variety of conditions tested for regeneration purposes, such as 30 s injections of solutions with high and low pH and ionic strength, complexing agents, surfactants, organic solvents, chaotropic substances and combinations thereof. A combination of LiCl and ethanediol was found to completely dissociate the inhibitors while still maintaining full analyte binding capacity of the surface.

Kinetics of the interaction between inhibitors and human renin

The kinetics for the interaction between the renin inhibitors was determined by a direct interaction assay where renin was immobilized on a sensor chip and the inhibitors injected as analytes. The association rates for the compounds were similar, but the dissociation rates differed significantly, with aliskiren and remikiren displaying very slow dissociation, as illustrated by the normalized sensorgrams for a single concentration of each of the three different inhibitors (Fig. 2).

In order to quantify these differences, the kinetic parameters for these interactions (Table 2) were determined from a series of inhibitor concentrations (data shown in Fig. 3) and by assuming a simple 1:1 Langmuir interaction. All compounds were well-described by this model.

The interaction studies showed that remikiren and aliskiren had a tight binding to the enzyme with subnanomolar affinity, while compound 1 had a tenfold lower affinity with a relatively fast dissociation rate. Aliskiren displayed a relatively fast association but had a very slow dissociation from renin. The association of remikiren was twice as fast, however a twofold faster dissociation rate makes the affinity for both compounds similar (0.2–0.3 nM). By contrast, compound 1 had the fastest association rate but dissociation from the enzyme was approximately 50-fold faster than for remikiren and aliskiren.

Table 1 Enzyme inhibition

Compound	K_i (nM)	IC_{50} (nM)
Compound 1	1.2	14
Remikiren	<0.04	0.18
Aliskiren	0.04	0.29

K_i—values were determined with an enzymatic assay using recombinant renin and a FRET substrate. IC_{50}—values were obtained with a plasma renin assay monitoring hydrolysis of angiotensin I. The values are means from at least two independent experiments

Discussion

An SPR biosensor assay for the study of interactions between renin and small molecules has been developed. Inhibitors failed to interact with renin immobilized by amine coupling, whereas aldehyde coupling of renin via glycosyl residues was found to produce a stable sensor surface with high analyte binding capacity. Renin is specifically glycosylated at Asn5 and Asn75 and immobilization by aldehyde coupling via glycans at these residues, which are distant from the active site, is not likely to affect binding of compounds (Carilli et al. 1988; Sielecki et al. 1989). On the contrary, the glycans

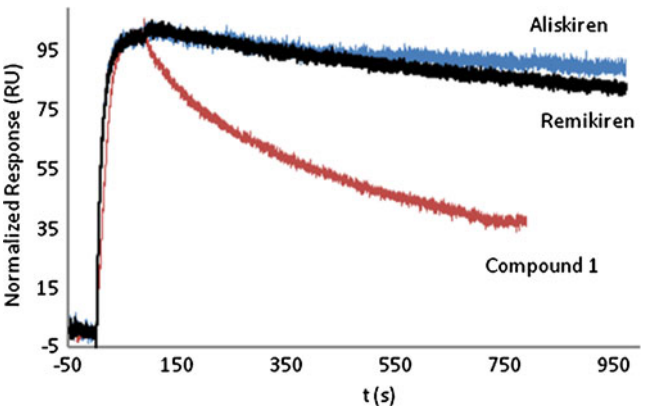

Fig. 2 Normalized sensorgrams for saturating concentrations of the three inhibitors injected over a renin surface. *Red* compound 1, 100 nM; *black* remikiren, 300 nM; *blue* aliskiren, 300 nM

Table 2 Interaction kinetics

Compound	k_{on} (x 10^5 $M^{-1}s^{-1}$)	k_{off} (x 10^{-3} s^{-1})	Residence time (min)	K_D (nM)
Compound 1	23±2	4.9±0.7	3.4	2.2±0.3
Remikiren	7.4±0.8	0.18±0.05	93	0.24±0.04
Aliskiren	4.0±0.2	0.11±0.04	152	0.30±0.09

The direct interaction between immobilized renin and inhibitors was determined using an SPR biosensor assay. A concentration series of the inhibitors were injected and the kinetic parameters were determined by global regression analysis of the sensorgrams (representative sensorgrams are shown in Fig. 3). The residence time was defined as $1/k_{off}$

Mean±standard deviation. n=3–6 independent measurements

probably provide an excellent handle for immobilization since direct amine coupling via lysine residues evidently distorted the protein structure in some way.

The anticipated in vivo efficacy of receptor antagonists or enzyme inhibitors and their duration of action can be difficult to predict based only on potency values obtained by in vitro assays (see Copeland et al. 2006 and Tummino and Copeland 2008 for recent reviews). It has been suggested that monitoring the residence time (which is the reciprocal of the dissociation rate constant, $\tau=1/k_{off}$) of an antagonist/inhibitor at its target receptor/enzyme may provide additional information on possible duration of drug efficacy in vivo and assist in developing compounds with long-lasting effects if desired (Tummino and Copeland 2008). For instance, when it comes to the RAS system, it has been proposed that administering angiotensin AT_1 receptor antagonists, which form slowly dissociating complexes with the AT_1 receptor, result in long-lasting clinical actions (Vauquelin and Van Liefde 2006). With respect to protease inhibitors, the slow dissociation of darunavir from HIV protease has been suggested to result in more efficient, broad-spectrum antiviral activity compared to other approved HIV protease inhibitors (Dierynck et al. 2007).

The current study demonstrates that the interaction kinetics differ substantially between renin inhibitors. Aliskiren, the only renin inhibitor approved for clinical use, has a rather long residence time of 2.5 h at the human renin enzyme. In contrast, the renin inhibitor compound 1, which displayed 1.2 nM potency at the enzyme level, quickly dissociated from the enzyme with a residence time of about 3 min.

If the marked differences in residence time between compounds correlate to efficacy duration in vivo (i.e., anti-hypertensive effects) warrants further investigation. However, when evaluating effect duration in vivo it is crucial to take pharmacokinetic properties into consideration along with kinetic parameters of enzyme binding such as residence time. Thus, to demonstrate an additional value in vivo with an enhanced residence time measured in vitro, carefully designed studies which monitor efficacy (blood pressure lowering) together with compound exposure levels at the assumed site of action (kidney and/or plasma) are required. Nevertheless, it has been demonstrated that the tachykinin NK_1 receptor antagonist aprepitant, with demonstrated prolonged residence time at the NK_1 receptor in vitro compared to other equipotent antagonists, produced longer efficacy duration in vivo (Lindström et al. 2007). The prolonged in vivo efficacy of aprepitant was not explained by differences in pharmacokinetics between compounds, but was more likely due to slowly dissociating receptor complexes.

In man, metabolism of aliskiren appears to be minimal, leading to terminal half-lives ranging between 23 and 36 h (Waldmeier et al. 2007). Thus, despite the low oral bioavailability (2–3%), aliskiren is able to produce anti-hypertensive effects lasting 24 h with anti-hypertensive effects in the same range as those produced by ACE inhibitors and ARBs (Oparil et al. 2007). However, the effects of aliskiren on renin activity and blood pressure last for several weeks after stopping treatment (Oh et al. 2007, Andersen et al. 2008). Our results confirm this hypothesis. Conceivably, when free plasma (or renal) levels of aliskiren are reduced below those required normally for efficacy; an enhanced residence time for the renin inhibitor complex may result in extended efficacy not necessarily reflected by plasma (or renal) levels of compound.

Recent studies demonstrate that aliskiren accumulates in renin-containing secretory granules in vitro (Krop et al. 2008) and the kidney/plasma ratio of aliskiren was 30–60-fold in rats in vivo (Feldman et al. 2008). This accumulation could be due to physicochemical properties of the compound keeping it accumulated in renin-containing granules in kidney tissue and thus possibly allowing for rebinding to the enzyme. However, our data support the suggestion that aliskiren is accumulated in kidneys at least in part by long-lasting interactions with renal renin. Indeed, so-called target-mediated drug disposition has been previously described for drugs displaying high affinity binding to a pharmacological target (Levy 1994).

Remikiren displayed similar K_D values as aliskiren and had a residence time of approximately 90 min. Remikiren

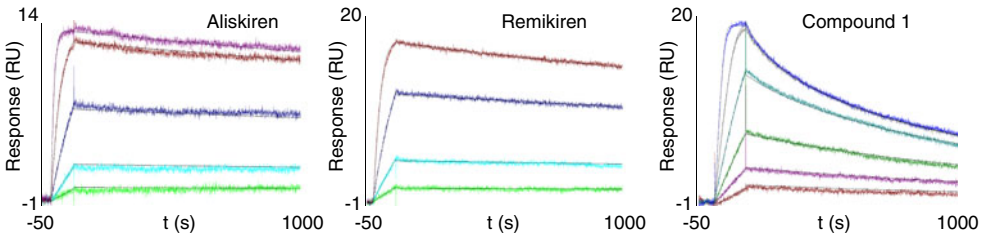

Fig. 3 Global fits of a model for a reversible 1:1-binding with mass-transfer to a concentration series of each inhibitor. Aliskiren 3.7–300 nM; remikiren 3.7–100 nM; compound 1 3.125–100 nM

was in clinical development in the early 1990s for the treatment of hypertension. The program was discontinued due to low oral bioavailability of the drug. Nevertheless, several clinical reports demonstrate that remikiren exerts sustained pharmacodynamic effects despite undetectable plasma drug levels (van den Meiracker et al. 1990, Kobrin et al. 1993, Kobrin et al. 1993, Viskoper et al. 1994). Hence, this sustained pharmacological action may be due to slow dissociation from the target, as demonstrated in the current study and/or target (kidney)-mediated drug disposition.

In conclusion, we demonstrate for the first time that human renin can be functionally immobilized, allowing the study of renin inhibitor interaction kinetics. The dissociation rate, and hence residence time, differed substantially between inhibitors tested. The relatively slow dissociation of remikiren and aliskiren may contribute to sustained pharmacological efficacy and target-mediated drug disposition in vivo.

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