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REVIEW SERIES

New insights into the renoprotective actions of the renin inhibitor aliskiren in experimental renal disease

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The renin–angiotensin–aldosterone system (RAAS) has a central function in the regulation of blood pressure. Aliskiren, the first direct renin inhibitor to be approved for the treatment of hypertension, blocks the RAAS at its point of activation. As renin inhibition acts at the top of the RAAS cascade, this mechanism has been proposed to offer advantages over existing modes of RAAS blockade. The RAAS is also considered to be a major factor in the pathogenesis of many renal diseases, especially diabetic nephropathy (DN), the main cause of end-stage renal disease. Existing therapies to block the RAAS slow the progression of DN, but they do not halt the disease. Therefore, more effective modes of interventions are needed. Studies to determine the efficacy of aliskiren in human renal disease are in progress. This review summarizes *in vivo* studies in which the efficacy of aliskiren was tested in experimental models of renal disease, and presents *in vitro* studies that provide insights into the possible mechanisms by which aliskiren confers renoprotection in animals. These works are discussed in the framework of the intrarenal RAAS and suggest that aliskiren may act by unique renoprotective mechanisms.
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INTRODUCTION

The renin–angiotensin–aldosterone system (RAAS) is an ancient pathway[1] that has evolved into a central mechanism by which mammalian blood pressure (BP) and fluid homeostasis are regulated. Research in this field started with the discovery in 1898 by Tigerstedt and Berman[2] that a renal extract when injected into rabbits induced a rapid increase in systemic BP. Subsequent work over many years established the RAAS as a pivotal contributor to cardio-renal diseases. Hence, inhibitors of the middle and distal portions of the RAAS pathway, angiotensin-converting enzyme (ACE) inhibitors (ACEI) and AT_1 receptor blockers, respectively, have become mainstay therapies for treating hypertension. In 2007, aliskiren, the first direct renin inhibitor (DRI), was approved for the treatment of hypertension. This heralded the therapeutic control of BP by inhibiting the RAAS at its first and rate-limiting step. However, RAAS blockade is also effective for treating renal disease[3–6] and the efficacy of aliskiren for this purpose is currently being investigated. Accordingly, the subject of this review is to summarize the preclinical evidence for renoprotection by aliskiren, and to discuss recently published information on the possible mechanism(s) for these benefits. As it is well recognized that there is a tissue as well as circulating RAAS,[7] the preclinical actions of aliskiren will be discussed in the context of the intrarenal RAAS and the potential for its inhibition by aliskiren.

INTRARENAL RAAS

Goormaghtigh[8] first proposed that renin was produced just outside of the glomerulus in juxtaglomerular (JG) cells. However, work over the past several decades indicates a broader pattern of *intra*-glomerular RAAS expression. Indeed, the kidney possesses a fully functional tissue RAAS[9–11] with expression along virtually the entire nephron.

Gene and protein expression of angiotensinogen (Aogen), renin and ACE has been reported in cultured glomerular mesangial cells[12–24] and podocytes.[25–28] Mesangial expression of Aogen and ACE has also been documented in renal tissue sections.[19,23]

Mesangial cells synthesize Ang II[22,24] and aldosterone, the latter apparently through an Ang II-dependent mechanism.[29] Podocytes also produce Ang II[25,26,28] and aldosterone.[30] Angiotensin type 1 receptors are expressed on mesangial cells[19,24,31] and podocytes[26,28,32] and mineralocorticoid receptors are also expressed by both cell types.[29,33,34]

The likely importance of the local glomerular RAAS relates to the pathogenesis of glomerulosclerosis and the loss of glomerular permselectivity observed in many glomerular diseases. In cultured mesangial cells, Ang II[35–37] and aldosterone[38,39] provoke synthesis of extracellular matrix (ECM) proteins that accumulate in glomerulosclerosis. In podocytes, Ang II has been shown to induce apoptosis,[40,41] cytoskeletal rearrangement[42] and nephrin loss[43] in podocytes, alterations that have been linked to albuminuria.[44] Aldosterone can

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also damage podocytes by inducing apoptosis and reducing nephrin gene expression.[30,33,45] Thus, through autocrine or endocrine mechanisms, Ang II and aldosterone may induce damage and/or pro-fibrotic pathways in mesangial cells or podocytes, and thus contribute to glomerular damage.

Renal tubular cells express all of the components of the RAAS. Renin mRNA and protein are expressed in the proximal and connecting tubules, as well as the collecting ducts.[19,46–54] Interestingly, mechanisms for regulating tubular renin seem to be independent of those in the JG cell.[47,48,50,52] Proximal tubular cells express mRNA[54–56] and protein[53,57] for ACE and Aogen.[58–61] *In vivo* evidence that proximal tubular cells may synthesize Ang II has also been reported.[62]

The above studies indicate the presence of a functional renal tubular RAAS, which likely has an important function in regulating sodium and fluid balance, and hence BP. However, the tubular RAAS may also be linked to the pathogenesis of tubulo-interstitial fibrosis. For instance, Ang II induces epithelial-mesenchymal transformation in tubular cells,[63] a process thought to presage renal fibrosis.[64]

RENOPROTECTIVE EFFECTS OF RENIN INHIBITION WITH ALISKIREN

Aliskiren is a competitive inhibitor of renin; it binds to the active site of the enzyme and thereby prevents access to its substrate, Aogen. In turn, activation of the RAAS cascade is blocked. Given the above evidence that the primary and damaging effector molecule (Ang II) of the RAAS is made at many sites throughout the kidney, and that inhibition of renin blocks the RAAS at the first and rate-limiting step in Ang II formation, it is reasonable to propose that a DRI may provide distinct renoprotective benefits in a variety of renal diseases. The ensuing discussion will outline the evidence that the DRI aliskiren ameliorates renal damage in experimental renal disease. However, let us first consider some aspects of aliskiren that bear on its use in experimental settings.

Testing aliskiren in animal models

Certain important characteristics of aliskiren impact its use in animal models. First, because aliskiren is a human renin inhibitor, it is a less potent inhibitor of rodent renin than human renin[65] (Table 1). Thus, although rodent models of renal disease are very commonly used, models that express rat renin are not ideal for testing aliskiren. This issue can be circumvented by using a transgenic rat model with an activated RAAS. The so-called double transgenic rat[66] expresses the human genes for renin and Aogen, and thereby provides a model in which a human DRI can inhibit human renin in a rodent. A second model, the TG(mRen-2)[27] (mRen-2) rat expresses the mouse *ren-2* gene,[67] and takes advantage of the capacity of mouse renin to cleave rat Aogen. Aliskiren is effective in this model as the IC_{50} of the drug against mouse renin is relatively low (Table 1). The organ damage seen in dTGR[68] and mRen-2 rats[69] is ameliorated by RAAS blockade, giving relevance to testing aliskiren in these models.

Table 1 Inhibitory activity of aliskiren against human and rodent renin

Species	*IC_{50} (nM)*
Human	0.6
Rat	80
Mouse	4.5

IC_{50}: concentration of inhibitor that inhibits 50% of enzyme activity.
IC_{50} values: human, rat[65] and mouse.[87]

A second relevant property of aliskiren is its low oral bioavailability in rodents, in which oral dosing of this DRI does not result in good efficacy. Rather, consistent efficacy is seen when aliskiren is administered to rodents by subcutaneously implanted osmotic minipumps.

Aliskiren protects against experimental renal disease

Hypertensive renal disease. The first evidence for renoprotection by aliskiren was reported in dTGR. The activated RAAS in these animals leads to severe hypertension with renal damage, including albuminuria, elevated serum creatinine, renal infiltration of macrophages, and ECM deposition.[68,70] In this model, aliskiren (0.3 or 3 mg kg^{-1} per day) normalized the BP and reversed existing renal damage, evidenced by normalizing albuminuria and serum creatinine levels. Furthermore, glomerular collagen IV accumulation and renal macrophage and lymphocyte contents were lowered.[71] This paper also showed a reduction in renal cortical Ang I and Ang II levels in aliskiren-treated dTGR, thus documenting an inhibitory effect of this DRI on the intrarenal RAAS. Moreover, Ang I-forming capacity of the sera from these rats was also significantly reduced. A subsequent report by this group[72] showed that treatment with aliskiren inhibited complement activation and cellular infiltration in kidneys of dTGR. These studies showed the efficacy of aliskiren in high Ang II-induced renal injury and suggested a beneficial effect of the drug in complement-dependent renal disease. Notably, an earlier study of dTGR showed that when the renin inhibitor remikiren and a non-RAAS blocking anti-hypertensive therapy were compared at comparable BP-lowering doses, remikiren showed stronger renoprotection,[70] suggesting BP-independent effects of renin inhibition.

In both the above studies,[71,72] AT_1 receptor blockers were included as positive controls; they were not intended to be comparator agents. These drugs were administered at different dosages and by different routes of administration than aliskiren. Although comparable renoprotective effects were observed with aliskiren and its comparator in each of these studies, conclusions on comparative efficacy *vs.* aliskiren could not be made.

In this regard, we tested in mRen-2 rats the effects of 4 weeks of treatment with aliskiren or enalapril, both administered through osmotic minipumps to achieve constant plasma levels of each drug, at doses that achieved comparable tail cuff BP control (Figure 1a) (Avigdor, Hu, Jen, and Feldman, unpublished data). Compared with vehicle controls, albuminuria was reduced similarly by both therapies (Figure 1b), as were plasma levels of Ang II and aldosterone (Table 2) at the end of the experiment. This study indicated that when drug delivery and BP lowering were similar, aliskiren induced comparable reductions in albuminuria and blockade of the circulating RAAS, including lowering plasma aldosterone.

Endothelial nitric oxide synthase (eNOS) regulates renal hemodynamics and renal function.[73] Deficiencies in eNOS lead to accelerated renal damage in models of renal disease,[74] highlighting the importance of nitric oxide in renal health. Recently, aliskiren was shown to inhibit the renal disease that develops in eNOS$^{-/-}$ mice.[75] Increases in albuminuria, glomerulosclerosis and renal macrophage infiltration observed in the vehicle-treated knockout mice were significantly reduced by aliskiren treatment (25 mg kg^{-1}), as were levels of glomerular superoxide and renal NADPH oxidase. Importantly, hydralazine given at a dose that lowered tail cuff BP similarly to aliskiren did not show these beneficial effects. This work showed that the renoprotective actions of aliskiren do not require eNOS-mediated pathways. Moreover, this protection seems to result at least partially from effects that are beyond BP control, possibly by inhibiting Ang II-induced damaging effects in the kidney.

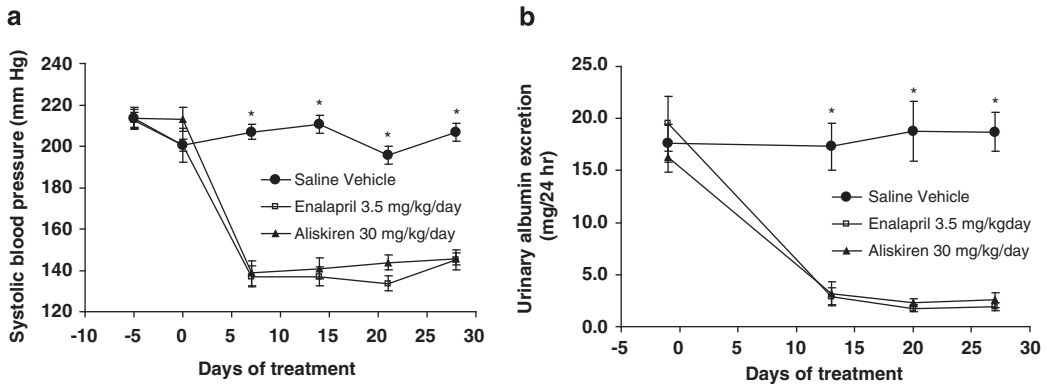

Figure 1 Effect of aliskiren and enalapril on blood pressure (**a**) and albumin excretion (**b**) in mRen-2 rats. mRen-2 rats were treated with aliskiren or enalapril by subcutaneous osmotic minipumps. Blood pressure was measured by the tail cuff method and urinary albumin levels were measured by ELISA. $^{*}P<0.05$ *vs.* vehicle.

Table 2 Effect of aliskiren enalapril on plasma Ang II and aldosterone levels in mRen-2 rats

		Plasma	
Group	N	*Ang II (pg ml^{-1})*	*Aldosterone (nM)*
Sprague–Dawley Mollegard	8	11.06 ± 0.55*	0.59 ± 0.10*
mRen-2+vehicle	6	25.3 ± 2.84**	1.32 ± 0.19**
mRen-2+enalapril *3.5 mg kg^{-1} per day*	8	6.9 ± 0.57	0.43 ± 0.04
mRen-2+aliskiren *30 mg kg^{-1} per day*	8	7.8 ± 1.01	0.51 ± 0.09

$^{*}P<0.05$ *vs.* all mRen-2 groups and $^{**}P<0.05$ *vs.* other mRen-2 groups. Plasma was sampled at the end of the experiment; values are ± s.e.m.

The anti-inflammatory effect of aliskiren observed in the kidneys of dTGR[71] and eNOS$^{-/-}$ [75] mice is not a renal-specific effect. Recently, Ino and coworkers[76] reported that at a non-BP-lowering dose, aliskiren reduced leukocyte adhesion in a murine vascular injury model. Furthermore, this effect of aliskiren was associated with a reduction in the injury-induced up-regulation of adhesion molecules on the vascular endothelium, pointing toward a possible mechanism for the reductions in inflammatory infiltrates noted above in the kidneys of dTGR[71] and mice.[75]

Diabetic nephropathy. Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the developed world.[77] The RAAS is thought to have a central function in the pathogenesis of this disease, evidenced by numerous studies showing that RAAS blockade by ACEIs and AT_1 receptor blockers slows the progression of DN.[3–6] However, these treatments do not halt the progression of this disease. Thus, the underlying mechanisms for progression of DN continue even in patients treated with accepted therapies. The intrarenal RAAS is thought to be activated during diabetes, based on *in vitro* and *in vivo* evidence.[21,22,24,25,48,54,55,69,78] Therefore, the effect of renin inhibition in diabetic conditions is of great interest.

In vitro effects of aliskiren in high glucose conditions. Albuminuria is the first clinically detectable change in renal function in DN, resulting from a reduction in permselectivity of the glomerular capillary wall. The glomerular permeability barrier consists of three layers, the outer most glomerular visceral epithelial cells (podocytes) that cover the (middle) glomerular basement membrane, which is lined on its luminal side by the inner layer, the fenestrated endothelium. Podocytes seem to have a key function in preventing the escape of plasma proteins across the glomerular capillary wall into the urine. Their involvement in the development of albuminuria and glomerulosclerosis in DN is increasingly accepted.[44,79]

During diabetes, podocytes are exposed *in vivo* to high glucose levels and stretch forces, the latter because of glomerular hypertrophy and hyperfiltration. When these conditions are modeled *in vitro* in mouse podocytes, the RAAS becomes activated. Indeed, podocytes subjected to mechanical stretch show Ang II-mediated apoptosis.[41] Aliskiren was tested for its effects on cultured mouse podocytes exposed to high glucose levels.[25] In such podocytes, high glucose induced increased expression of renin mRNA and protein, and Ang II formation was detected. The latter was not inhibited by the ACEI captopril, but it was suppressed by aliskiren and the non-selective chymase inhibitor chymostatin. These findings suggest that high glucose conditions activated the podocyte RAAS at the level of renin, leading to increased Ang II formation. If we can assume that captopril actually entered the podocytes in this study, the results further indicate that Ang II formation in these cells was mediated primarily by non-ACE pathways (that is, chymase). To the extent that the up-regulation of glomerular chymase in diabetic kidneys[18] may suggest a function for ACE-independent Ang II formation in DN, renin inhibition may have a protective advantage over ACEI in this disease. Thus, these *in vitro* results may be an example in which blocking the RAAS at the top of the cascade (that is, at renin) may achieve different outcomes than by inhibiting elsewhere in the pathway.

The loss of podocytes from the glomerular capillary wall is associated with the development of albuminuria[80] and glomerulosclerosis,[81] and apoptosis of podocytes has been suggested as a mechanism for such loss.[44] Phillips and coworkers[82] reported that aliskiren attenuated the high glucose-induced increase in cleaved caspase-3 in cultured mouse podocytes, suggesting a protective effect of aliskiren against podocyte apoptosis in a high glucose milieu. Moreover, these authors showed that aliskiren inhibited the high glucose-induced increase in fibronectin mRNA and protein in these cells.

The above two *in vitro* studies in podocytes have potential clinical significance because they suggest that aliskiren can induce effects that relate directly to protecting the glomerular filtration barrier. Aliskiren has a molecular weight (551.8) small enough to be filtered by the glomerulus, and indeed the drug appears in the urine.[83] Thus, *in vivo* exposure of podocytes to aliskiren is predicted. As podocytes express a functional RAAS,[25–28] renin inhibition in these cells by aliskiren may

provide a cellular mechanism (that is, podocyte protection) for the anti-albuminuric effect observed in human beings[84,85] as well as animals.[71,75,86–88]

In vivo effects of aliskiren in experimental DN. Transgenic mRen-2 rats with superimposed STZ-induced diabetes develop albuminuria, glomerulosclerosis and tubulo-interstitial fibrosis,[69] similar features as in human DN. Aliskiren (10 mg kg^{-1} per day) and the ACEI perindopril (0.2 mg kg^{-1} per day) were compared for their renoprotective effects in this model.[88] The chosen dose of perindopril lowered tail cuff BP 36 mm Hg more than in aliskiren-treated STZ-mRen-2 rats. However, despite this difference, aliskiren attenuated albuminuria and glomerulosclerosis to similar degrees as perindopril. Interestingly, levels of albuminuria correlated well with BP in vehicle- and perindopril-treated mRen-2 rats, but not in aliskiren-treated rats. Moreover, histological evaluation showed that aliskiren conferred significantly greater protection against tubulo-interstitial fibrosis than perindopril. These two differential effects of aliskiren *vs.* perindopril may reflect a BP-independent renoprotective effect of aliskiren, possibly by inhibiting the intrarenal RAAS to a greater extent than the ACEI.

In another study with diabetic mRen-2 rats in which BP was monitored continuously by telemetry, aliskiren at 10, 30 and 60 mg kg^{-1} per day dose dependently reversed existing hypertension and prevented the development of albuminuria over the 10 weeks study.[87] At the end of this study, renal cortical gene expression of collagen I and the pro-fibrotic growth factor, transforming growth factor-β (TGF-β), in the mRen-2 rats were significantly reduced in the aliskiren *vs.* the vehicle group.

In this experiment, treatment of diabetic mRen-2 rats with the high-dose (60 mg kg^{-1} per day) aliskiren was discontinued about 3 weeks into the study, but the animals were monitored for the remainder of the 10 weeks experiment (Webb, Zhou, Feldman, unpublished data). Withdrawal of aliskiren treatment was accompanied by an increase in BP and the development of albuminuria. The rise in BP was gradual, taking about 10 days to reach vehicle-control levels. Moreover, at the end of 10 weeks, gene expression of renal cortical TGF-β was still suppressed, despite stopping treatment with aliskiren about 7 weeks earlier. It is unknown whether this suppression of TGF-β reflects a continued presence or persistent effect of aliskiren in the kidneys, or whether RAAS inhibition early in the development of the hypertensive phenotype of this model confers long-term effects on growth factor expression.

Aliskiren has also shown renoprotective effects in db/db mice, a model of type 2 diabetes.[86] In this study, treatment with aliskiren ameliorated the albuminuria and glomerulosclerosis seen in the vehicle-treated mice. These benefits were accompanied by reductions in expression of TGF-β, collagen IV and nephrin, as well as p22phox and NADPH oxidase activity.

Collectively, the above *in vitro* and *in vivo* studies suggest that the functional and structural renoprotection conferred by aliskiren in experimental renal disease may be explained by inhibition of the intrarenal RAAS, which may result in BP-independent renoprotective mechanisms (for example, anti-inflammatory, anti-oxidant) that reduce ECM accumulation and possibly mitigate against cellular (for example podocyte) damage. These studies also provide hints of persistent renoprotective effects of this DRI.

POTENTIAL NOVEL MECHANISMS OF ALISKIREN'S RENOPROTECTIVE ACTIONS

Given that aliskiren blocks renin, it is not surprising that this drug has renoprotective effects in the experimental settings described above. Rather, the intriguing question is: 'does a DRI, by blocking the RAAS at the first and rate-limiting step, impart a specific renoprotective mechanism?' New evidence is emerging that suggests this may be the case.

Aliskiren localizes in the kidney

The kidney is the main source of renin; this organ contains a fully functional RAAS distributed over virtually all regions of the nephron, as described earlier. Consequently, as this pathway has been linked closely with renal damage, a distinct renoprotective advantage is expected from blocking the intrarenal RAAS. For a drug to inhibit the intrarenal RAAS and protect against organ damage, an obvious requirement is that it partitions to an appropriate renal compartment. On the basis of the experiments discussed below, this requirement seems to be met by aliskiren.

Sprague–Dawley rats were treated with 10 mg kg^{-1} per day aliskiren for 2 weeks by osmotic minipumps, and their plasmas and kidneys were studied for the presence of the drug. Renal levels of aliskiren were 46-fold over plasma levels, indicating that renal localization of aliskiren occurred, but was not due simply to equilibration from the plasma.[87] Other renin inhibitors have shown a similar renophilic property.[89] Although this study showed that aliskiren partitions to the kidney, it did not indicate the renal compartment in which aliskiren localized.

Therefore, in another experiment, the renal topographical localization of aliskiren was explored by administering ^{14}C-aliskiren intravenously to normotensive rats and harvesting their kidneys 2 h afterward. By light microscopy, cryostat sections revealed heavy autoradiographic labeling in 100% of glomeruli on each renal section, indicating extensive partitioning of aliskiren throughout these structures[87] (Figure 2a). Moreover, images suggestive of the presence of aliskiren in JG cells of the afferent arterioles were obtained, although such localization was not identified conclusively in these relatively thick cryostat sections. However, the possibility that aliskiren can enter vascular structures was shown clearly by the presence of label in larger caliber intrarenal arteries in these kidneys (Figure 2b), and preliminary evidence from recent studies suggests that in fact, aliskiren may localize in the afferent arterioles (Feldman *et al.*, paper in preparation). However, in contrast to glomeruli, autoradiographic evidence for the presence of aliskiren in the tubulo-interstitium was not observed within the short duration of exposure in this experiment.[87]

The above studies show that aliskiren partitions to the kidneys and localizes in the renal vasculature, possibly in structures known to contain renin. Moreover, extensive partitioning of aliskiren to the glomeruli suggests that it can access mesangial cells and podocytes, potentially inhibiting the RAAS in these cells and conferring structural and functional renoprotection observed in rodents.[71,75,86–88] Light microscopic studies with labeled aliskiren have not been conducted in disease models in which changes in (extraglomerular) vascular permeability may facilitate permeation of the drug to the tubulo-interstitium, with potential access to tubular cells and fibroblasts. However, it seems reasonable to speculate that this may occur, and it may have relevance to the extent that enhanced expression of tubular renin may be linked to the development of tubulo-interstitial fibrosis.[47]

Not only does aliskiren partition to the kidneys in rats, its renal presence is prolonged. Two lines of evidence support this concept. First, dTGR were treated with aliskiren (3 mg kg^{-1} per day) for 2 weeks, during which time BP (tail cuff) and albuminuria were significantly reduced *vs.* vehicle controls. After a 3-week washout period, renal and plasma levels of aliskiren were measured. Aliskiren

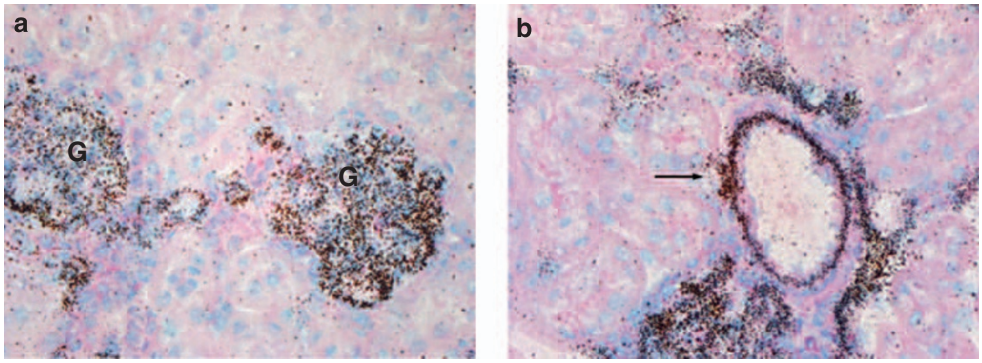

Figure 2 Autoradiographic localization of aliskiren in rat kidneys. Hanover–Wistar rats were injected intravenously with ^{14}C-aliskiren and 2 h later the kidneys were removed for autoradiography. (**a**) Two glomeruli (G) show extensive autoradiographic labeling, indicating the presence of aliskiren. (**b**) A renal cortical artery (arrow) shows extensive autoradiographic labeling in vascular wall. (Reproduced with permission from *Lippincott Williams & Wilkins.*[87])

was not detectable in the plasma from these rats, but drug levels in the kidney were still above the IC_{50} for inhibiting human renin.[90] Second, Vaneckova and coworkers[91] treated mRen-2 rats with aliskiren (10 mg kg^{-1} per day) for 4 weeks, during which time BP (telemetry) was normalized. At the end of this treatment period, plasma and renal Ang II levels were lowered to those in non-hypertensive vehicle controls, confirming the observations in dTGR[71] that treatment with aliskiren reduces renal Ang II content. At this time, aliskiren treatment of mRen-2 rats was stopped for 12 days washout. Importantly, after the washout period, plasma and renal Ang II levels were still reduced to vehicle-control levels.

Taken together, the above studies showed that aliskiren partitions to the kidney and exhibits a prolonged renal residence. These studies also suggest that aliskiren inhibits the intrarenal RAAS, and that such inhibition is enduring. In view of the growing evidence that 24-h BP control predicts better outcomes for target organ protection,[92–94] such long-acting inhibition of the intrarenal RAAS may be especially important in conditions in which this pathway is activated, such as believed to occur in DN.[78]

Aliskiren may bind to intrarenal renin

The autoradiographic images discussed above[87] provoke questions regarding the intracellular fate of aliskiren. Whether labeled aliskiren found in the kidneys of rats represents renin-bound inhibitor is an important issue. It seems doubtful that all of the labeled aliskiren observed in glomeruli in these studies was bound to renin: it is highly unlikely that so much renin was present in the glomeruli of the normotensive rats used in this study.[69,95,96] Moreover, it seems similarly implausible that exposure to aliskiren for 2 h in normotensive rats could have induced recruitment of such high levels of glomerular renin, as has been shown in pre-glomerular vessels after chronic RAAS blockade,[97] especially as aliskiren is a relatively weak inhibitor of rat renin (Table 1). However, some of the label seen in glomeruli may have reflected aliskiren bound to renin that possibly was present in the mesangial matrix. In addition, as aliskiren can penetrate cultured cardiomyocytes,[98] the drug may have entered mesangial cells and podocytes, both of which express renin[20,24–26] to which aliskiren could bind. Furthermore, the unequivocal evidence for labeled aliskiren in the walls of renal vessels prompts the proposal that this DRI can enter JG cells, and possibly incorporate into forming or formed renin granules. Recent *in vitro* studies provide evidence for the latter possibility.

Cultured JG cells do not store renin,[99] making them unsuitable for studying the intracellular renin-binding capacity of aliskiren. However, a human mast cell line synthesizes and secretes (pro)renin.[100] Krop and coworkers[101] used these cells to ask whether aliskiren can incorporate into renin granules. They found that mast cells that had been incubated for 7 days with aliskiren secreted renin that displayed inhibited enzyme activity (that is, inhibited Ang I-forming capacity). These data show that aliskiren can access intracellular renin, bind or incorporate into renin granules, and remain associated with the enzyme through the secretory process. Similar results were seen with prorenin, which is constitutively released from HMC-1 cells.[102] This study lays the groundwork for understanding the intracellular fate of aliskiren. Moreover, the implications from this work and the autoradiographic data are that aliskiren may provide an unusual example in which an inhibitor incorporates into its (still intracellular) target even before the latter is secreted. In this case, (pro)renin would be secreted from JG cells to the plasma or the tubulo-interstitium[103] in an already inhibited state. The renin activity in this (pro)renin-aliskiren complex would be inhibited: this renin (or prorenin, which may undergo activation at tissue sites) could not cleave Aogen, and the tissue and systemic RAAS cascades would not be activated. As this mechanism should operate independently of plasma drug levels,[101] it may help to explain the prolonged BP benefits observed in rats discussed earlier, and in patients in whom aliskiren treatment has been stopped.[104]

Aliskiren, prorenin and the (pro)renin receptor

The recent discovery of a receptor for renin and prorenin, the (P)RR,[105] has added an important new dimension to the tissue RAAS.[106–108] In the kidney, gene and/or protein expression of the (P)RR has been reported in the glomerular mesangium,[109] vascular wall[87,109] and tubular cells.[87,110] Gene and protein expression for the (P)RR has also been reported in podocytes.[111–113]

Stimulation of the (P)RR initiates Ang II-dependent and -independent effects. The former include a gain in catalytic activity for renin bound to the (P)RR (*vs.* unbound renin). This effect amplifies the formation of Ang II at the cell surface.[109] Furthermore, on binding to the (P)RR, prorenin, which is normally inactive, becomes catalytically active (it can cleave Aogen to form Ang I), possibly because of a conformational change induced by the binding.[109,114–116] The Ang II-independent effects of (P)RR stimulation include activation of extracellular signal-regulated kinase1/2,[109,117,118] and heat shock protein 27[119] and phosphatidylinositol-3 kinase p85α[120] pathways. The production of TGF-β and plasminogen-activator-1 is also increased, as are gene expression and production of collagen I and fibronectin, respectively.[117,121] Thus, through the (P)RR, renin and

prorenin can induce direct effects on cells. Continuing work on this receptor has led to the isolation and characterization of a soluble form of the (P)RR,[111] the significance of which remains to be elucidated.

As the (P)RR can activate prorenin, it has been suggested that this receptor may have an important function in the activation of prorenin at tissue sites, with consequent Ang II-mediated tissue damage.[122] Prorenin is inactive because a 43 amino-acid prosegment covers the enzyme's active site. Incubation of prorenin with a renin inhibitor under the appropriate conditions permits access of the inhibitor to the active site of prorenin, while the prosegment, in equilibrium between 'covering' and 'lifted' conformations, is lifted from its position covering the active site.[123] *In vitro* studies showed that when incubated with prorenin, aliskiren binds to the active site of the enzyme[87] and can inhibit Ang I formation that would normally occur from non-proteolytic [(P)RR-mediated] activation of prorenin.[116] This finding is significant because activation of prorenin at tissue sites might occur by (P)RR-mediated mechanisms or by proteolytic cleavage of the prosegment. The latter may occur in tissues in which inflammatory or dead/dying parenchymal cells may release proteases such as cathepsin G or elastase,[124] which are capable of cleaving the prosegment. In either case, the Ang I-forming capacity of prorenin and the subsequent formation of Ang II (in the presence of ACE or chymase) would be blocked by bound aliskiren. The ability of aliskiren to inhibit prorenin activation has additional relevance because circulating levels of prorenin are about 10-fold those of renin.[125] This represents high potential renin activity. In addition, plasma prorenin levels can be substantially elevated in diabetes[126] and are strongly associated with microvascular complications of this disease.[127] Thus, to the extent that activation of prorenin at tissue sites may contribute to local Ang II formation and tissue damage, aliskiren may provide tissue-protective effects. However, it must be noted that the concept of prorenin-induced tissue injury is not fully established; in two models of prorenin over-expressing mice, cardio-renal damage was not observed.[128,129]

The potential clinical significance of the (P)RR is highlighted in a set of studies by Ichihara and coworkers (reviewed elsewhere[130]) that suggest a central function for this receptor in the pathogenesis of DN. This body of work is particularly provocative because the authors have reported dramatic benefits in STZ-diabetic rats with a putative (P)RR blocker. However, attempts by other investigators to reproduce the cellular effects of the blocker, and its *in vivo* benefits (although in non-diabetic models of renal damage), have largely been unsuccessful.[115,118,131,132] Replication by others of the experimental designs used by Ichihara and coworkers may clarify some of the questions on the function of the (P)RR in DN and in tissue damage in general.

What is the effect of aliskiren on the function of the renal (P)RR? Aliskiren does not inhibit binding of (pro)renin to the (P)RR,[87,118] nor does it prevent (pro)renin-induced cell signaling.[87,118,120] However, in STZ-diabetic mRen-2 rats treated with aliskiren for 10 weeks, *in situ* hybridization on renal sections revealed a clear reduction in gene expression of the (P)RR in the glomeruli and tubular cells compared with vehicle-control diabetic mRen-2 rats.[87] Interestingly, *in vitro* studies showed that aliskiren did not affect gene expression of (P)RR in mesangial cells. These data suggest that aliskiren can

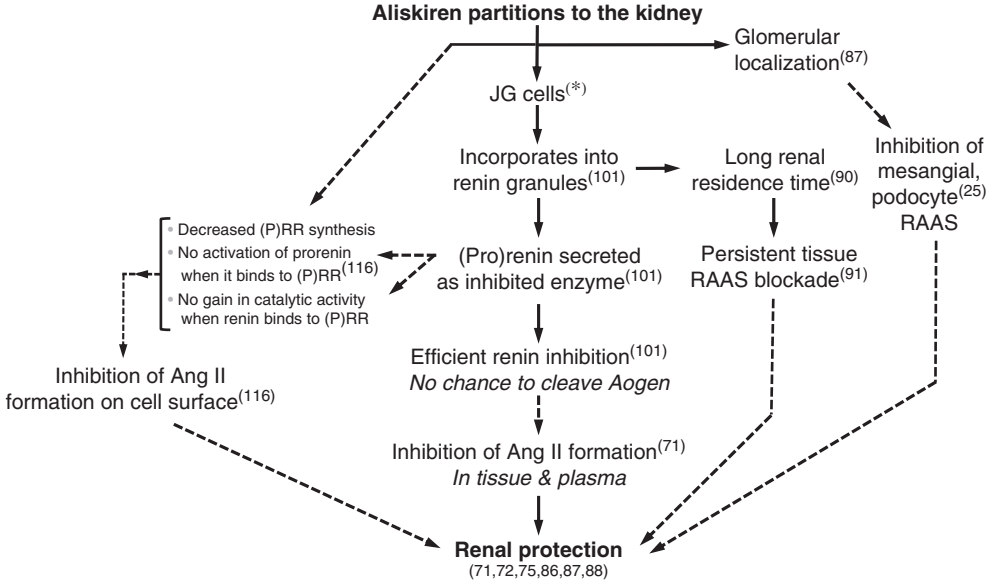

Figure 3 In this working model of the renoprotective actions of aliskiren, outcomes for which *in vitro* or *in vivo* evidence exists are referenced (see References); no reference indicates a logical, but still speculative outcome. Solid arrows indicate the existence of *in vitro* or *in vivo* evidence to support a causal link between the events. Dotted lines denote a logical, but as yet, unproven causal link between events. * indicates Feldman DL *et al.*, manuscript in preparation. Once aliskiren localizes in the kidney (for example, JG cells), it may incorporate into renin granules or prorenin and stay in these cells until released with (pro)renin. This renal localization would lead to a longer renal residence time than if unbound, and ultimately would lead to prolonged local RAAS blockade. Aliskiren that deposits in the glomeruli may inhibit mesangial and podocyte renin, inhibiting Ang II-mediated glomerular ECM accretion and loss of permselectivity. Renin or prorenin bound to aliskiren in JG cells would be released as inhibited enzymes and would theoretically have no abilities to cleave Aogen. The formation of Ang II would be inhibited, maintaining a quiescent local (tissue) and systemic RAAS. If aliskiren reduces synthesis of the (P)RR as a result of suppressing its gene expression, lower receptor density on the cell surface would be expected, reducing the opportunity for (pro)renin to bind to diminished numbers of receptors. Once released from JG cells, inhibited renin could still bind to the (P)RR, but could not gain the amplified catalytic activity that has been described after such binding. Similarly, prorenin released as bound to aliskiren could not become activated by proteolytic or non-proteolytic mechanisms. These actions on renin and prorenin would reduce Ang II formation at the cell surface. Extrarenal tissues that take up already inhibited renin would also theoretically derive protection from these mechanisms. Finally, in addition to the mechanisms described here, renoprotection would also derive from aliskiren binding to (pro)renin in the plasma.

suppress mesangial cell gene expression of the (P)RR *in vivo*, but probably not through a direct cellular effect. Regardless, if this reduced gene expression also leads to lower (P)RR density at the cell surface, a reduction in Ang II-dependent and -independent effects could be expected from administration of aliskiren. This topic deserves more investigation.

In addition to suppressing gene expression of the (P)RR, treatment of diabetic mRen-2 rats with aliskiren also led to a reduction in renal cortical mRNA abundance for TGF-β and collagen I.[87] It is tempting to invoke a causative relationship between reductions in gene expression of the (P)RR, TGF-β and collagen I in the above study. Indeed, elegant *in vitro* experiments by Huang and coworkers[18] established a strong link between renin-induced production of TGF-β and ECM production in cultured mesangial cells, whereas blockade of the (P)RR with siRNA significantly inhibited this response. However, a similar causative conclusion from the *in vivo* study[87] awaits a more robust proof.

Working model of renoprotective mechanism of action of aliskiren

From the data presented heretofore, it is possible to propose a working model for the mechanism(s) by which aliskiren protects the kidneys in experimental settings (Figure 3). Although this model is necessarily speculative, it provides testable hypotheses.

Summary

Although the idea to inhibit renin as a means of blocking the RAAS originated from studies that were conducted over 100 years ago,[2] it is only recently that the effects of inhibiting this enzyme have been studied in depth. There is still much to learn about the mechanism of action of renin inhibition, and how it differs from those of ACEI and AT_1 receptor blockers. The possibility that aliskiren binds to intracellular (pro)renin, leading to release of already inhibited enzyme seems to be a novel mechanism. However, it still must be documented that this occurs *in vivo*, and in fact that such a mechanism confers a benefit for organ protection. Toward this end, preclinical studies offer the means by which aliskiren's mode of action can be revealed more fully, thus generating hypotheses that can be tested in patients.

CONFLICT OF INTEREST

David L Feldman is a full-time employee of Novartis Pharmaceuticals.

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