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Structure-based design of aliskiren, a novel orally effective renin inhibitor

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Abstract

Hypertension is a major risk factor for cardiovascular diseases such as stroke, myocardial infarction, and heart failure, the leading causes of death in the Western world. Inhibitors of the renin–angiotensin system (RAS) have proven to be successful treatments for hypertension. As renin specifically catalyses the rate-limiting step of the RAS, it represents the optimal target for RAS inhibition. Several peptide-like renin inhibitors have been synthesized previously, but poor pharmacokinetic properties meant that these compounds were not clinically useful. We employed a combination of molecular modelling and crystallographic structure analysis to design renin inhibitors lacking the extended peptide-like backbone of earlier inhibitors, for improved pharmacokinetic properties. This led to the discovery of aliskiren, a highly potent and selective inhibitor of human renin *in vitro*, and *in vivo*; once-daily oral doses of aliskiren inhibit renin and lower blood pressure in sodium-depleted marmosets and hypertensive human patients. Aliskiren represents the first in a novel class of renin inhibitors with the potential for treatment of hypertension and related cardiovascular diseases.

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Hypertension is a major risk factor for cardiovascular diseases such as stroke, myocardial infarction, and heart failure with recent estimates by the World Health Organization suggesting that hypertension was responsible for 7.1 million deaths worldwide (13% of total deaths) in

the year 2000 (World Health Report, 2002). Despite the known risks associated with hypertension, the majority of hypertensive patients do not have their blood pressure controlled to target levels [1], indicating a clear need for new approaches to the management of hypertension.

The renin–angiotensin system (RAS) is a key regulator of blood pressure and body fluid volume, and acts primarily via the effects of the octapeptide hormone, angiotensin II (Ang II). Ang II is formed in a two-step

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process: the aspartic peptidase renin cleaves the Leu10–Val11 peptide bond of angiotensinogen to form angiotensin I (Ang I), which is then converted by angiotensin converting enzyme (ACE) into Ang II. Excessive RAS activity is believed to be an underlying cause of many pathological states, because Ang II increases blood pressure and exerts direct growth-promoting effects on cardiac [2] and renal [3] tissue that lead to end organ damage. Indeed, RAS inhibitors, such as ACE inhibitors and Ang II receptor blockers (ARBs), have proven to be successful treatments for hypertension and heart failure [4,5].

Renin inhibitors prevent the formation of Ang I and Ang II [6,7] and so may act differently from ARBs, which increase angiotensin peptide levels, and ACE inhibitors, which increase Ang I levels and do not block ACE-independent Ang II production [8,9]. A variety of stable peptide-like analogues of the scissile peptide bond of angiotensinogen have been developed over the past 20 years [7,10], and were shown to inhibit renin and lower blood pressure (BP) following intravenous administration to sodium-depleted marmosets [11]. Moreover, studies in humans showed that intravenously administered renin inhibitors lowered BP at least as effectively as ACE inhibitors [12–14]. Poor pharmacokinetic properties (poor absorption and rapid elimination) following oral administration in humans meant, however, that the renin inhibitors developed to date have not been clinically useful drugs [15].

We employed a combination of crystal structure analysis of renin–inhibitor complexes and computational methods to design novel, low molecular weight renin inhibitors without the extended peptide-like backbone of previous inhibitors and with favourable pharmacokinetic properties after oral administration to monkeys. Our design approach led to the development of aliskiren, the first in a new class of orally effective, non-peptide renin inhibitors which represent novel potential treatments for hypertension and related cardiovascular diseases.

Materials and methods

X-ray structure analysis. Recombinant glycosylated human renin was co-crystallized with aliskiren, and X-ray diffraction data for the aliskiren–renin complex crystals were collected and processed as described previously [16]. Figures were generated using MOLSCRIPT [17], Raster 3D [18,19], and the GRASP programs [20].

Enzyme assays. The inhibitory potency of aliskiren against human renin was studied *in vitro* as described previously [21]. Human recombinant renin (0.33 ng/ml) was incubated with a synthetic tetradecapeptide substrate (TDP, 13.33 μ M) corresponding to the 14 amino terminal amino acids of human angiotensinogen, in 0.33 M Tris buffer, pH 7.2, containing 1% (w/v) human serum albumin (HSA) and 0.1% neomycin sulphate for 1 h at 37°C. Total volume of the incubation solution was 60 μ l. The enzymatic reaction was stopped by adding 1 ml ice-cold 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1% HSA. The

Ang I generated during the incubation was measured by radioimmunoassay [22].

Aliskiren was tested against human cathepsin D, E and pepsin using the synthetic peptide Lys–Pro–Ile–Glu–Phe–Nph–Arg–Leu as substrate, and against HIV-1 peptidase using the synthetic peptide Lys–Ala–Arg–Ile–Nle–Nph–Glu–Ala–Nle–NH₂ as substrate [23].

Species specificity of aliskiren was tested by determining the IC₅₀ for inhibition of endogenous renin in plasma from human, marmoset, rat, dog, rabbit, cat, pig, and guinea pig. Plasma collected on EDTA was incubated with the angiotensinase inhibitor, 2,3-dimercaptoethanol (5 mM), at pH 7.2 (0.5 M Tris [hydroxymethyl]aminomethane acetate buffer) in the absence or presence of aliskiren (0.1 nM–10 μ M) for 1 h at 37°C. The Ang I generated was quantified by radioimmunoassay [22].

Blood pressure and heart rate measurements in marmosets. Blood pressure (BP) and heart rate (HR) were measured by telemetry in conscious, freely moving marmosets (250–350 g in weight) [24]. Briefly, pressure transmitters (AM Unit, model TA11PA-C40, Data Sciences, St. Paul, Minnesota) were implanted into the peritoneal cavity under aseptic conditions and light anesthesia, and the sensor catheter was placed in the aorta below the renal artery pointing upstream [24]. Marmosets were allowed to recover for at least 4 weeks before any experiment.

Marmosets were maintained on a low sodium diet (pellets with Na⁺, 9 mmol/kg and K⁺, 128 mmol/kg, NAFAG, Gossau, Switzerland) supplemented with fruit and with free access to water for 1 week before, and during, experiments. BP and HR were measured for 1 week before drug administration and for 4 days after the final dose. Marmosets received aliskiren, 3 or 10 mg/kg, or vehicle (distilled water), orally by gavage once daily for 8 days. Mean values of MAP and HR were calculated over 1 h periods, and changes in BP and HR were calculated by comparison of each value in the pre- and post-administration diurnal profiles with each animal acting as its own control.

Determination of PRA and plasma renin levels in marmosets. Plasma samples were taken before administration of the first dose, and on day 8 of drug administration, 2 and 24 h after the last dose. Blood samples were collected by direct puncture of a femoral vein using EDTA as an anticoagulant. PRA was measured by the antibody trapping method [25]; for each animal, percentage inhibition of PRA at each time point was calculated using the pre-treatment PRA as the baseline. Plasma concentrations of active and total renin were measured by IRMA [26].

Blood pressure and heart rate measurement in hypertensive patients. Eight patients aged 18–70 years with mild-to-moderate hypertension (mean daytime systolic BP \geq 140 mmHg) of general good health participated in the study. All patients provided written informed consent prior to study participation, and the study protocol was approved by the Irish Medicines Board and the Beaumont Hospital Ethics Committee (Beaumont Hospital, Dublin 9, Ireland). The study was open-label and sequential with respect to dose escalation for safety reasons. Following a 2-week wash-out period, patients were treated for 4 weeks with aliskiren, 75 mg. Safety and tolerability were evaluated before administering a further 4 weeks of treatment with aliskiren, 150 mg. One gelatin capsule was administered per day with 200 ml of water, 30 min before breakfast.

Ambulatory blood pressure measurement (ABPM) was performed using a Spacelabs 90207 ABPM (Spacelabs Medical, Issaquah, West Virginia), with monitors programmed to measure BP at 30 min intervals during the day and night. Data were analysed using the software package DABL [27], with daytime defined as 09:00–21:00 h and nighttime as 01:00–06:00 h. ABPM was performed prior to treatment (baseline) and after each aliskiren treatment period.

Determination of PRA in humans. Venous blood samples were collected via intravenous catheter prior to the first administration of aliskiren, and 24 h after administration of the final dose of aliskiren, 75 mg, and the final dose of aliskiren, 150 mg. Plasma samples were stored at –30°C until analysed for PRA according to methods described previously [28].

Results

Structure-based drug design of aliskiren

Molecular modelling methods were applied in order to design compounds lacking the P1–P4 spanning backbone of previous peptide inhibitors that would instead optimally exploit the extended hydrophobic surface of the large S3–S1 cavity of renin. This design concept utilized a dipeptide-like hydroxyethylene transition state mimetic with a directly linked P3–P1 pharmacophore, resulting in the synthesis of novel inhibitors such as **1** (Fig. 1) [29]. **1** inhibited purified recombinant human renin at nanomolar concentrations, but showed reduced activity in the presence of plasma. Computational modelling and X-ray crystallographic resolution of enzyme–inhibitor complexes guided lead optimization [16] with the aim of identifying inhibitors with improved potency in the more physiologically relevant plasma renin assay.

The tertiary butyl residue in **1** was replaced by the smaller, more polar OMe group despite the hydrophobic nature of the S3 specificity site, thus forming less lipophilic compounds (**2**) but without compromising in vitro activity. A further major breakthrough was made with the incorporation of various alkylether aromatic sidechains (**3** and **4**). X-ray structure analysis showed that these compounds interact with a distinct sub-pocket, S3^{SP}, oriented perpendicular to the substrate binding cleft of renin, that is not occupied by substrate-derived inhibitors such as CGP 38560 [30]. Exploitation of this hitherto unrecognized site, by optimization of the mostly hydrophobic interactions within the S3^{SP} sub-pocket, dramatically enhanced binding affinity for renin, and selectivity over related aspartic peptidases [16]. The methoxypropoxy sidechain in **4** appears optimal with regard to length and the position of the distal ether oxygen as H-bond acceptor from Tyr 14 of the S3^{SP} sub-pocket.

At the P1' position, substitution of the methyl group (**4**) for an isopropyl moiety (**5**) had little effect on in vitro

activity, but improved duration of action in preliminary studies investigating the effects of oral administration to marmosets. Extensive modifications of the P2' portion including replacement of the *n*-butyl sidechain of **4** with basic (**6**) or neutral polar residues (**7** and **8**) produced inhibitors with similar in vitro potencies. The optimization process resulted in **9**, with the terminal carboxamide group involved in an additional H-bonding interaction with Arg74 and insertion of the geminal methyl residues into the P2' sidechain providing hydrophobic van der Waals interactions with the S2' site of renin, increasing affinity into the sub-nanomolar range. Compound **9** as its hemi-fumarate salt is the non-peptide, small molecule, transition-state mimetic human renin inhibitor aliskiren (SPP-100; Fig. 2A).

Interaction of aliskiren with human renin

A global perspective of aliskiren bound to the extended active site of the recombinant human protein fold is shown (Fig. 2B). Aliskiren acts as a transition state mimetic, inhibiting renin via hydrogen bonding of both the central hydroxyl group and amino function to the catalytic Asp32 and Asp215 residues [16]. With no large P4–P2 spacing backbone, aliskiren does not interact with the S2 or S4 binding sites of renin, unlike peptide-like renin inhibitors such as CGP 38560. The altered hydrogen bonding pattern and conformational shifts for the P1–P2' portion of aliskiren, compared with previous inhibitors [31], are clearly illustrated by overlaying aliskiren with the enzyme-bound conformation of CGP 38560 (Fig. 2C) [21]. The P3–P1 pharmacophore of aliskiren is accommodated by the complementary large hydrophobic S3–S1 'superpocket,' constituted in part by the 'flap' region in its closed conformation, with the S3^{SP} sub-pocket accommodating the aromatic alkoxy sidechain (Fig. 2D).

Physical and chemical properties of aliskiren

In contrast to most peptide-like renin inhibitors, aliskiren is a rather hydrophilic molecule ($\log P_{\text{oct/water}} = 2.45$ at pH 7.4) with favourable physico-chemical properties including high aqueous solubility (>350 mg/ml at pH 7.4) that were considered an important prerequisite for improved oral bioavailability. Aliskiren has as its free base ($pK_a = 9.49$) the molecular formula C₃₀H₅₃N₃O₆ and a molecular mass of 551.8 g/mol (609.8 g/mol as hemi-fumarate salt).

Potency and enzyme specificity of aliskiren in vitro

The in vitro enzyme specificity of aliskiren for human renin over other human aspartic peptidases and HIV-1 peptidase was tested (see Materials and methods). Aliskiren is a potent (sub-nanomolar IC₅₀ value), tight-

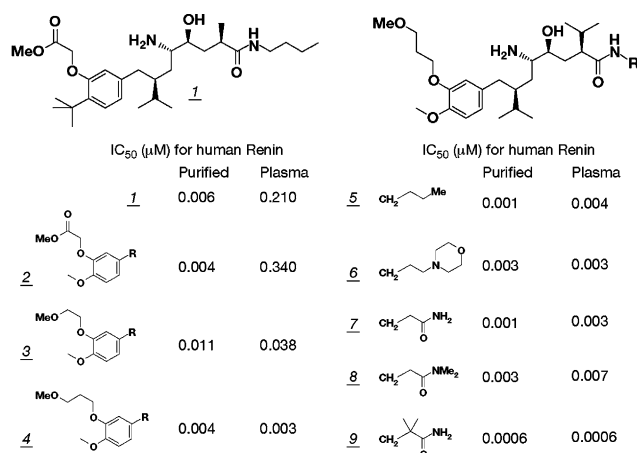


Fig. 1. Structure–activity optimization of renin inhibitor compounds.

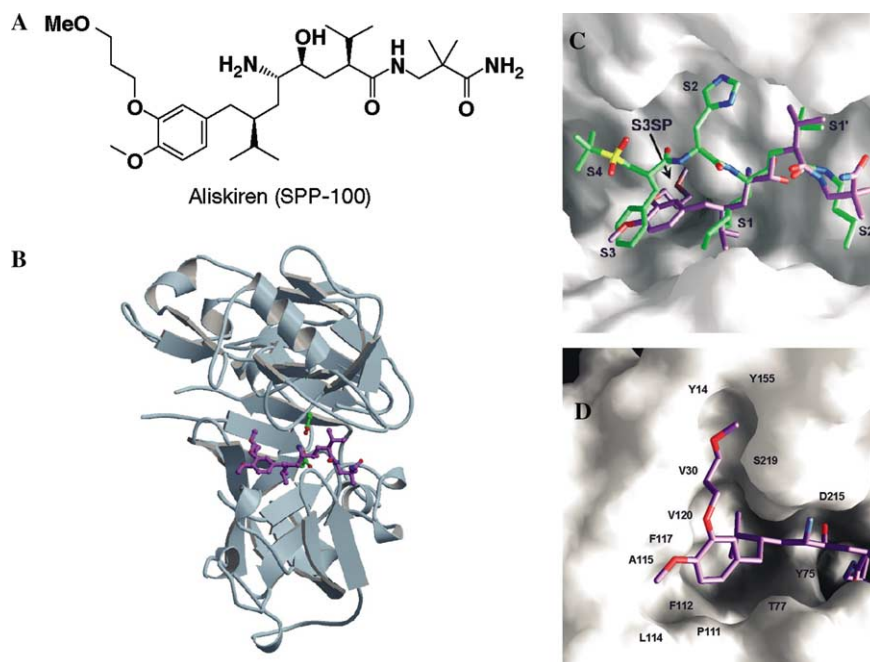


Fig. 2. Aliskiren (SPP-100), a highly selective, tight-binding inhibitor of human renin. (A) Chemical structure of aliskiren (SPP-100; IUPAC name 2(*S*),4(*S*),5(*S*),7(*S*)-*N*-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)-phenyl]-octanamide). (B–D) X-ray crystal structure representations of aliskiren co-crystallized with recombinant glycosylated human renin at 2.2 Å resolution. (B) General view of aliskiren (ball-and-stick representation, with all bonds to carbon atoms shown in purple) in the binding complex with human renin; the enzyme β -strands are represented as arrows and α -helices as ribbons. Aliskiren can be seen to occupy the S3 to S2' subsites, thereby blocking the catalytic function of the enzyme. (C) Close-up view of aliskiren (purple) binding to the active site of human renin with the enzyme specificity pockets S4 to S2' being indicated. Superposition with the peptidic inhibitor CGP 38560A [30,39] (green) indicates the differences in binding interactions for aliskiren (see text for details). (D) Extended view of aliskiren binding to the large hydrophobic S1–S3 site, and the newly discovered non-substrate pocket S3^{SP} of renin, accommodating the methoxyalkoxy sidechain of the inhibitor essential for strong binding. In this view, the active site is rotated by about 90° relative to the horizontal axis as compared to (A) and (B). Ser219 inducing a H-bond interaction via its sidechain OH group to the inhibitor is indicated. Note also the positioning of the polar OMe moiety filling the hydrophobic S3 pocket of the enzyme.

binding competitive inhibitor of purified renin, but showed more than 10,000-fold lower affinity for related aspartic peptidases (Table 1A). The *in vitro* inhibition by aliskiren of the activity of endogenous renin on angiotensinogen in plasma from a variety of species was also determined [21]. Aliskiren is one of the most potent renin inhibitors yet identified ($IC_{50} = 0.6$ nM), with high species specificity for primate renin (Table 1B).

At a concentration of 10 μ M, aliskiren exhibited little or no effect on a series of neurotransmitter receptors, including α_1 -, α_2 -, and β -adrenoceptors, 5-HT, histamine, opiate, benzodiazepine and adenosine receptors, muscarinic cholinergic receptors, and AMPA, kainate or NMDA glutamate receptors (J. Wood, personal communication).

Oral aliskiren inhibits plasma renin activity and lowers blood pressure in sodium-depleted marmosets

The effects of 8 daily, oral doses of aliskiren, 3 or 10 mg/kg, on blood pressure (BP), heart rate (HR, both monitored by telemetry), and plasma renin were tested in

Table 1
Potency, enzyme specificity, and species specificity of aliskiren *in vitro*

	IC_{50} (nM)
(A) Enzyme	
Renin	0.6
Cathepsin D	5000
Cathepsin E	>10,000
Pepsin	>10,000
HIV-1 peptidase	>10,000
(B) Mammalian species	
Human	0.6
Marmoset	2
Rat	80
Dog	7
Rabbit	11
Cat	8500
Pig	150
Guinea pig	63

(A) IC_{50} values (50% inhibitory concentrations) for inhibition by aliskiren (**9**, hydrochloride salt) of the *in vitro* activity of various human aspartic peptidases and HIV-1 peptidase. (B) IC_{50} values for inhibition by aliskiren (**9**, hydrochloride salt) of the *in vitro* activity of endogenous renin on angiotensinogen in plasma from a variety of mammalian species.

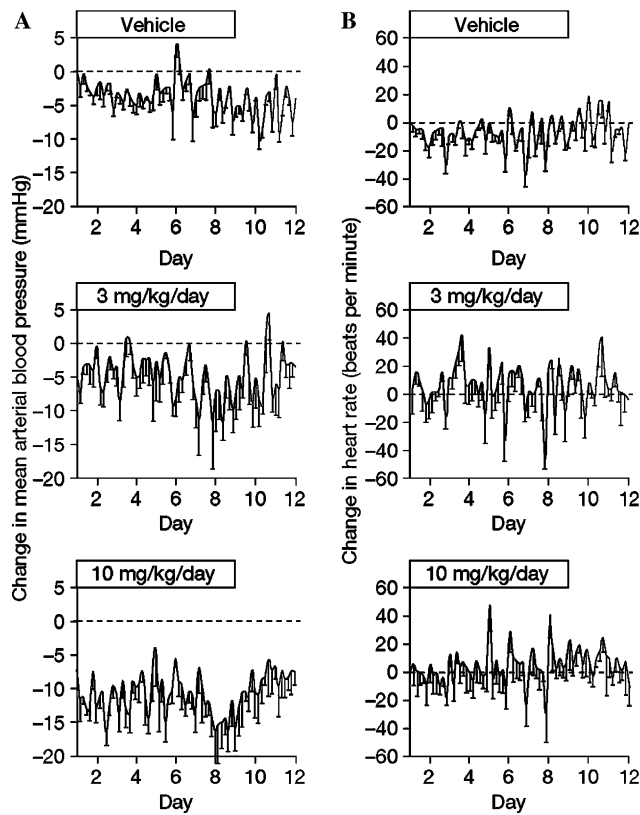


Fig. 3. Aliskiren lowers blood pressure without affecting heart rate in sodium-depleted marmosets. The effects of once daily oral doses of aliskiren (9, hydrochloride salt), 3 mg/kg (middle panel, $n = 4$) or 10 mg/kg (lower panel, $n = 10$), or vehicle control (upper panel, $n = 8$) on (A), mean arterial BP and (B), HR in sodium-depleted marmosets. BP and HR were measured by telemetry and changes in BP and HR were calculated using each animal as its own control as described in Materials and methods. Data are presented as mean values and mean \pm SEM values are presented every 4 h for clarity.

mildly sodium-depleted marmosets. Aliskiren, 3 mg/kg, lowered BP by approximately 10 mmHg within 2 h of administration on day 1, with BP recovering to pretreatment values after approximately 20 h (Fig. 3A). Aliskiren, 10 mg/kg, lowered BP by a maximum of 13 ± 2 mmHg on day 1 and BP was still reduced by 6 ± 1 mmHg at the time of dosing on day 2. Recovery of BP at 24 h decreased during the treatment period, hence by day 8, BP was maximally reduced by 16 ± 2 mmHg (Fig. 3A). No significant changes in HR were seen with aliskiren, 3 mg/kg, and only transient increases in HR occurred within the first 2 h after administration of aliskiren, 10 mg/kg (Fig. 3B). There was no rebound increase in BP following the end of treatment with either dose of aliskiren.

Plasma concentrations of active and total renin at baseline were similar in all treatment groups (Figs. 4A and B). On day 8, total and active renin levels increased after administration of either dose of aliskiren, whereas small decreases in renin levels were observed in vehicle treated marmosets ($n = 7$). The time course of changes

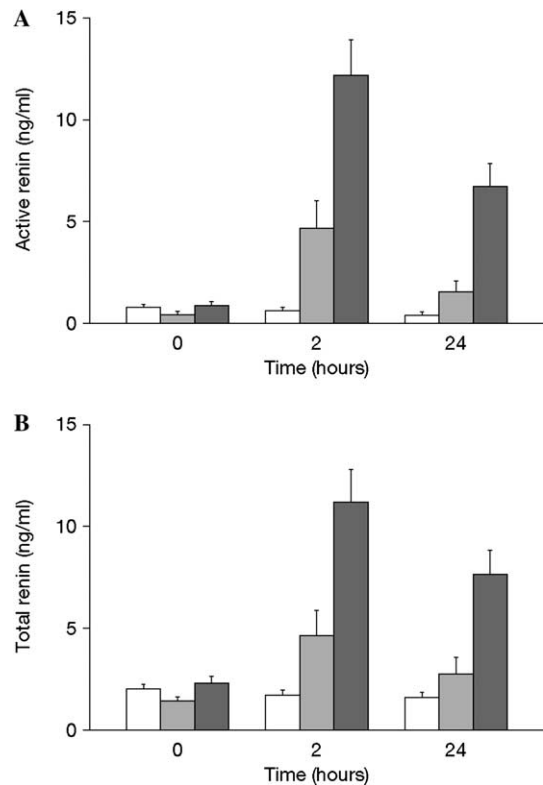


Fig. 4. Aliskiren increases plasma immunoreactive renin levels in sodium-depleted marmosets. Effects of once daily oral doses of aliskiren (9, hydrochloride salt), 3 mg/kg (light bars, $n = 5$) or 10 mg/kg (dark bars, $n = 10$), or vehicle control (open bars, $n = 7$) on (A) plasma active renin levels and (B) plasma total renin levels in sodium-depleted marmosets. Plasma samples were taken before administration of the first dose (baseline), and on day 8, 2 and 24 h after the last dose, and plasma renin levels were determined as described in Materials and methods. Data are presented as means \pm SEM.

in BP, and plasma active and total renin levels was similar, with dose-dependent elevations 2 h after administration that had decreased by 24 h (Fig. 4A). In contrast, plasma renin activity (PRA), measured by the antibody trapping method, was completely inhibited 2 and 24 h after administration of aliskiren. Most methods for measuring PRA overestimate inhibition by renin inhibitors *ex vivo*, however, due to complete dissociation of the inhibitor from plasma proteins under *in vitro* assay conditions [32]. No grossly detectable adverse effects of aliskiren on the marmosets were observed at any time during the study.

Once-daily oral doses of aliskiren inhibit PRA and lower ambulatory BP in hypertensive human patients

The effects of aliskiren on 24-h ambulatory BP and PRA were assessed in eight patients with mild-to-moderate hypertension in an open-label, 8-week dose escalation study. Twenty-four hour ambulatory BP measurements were performed at baseline, and after 4 weeks of treatment with aliskiren, 75 mg once daily, and aliskiren, 150 mg

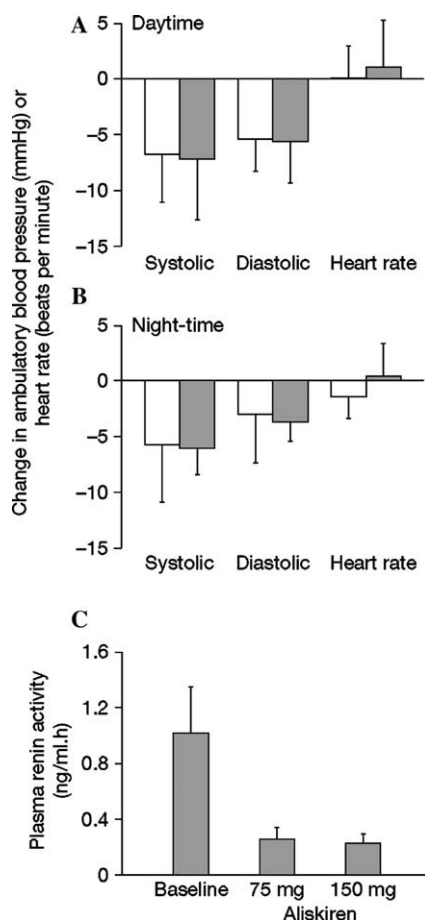


Fig. 5. Aliskiren effectively inhibits plasma renin activity and lowers blood pressure in hypertensive human patients. The effects of once daily oral doses of gelatin capsules containing aliskiren (9, hemifumarate salt), 75 mg for 4 weeks ($n = 8$), and aliskiren, 150 mg for 4 weeks ($n = 7$), in hypertensive human patients. (A, B) Mean changes in (A) daytime and (B) night-time ambulatory systolic and diastolic BP and heart rate after treatment with aliskiren, 75 mg (open bars; daytime, $n = 8$; night-time, $n = 7$) or 150 mg (filled bars; $n = 7$ for all). Values are presented as means \pm SEM. (C) Plasma renin activity at baseline ($n = 8$) and after treatment with aliskiren, 75 mg ($n = 7$) or aliskiren, 150 mg ($n = 7$). Values are presented as means \pm SEM. No data were available for one patient at the 75 mg dose.

once daily. Mean daytime systolic BP at baseline was 155 ± 11 (SD) mmHg and was lowered to 148 ± 14 mmHg after 4 weeks of treatment with aliskiren, 75 mg ($n = 8$). A further 4 weeks of treatment with aliskiren, 150 mg, administered to seven patients, resulted in a further reduction to 144 ± 18 mmHg. Reductions in mean daytime diastolic BP, and mean night-time systolic and diastolic BP, were observed (Figs. 5A and B). No significant changes in either daytime or night-time HR occurred during treatment with either dose of aliskiren. The reductions in BP paralleled PRA inhibition (Fig. 5C). Thus aliskiren, 75 mg for 4 weeks, reduced PRA to $34 \pm 7\%$ of baseline levels ($n = 7$), with 4 weeks of further treatment with aliskiren, 150 mg, giving an additional reduction to $27 \pm 6\%$ of baseline activity ($n = 7$).

Aliskiren was well tolerated in all patients, with no clinically significant changes in clinical chemistry or haematology. Adverse events were reported by four of the eight patients and were mild to moderate in intensity. Three patients had infections (urinary tract or respiratory). Reports of headache in two of the eight patients were present before the study started; one patient dropped out of the study at their own request after completion of the 75 mg regimen. One patient reported nausea during treatment with aliskiren, 75 mg.

Discussion

We employed a combination of molecular modelling and crystallographic structure analysis to design aliskiren, the first in a novel class of potent, orally effective non-peptide renin inhibitors. Aliskiren is a highly potent inhibitor of human renin, with an IC_{50} of 0.6 nM for in vitro inhibition in plasma. This compares favourably with previous generation renin inhibitors such as Ro 42-5892 (remikiren; $IC_{50} = 0.8$ nM), A-72517 (zankiren; 1 nM), and A-64662 (enalkiren; 14 nM) [7]. Aliskiren also shows high specificity for human renin, with almost no inhibitory effect against other aspartic peptidases such as cathepsin D and pepsin.

In contrast to all previous inhibitors, aliskiren is much more potent and long acting in vivo after oral administration; after once-daily oral dosing it effectively lowers BP over 24 h without altering HR in sodium-depleted marmosets and in hypertensive patients. The rapid rise in plasma immunoreactive renin levels observed following aliskiren administration is a well-known indicator of RAS inhibition [33], caused by removal of the normal feedback inhibition of Ang II on renin release [34]. Importantly, the rise in plasma renin levels did not compromise the ability of aliskiren to provide sustained PRA inhibition and BP lowering, in accordance with the sustained inhibition of Ang II production observed over an 8-day period with once-daily oral doses of aliskiren, 40–640 mg, in healthy humans [35].

Aliskiren effectively lowered BP in hypertensive patients in the open-label study reported here. BP measurements in humans were carried out using ambulatory BP monitoring, a technique that provides more accurate and reproducible results compared with discrete BP readings [36]. Notably, increasing the dose of aliskiren from 160 to 640 mg resulted in increased renin inhibition in healthy humans [35], hence even larger BP reductions may be anticipated with higher doses of aliskiren than those employed in our pilot study.

Poor patient compliance is a major problem with existing antihypertensive therapies, with more than half of patients typically discontinuing a new course of therapy within 6 months [37]. The antihypertensive class that exhibits the best adherence to therapy is the ARBs

[38], due to their placebo-like tolerability and once-daily oral dose regimen. It is therefore important to note that a recent study in 226 patients with mild-to-moderate hypertension showed that aliskiren, 300 mg once daily, lowered BP with similar potency and tolerability compared with the ARB losartan, 100 mg once daily (E. O'Brien, manuscript submitted).

In conclusion, aliskiren is a potent and selective inhibitor of human renin at sub-nanomolar concentrations. We have shown that once-daily treatment with oral doses of aliskiren is well tolerated, providing effective 24-h renin inhibition and BP lowering in hypertensive patients. Aliskiren therefore represents the first in a novel class of renin inhibitors discovered with the aid of molecular modelling, with the potential for highly effective treatment of hypertension and related cardiovascular diseases.

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