The direct renin inhibitor aliskiren localizes and persists in rat kidneys

David L. Feldman,1 Liang Jin,1 Hong Xuan,1 Elke Persohn,1 Wei Zhou,1 Helmut Schuetz,1 Joon-Keun Park,2 Dominik N. Muller,3 and Friedrich C. Luft3

1Novartis Institutes for Biomedical Research, East Hanover, New Jersey; 2Clinic for Nephrology and Hypertension, Hannover Medical School, Hannover, Germany; and 3Experimental and Clinical Research Center, Charité Medical Faculty and the Max-Delbrück Center for Molecular Medicine, Berlin, Germany

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The direct renin inhibitor aliskiren localizes and persists in rat kidneys. Am J Physiol Renal Physiol 305: F1593–F1602, 2013. First published August 7, 2013; doi:10.1152/ajprenal.00655.2012.—The aims of this study were to 1) determine whether renal localization of aliskiren and its antihypertensive and renoprotective effects persist after administration of the drug is stopped and 2) define the renal localization of aliskiren by light microscopy autoradiography. Hyper-tensive double transgenic rats (dTGR) overexpressing genes for human renin and angiotensinogen were treated with aliskiren (3 mg-kg−1-day−1 sc; osmotic minipumps) or enalapril (18 mg/l in drinking water). After a 2-wk treatment, dTGR were assigned to either continued treatment with aliskiren (“continued”), or to cessation of their respective treatment (“stopped”) for a 3-wk washout. One week of treatment with aliskiren and enalapril reduced blood pressure and albuminuria vs. baseline. After cessation of either treatment, blood pressure had returned to pretreatment levels and albuminuria remained relatively low for 1 wk, but rose thereafter similarly in both groups. In contrast, renal mRNA for transforming growth factor-β and renal collagen IV was reduced by aliskiren (continued and stopped groups), but not after cessation of enalapril. Similar patterns were found for collagen IV protein expression. Even 3 wk after stopping aliskiren treatment, renal levels of the drug exceeded its IC50, whereas enalaprilat was not detected. To localize aliskiren accumulation, Wistar rats were treated with [3H]-aliskiren for 7 days. Autoradiography demonstrated specific labeling in glomeruli, arterioles, and afferent arterioles as well as in the distal nephron. Labeling could still be observed even after 7 days’ washout. These results suggest that the renophilic properties of aliskiren are different from enalapril and could have contributed to the renoprotective mechanism of this renin inhibitor.

aliskiren; renin; dTGR; kidney

THE DIRECT RENIN INHIBITOR (DRI) aliskiren displays antihypertensive (14) and antialbuminuric (15) effects in humans and in animal models (7, 17, 19) even after treatment is discontinued (19). This persistent effect of aliskiren could result from the renophilic nature of this drug (7). Since the site of synthesis of most circulating renin is the kidney (4), localization of a renin inhibitor to renin-producing renal cells might permit inhibition of renin at an early stage of the enzyme’s life cycle and thereby facilitate effective blockade of the renin-angiotensin-aldosterone system (RAAS).

Double transgenic rats (dTGR) overexpressing the human renin and human angiotensinogen genes exhibit high ANG II levels in the circulation as well as in target organs such as the heart and the kidney (12). Untreated dTGR develop hypertension, albuminuria, and cardiac hypertrophy and die between weeks 7 and 8. Since the IC50 of aliskiren against human renin (25) is much lower compared with rat and mouse renin (7, 25), the humanized TGR are highly suitable for testing the renoprotective actions of this DRI.

The current work was undertaken with two purposes. First, we sought to determine in dTGR whether aliskiren is retained in the kidney after administration of the drug is stopped and to what extent its antihypertensive and renoprotective effects persisted during this post-treatment residence. Second, toward unveiling possible mechanisms of the anticipated renal retention of aliskiren, we investigated the renal localization of this DRI by light microscopy autoradiography (LMAR) after various times following administration to rats.

METHODS

General Procedures

Animals. All procedures involving animals were conducted in compliance with the standards of the Novartis Animal Care and Use Committee. Male dTGR were obtained from the Research and Consulting Company (RCC; Fullsindorf, Switzerland) and subsequently bred at Novartis. Animals were about 5.5 wk old at the start of the study. This age was chosen to avoid the presence of existing renal disease at the start of the study. Male Wistar rats (RCC and Taconic Farms, Germantown, NY) were 6–8 wk old at the time of use.

In Vivo Procedures

Administration of aliskiren by minipumps. Aliskiren was administered via subcutaneously (sc) implanted osmotic minipumps (model 2004, Alzet, Bacoaville, CA), which were implanted while rats were anesthetized with 2.5% isoflurane. Treatment was ceased by removing the minipump under isoflurane anesthesia.

Measurement of blood pressure, urinary albumin excretion, and plasma creatinine in dTGR. Systolic blood pressure (BP) was measured by the tail-cuff method using a 24-channel blood pressure system (MOD 56–1, ITTC Life Science, Woodland Hills, CA). BP was measured between 10:00 and 11:00 a.m. following each overnight urine collection. For each rat, three to four BP measurements per session were taken, and a mean value was computed.

Plasma creatinine levels from terminal blood samples were measured using a Hitachi 917 Autoanalyzer. Urine was collected over 24 h from rats in metabolism cages with free access to food and water. Urinary albumin concentration was measured with a competitive ELISA using a sheep anti-rat albumin capture antibody and horseradish peroxidase-conjugated sheep anti-rat IgG (both from Bethel Laboratories, Montgomery, TX).

Terminal procedures

At autopsy, rats were anesthetized with isoflurane and blood was withdrawn from the inferior vena cava (study 1) or by puncturing the heart or sublingual vein (study 2). Plasma was prepared and stored at...
−70°C until assayed for respective parameters. The left kidney was removed, deparaffinized, immediately snap frozen in liquid nitrogen, and stored at −70°C.

**Blood and tissue processing for drug analysis in dTGR.** Measurement of aliskiren and enalaprilat concentrations in plasma and kidney homogenates was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The lower limit of quantification of aliskiren and enalaprilat in plasma and kidney homogenate was 1.00 ng/ml.

Renal gene expression in dTGR. For analysis of renal gene expression by quantitative real-time RT-PCR, total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA) and transcribed with TaqMan 2X universal PCR master mix (catalog no. PN 4304437, Applied Biosystems, Carlsbad, CA). The reaction was conducted in an ABI 7900 sequence detection system (Foster City, CA). Messenger RNA expression was standardized to 18s rRNA, and the comparative CT method (ΔΔCT) was used for relative quantification of gene expression.

Primers and probe sets for rat TGF-β (Applied Biosystems) were as follows: forward: CATGACATGAGCCAGAGGCTTTC; reverse: CAGTGTGGAGGGCTTTCACA; and FAM probe: TGTCCTGGACTGGC-CACCCCC. Rat collagen IV primers and probe sets (Sigma-Genosys, The Woodlands, TX) were as follows: forward: CCAAGAGGT-TATTGGGGAGA; reverse: GACAGAGAACAGAGGGCACA; and FAM probe: TCTGCAGACCTGGTCTCCC.

**Light Microscopy Autoradiography and Plasma Radioactivity**

Cryostat sections of kidneys (~10 µm thick) were placed on slides previously dipped in ILFORD K5 emulsion (no. 1355127) and stored in the dark at 4°C. The sections were exposed and then developed, fixed, and finally stained with methyl green-pyronin.

Specific radiolabeling by light microscopy was identified by more silver grains over the cells than the background without tissue. Labeling was graded minimal (1), slight (2), moderate (3), marked (4), or striking (5). In these sections, afferent arterioles were defined by the thicker vascular wall and longer, straighter length compared with efferent arterioles. Distal convoluted tubules were distinguished from proximal convoluted tubules by the absence of a brush border, the presence of a larger more clearly defined lumen, more nuclei per cross section, and pale cytoplasm in the former. In addition, profiles of distal tubule are less numerous than proximal tubules since the former is a shorter segment (26).

Radioactivity in plasma samples was determined from duplicate plasma samples (~0.15 ml each) by scintillation counting with IRGA-SAFE-LUS (Packard BioScience, Groningen, The Netherlands). Radiometry was performed in Liquid Scintillation System 2700 TR (Packard Instrument, Meriden, CT).

**Immunohistochemistry**

Renin in kidneys from aliskiren-treated Wistar rats. Renal sections fixed in Bouin’s solution were processed for routine paraffin embedding. Paraffin sections (4 µm thick) were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide diluted in TBS (50 mmol/l Tris-HCl, 154 mmol/l NaCl, pH 7.4) for 30 min at 25°C. Nonspecific antibody binding was blocked by incubation with a solution of 5% skim milk powder in TBS for 1 h at 25°C. The sections were incubated with rabbit polyclonal antibody to human renin (lot III, 1:200, a noncommercial antibody) in TBS containing 5% Carnation milk powder for 16 h at 4°C. The sections were then washed in TBS and incubated for 1 h at 25°C with horseradish peroxidase-linked donkey anti-rabbit IgG (catalog no. NA934V, GE Healthcare, Buckinghamshire, UK). This step was followed by washing in TBS. Immunoreactivity was detected with 0.025% diaminobenzidine and 0.03% hydrogen peroxide in Tris-buffer (50 mM, pH 7.4) for 8 min at 25°C. No staining was detected when the first antibody was omitted.

Collagen IV in dTGR. Coronal sections of kidneys from dTGR were fixed in methyl Carnoy’s solution and routinely processed for paraffin embedding. Two-micrometer-thick sections were stained for collagen IV by indirect immunofluorescence. Nonspecific binding sites were blocked with 10% normal donkey serum (Jackson Immuno-Research, West Grove, PA) for 30 min. Sections were then incubated with the primary antibody, goat anti-human type IV collagen (Southern Biotech, Birmingham, AL) for 1 h. This antibody strongly cross reacts with rat collagen IV. After being washed with TBS, sections were further incubated with Alexa Fluor 568-conjugated anti-goat secondary antibody (Invitrogen, Paisley, UK) for 1 h. All incubations were performed in a humid chamber at room temperature. Specimens were viewed using a Zeiss Axioplan-2 imaging microscope with the computer program AxioVision 4.8 (Zeiss, Jena, Germany). The microscopist was unaware of the treatment group assignment. The extent of specific fluorescence staining in glomeruli and the tubulointerstitium (combined) was graded as follows: 1 = very mild, 2 = mild, 3 = moderate, 4 = intense, and 5 = very intense.

**Experimental Designs**

**Study 1:** effects of stopping aliskiren treatment on blood pressure, renal damage, and renal retention of aliskiren in dTGR. Double transgenic rats were treated with either aliskiren or, for comparison, enalapril for 2 wk. At the end of this period (at 7.5 wk old), treatment with aliskiren was either continued for 3 wk (aliskiren-continued group; total treatment 5 wk; n = 11), or stopped for a 3-wk washout period (aliskiren-stopped group; n = 11). After 2-wk treatment with enalapril, a similar washout period began (enalapril-stopped group; n = 11). Aliskiren was administered at 3 mg·kg⁻¹·day⁻¹, Enalapril was administered in the drinking water at 18 mg/l, which resulted in a consumption of 4.2 ± 0.6 mg·kg⁻¹·day⁻¹ over the treatment period. This dose was chosen to achieve BP-lowering comparable to that in the aliskiren group.

During the study, BP and urinary albumin excretion (UAE) were measured periodically. All dTGR were euthanized at ~10.5 wk of age, that is, after 1) a 5-wk treatment with aliskiren or 2) a 2-wk treatment with either aliskiren or enalapril plus a 3-wk washout. At autopsy, blood was sampled for measurement of plasma levels of aliskiren, enalapril, and creatinine. Kidneys were removed for measurement of the concentration of aliskiren, enalapril, and for assessment of expression of TGF-β and collagen IV.

Without antihypertensive (RAAS blockade) treatment, dTGR die between 7 and 8 wk of age. Therefore, we used a separate group of untreated, 7-wk-old dTGR controls (n = 14) for comparison with the treated dTGR.

**Study 2:** renal localization of [³H]-aliskiren after continued and interrupted treatment. In a second experiment, we determined the renal localization pattern of radiolabeled aliskiren after continued treatment with the drug, or after a washout period analogous to study 1. Rats were treated with either a single intravenous (iv) dose of an aqueous solution of [³H]-aliskiren (5 mg/kg, 24.5 MBq/mg) into the femoral vein or a continuous infusion via sc implanted osmotic minipumps (Alzet 2ML1) containing an aqueous solution of 12.5 mg [³H]-aliskiren (8 MBq/mg) at 5 mg·kg⁻¹·day⁻¹. Rats receiving iv doses of aliskiren were euthanized 2 h, 3 days, or 7 days postinjection. Rats infused with aliskiren were killed after 7 days of infusion or after a 7-day washout that followed removal of the minipump after the 7 days of infusion. At death, the kidneys were removed, snap frozen in liquid nitrogen, and stored at ~80°C until processed for autoradiography.

**Study 3:** immunohistochemical localization of renin in rats treated with aliskiren. We sought to assess whether the presence of aliskiren in glomeruli demonstrated by LMAR could be explained by binding to endogenous glomerular renin. It was not possible to apply immunohistochemistry (IHC) for renin to the frozen renal sections used for autoradiographical analysis due to the risk that the tritium label would
wash away during the staining process. However, we reasoned that the demonstration of glomerular renin by IHC would support the idea of such binding. Therefore, separate groups of male Wistar rats were treated with vehicle or 5 mg/kg unlabeled aliskiren via minipumps for 1 wk or for 1 wk followed by a 1-wk washout, and kidneys were collected and processed for IHC staining of renin.

Statistics

Data are expressed as means ± SE. Differences between groups were determined by one- and two-way ANOVA, followed by Fisher’s, Dunn’s, or Tukey’s all pairwise comparison tests using SigmaStat (Jandel, San Rafael, CA). Areas under the curve were computed for individual animals using the trapezoidal rule, and group means ± SE are reported. P < 0.05 was considered statistically significant. Data representing UAE were log-transformed before statistical analysis, but original data are reported in the text and graphs.

RESULTS

Effect of Stopping Aliskiren and Enalapril in dTGR (Study 1)

Effect on BP. Baseline BP recorded in dTGR at 5.5 wk indicated the presence of hypertension, with no significant differences between groups. However, by 1 wk after the start of treatments (6.5 wk old), BP was normalized in dTGR treated with aliskiren or enalapril (P < 0.05 vs. baseline; Fig. 1A). The degree of BP lowering by aliskiren and enalapril was comparable and was sustained for the duration of the respective treatments. In contrast to treated dTGR, BP in a separate group of untreated dTGR rose to a group mean of 242 ± 13 mmHg by 7 wk of age, after which these animals were euthanized.

BP remained low in aliskiren-continued dTGR. However, in aliskiren- and enalapril-stopped groups BP rose and surpassed baseline levels 1 wk after treatments were stopped (Fig. 1A). By 8.5 wk, BP levels in enalapril-stopped dTGR approximated those of untreated 7-wk-old dTGR. In contrast, at 8.5 wk of age the BP in aliskiren-stopped dTGR was 38 mmHg lower (P < 0.005) than enalapril-stopped dTGR. Moreover, at this time no statistically significant differences were noted in BP between aliskiren-stopped dTGR vs. their baseline levels, whereas BP in enalapril-stopped dTGR was significantly higher than baseline (P < 0.05). Despite the slower rise in BP in the aliskiren-stopped group, the overall BP burden to which aliskiren- and enalapril-stopped dTGR were exposed during the study was not significantly different, as assessed by the mean area under the BP vs. time curves...
computed from individual dTGR: aliskiren-continued = 590.1 \pm 18.3 (P < 0.05 vs. other groups); aliskiren-stopped = 842.5 \pm 21.8; enalapril-stopped = 862 \pm 29.6.

Effect on urinary albumin excretion. At the start of the study, UAE did not differ significantly between groups. In untreated dTGR, UAE rose progressively with age (Fig. 1B). During the treatment of dTGR with either aliskiren or enalapril, UAE was within normal limits (range during treatment for aliskiren-continued dTGR = 0.8–1.5 mg/24 h; for enalapril = 0.8–3.0 mg/24 h). For comparison, in UAE in untreated 10-wk-old Sprague-Dawley rats was 1.6 \pm 1.1 mg/day. Upon cessation of drug treatments UAE rose (Fig. 1B). However, even at the end of the 3-wk washout period, UAE in aliskiren- or enalapril-stopped dTGR was still 36% lower than that of untreated 7-wk-old dTGR. Thus treatment with aliskiren or enalapril appeared to impart a temporary antialbuminuric effect even after treatment was stopped.

In Fig. 1C, BP and UAE data are plotted to show the temporal relationship between these parameters in aliskiren-treated dTGR. Values for BP and UAE in untreated dTGR are shown for comparison. For clarity, only data for aliskiren-stopped dTGR are shown, since those for enalapril were similar to aliskiren. After ceasing aliskiren treatment, UAE rose later than BP. Moreover, in contrast to BP, UAE did not reach the high levels of 7-wk-old dTGR. Thus, while BP levels in aliskiren-stopped dTGR at 10.5 wk were similar to those in 7-wk-old untreated dTGR (aliskiren-stopped group = 240 \pm 7 vs. untreated dTGR = 242 \pm 13), UAE in the former animals was only 64% of the mean levels in untreated dTGR.

In summary, at the doses administered, aliskiren and enalapril resulted in comparable suppression of UAE, and after stopping the respective treatments the development of albuminuria lagged behind the restoration of hypertension.

Effect on renal function. Plasma creatinine (CrPL) was measured at the end of the study as an index of renal function. Terminal values for CrPL indicated a trend toward improved renal function in aliskiren-continued dTGR vs. in untreated cohorts while mean plasma creatinine in aliskiren-continued dTGR was lower than in enalapril-stopped dTGR (Fig. 1D; P < 0.05). Evidence for continued protection of renal function after treatment with aliskiren or enalapril was not obtained (i.e., vs. untreated controls), although CrPL in aliskiren-stopped dTGR tended to be slightly lower than in enalapril-stopped cohorts, while that in enalapril-stopped dTGR tended toward higher levels than in untreated controls.

Effect on profibrotic renal gene expression and collagen IV accumulation. At the end of the study, renal gene expression for TGF-β in aliskiren-continued dTGR was lower (P < 0.005) than in untreated dTGR (Fig. 2A). Moreover, TGF-β expression was significantly lower in aliskiren-stopped dTGR vs. untreated dTGR (P < 0.01), whereas in the enalapril-stopped group this difference was numerically, but not significantly different from untreated dTGR. Renal gene expression of TGF-β in aliskiren-stopped vs. enalapril-stopped dTGR was not significantly different.

Renal levels of mRNA for collagen IV in aliskiren-continued dTGR were significantly lower than in untreated dTGR and enalapril-stopped dTGR (P < 0.05) (Fig. 2B). Of note, levels of mRNA for collagen IV from aliskiren-stopped dTGR were significantly lower compared with untreated dTGR and with the enalapril-stopped group.

Immunofluorescence microscopy for collagen IV (Fig. 3, A–E) revealed intense glomerular and tubulointerstitial staining in dTGR control rats. Continued treatment with aliskiren markedly reduced collagen IV content in both renal compartments vs. untreated dTGR (52%; P < 0.05). In aliskiren-stopped dTGR, expression of collagen IV was reduced by 28% vs. untreated dTGR (not statistically significant) but was not significantly different from that in aliskiren-continued dTGR. Collagen IV expression in enalapril-stopped dTGR was reduced by 20% vs. untreated dTGR but was greater (P < 0.05) than in aliskiren-continued dTGR. Thus, in contrast to collagen IV mRNA, while statistically significant reductions in collagen IV protein were not observed in the stopped groups vs. untreated dTGR, trends toward such reductions were evident.

Renal content of aliskiren and enalaprilat. Plasma and kidneys were analyzed for their contents of aliskiren and enalaprilat, the active form of enalapril, at the end of the treatment periods (Table 1). The mean level of renal aliskiren in aliskiren-continued dTGR was 52-fold greater than in the
plasma, indicating renal partitioning beyond simple equilibrium with the plasma. In aliskiren-stopped dTGR, renal levels of the drug were greatly reduced vs. in aliskiren-continued dTGR but were still 100-fold greater than the IC50 (0.6 nM) for the drug, despite being undetectable in the plasma of these rats at the time of euthanasia. Enalaprilat was undetected in plasma and kidney at the end of the study.

Renal Localization of Aliskiren After Continuous and Interrupted Treatment (Study 2)

We have previously used LMAR to show glomerular and vascular localization of aliskiren on renal sections after a single iv dose of the radiolabeled drug (7). To gain insight into the mechanism of renal retention of aliskiren in dTGR, we used LMAR to determine the renal localization sites of aliskiren after various times following dosing. Cryostat sections of kidneys from rats treated with aliskiren by iv or sc minipump administration showed specific labeling (silver grains), indicating the presence of aliskiren. Delivery of [3H]-aliskiren by either route resulted in similar intrarenal distribution patterns and intensity of labeling and therefore will be described together. In these 10-μm-thick cryosections, it was not possible to distinguish between distal tubules and collecting ducts; therefore, these two tubular compartments are collectively termed distal nephron segments (DNS) (18). Widespread specific labeling was observed in vascular and tubular compartments in each renal cryosection (Fig. 4). Silver grains were observed mainly over glomeruli and the walls of interlobular arteries, arterioles, capillaries, and veins. Label was also evident in cortical DNS (Fig. 4, B and C). Sometimes labeled tubular profiles were seen close to glomeruli, but whether these profiles represented the macula densa could not be confirmed because of the difficulty in discriminating between various segments of DNS. Labeling over background level was not detected in proximal tubules (Fig. 4 B) or the papilla. The interstitium was devoid of labeling. In the inner and outer medulla, labeling appeared to be restricted to the thick ascending limb of Henle or medullary collecting ducts, neither of which could be distinguished in these frozen sections (Fig. 4D).

One hundred per cent of glomeruli in each section were labeled for aliskiren. Within glomeruli, labeling appeared diffusely distributed; it was not possible to identify the glomerular region or cell type with which silver grains were associated (Fig. 4B). There was also labeling observed near the region of the juxtaglomerular apparatus (JGA) in the extraglomerular

Table 1. Levels of aliskiren and enalaprilat in plasma and kidney

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Drug Levels, nM</th>
<th>Kidney/Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Kidney</td>
</tr>
<tr>
<td>Aliskiren-continued (n=11)*</td>
<td>69 ± 34</td>
<td>3,579 ± 414</td>
</tr>
<tr>
<td>Aliskiren-stopped (n=11)</td>
<td>BLQ</td>
<td>73 ± 36</td>
</tr>
<tr>
<td>Enalapril-stopped (n=11)</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
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Values are means ± SE. BLQ, below levels of quantification. *n =10 for kidney.

Fig. 3. Renal collagen IV accumulation was assessed by indirect immunofluorescence microscopy in dTGR that were untreated (A) or treated with aliskiren for 5 wk (B) or aliskiren (C) or enalapril (D) for 2 wk, after which treatments were stopped for 3 wk. Extent of glomerular and tubulointerstitial staining was graded (E) as 1 = very mild, 2 = mild, 3 = moderate, 4 = intense, or 5 = very intense.
Fig. 4. A–F: light microscopy autoradiographic localization of [3H]-aliskiren in kidneys of Wistar rats. A: overall extent of labeling for [3H]-aliskiren in renal cortex and medulla is shown. Widespread labeling for aliskiren is seen in glomeruli (arrows), tubules, and vessels, the latter 2 being indistinguishable at this magnification. Inner stripe of the outer medulla (*) is uniformly labeled. A single intravenous (iv) dose of [3H]-aliskiren was administered; kidneys were harvested 2 h afterward. Original magnification ×25. B: labeling for [3H]-aliskiren is seen over distal nephron segments (DNS) but not proximal tubules (PT). Label is also present over glomerular tuft (G), in the extraglomerular mesangium (*), and in the wall of what appears to be an afferent arteriole [possibly juxtaglomerular (JG) cells; arrow]. EA, efferent arteriole. A single iv dose of [3H]-aliskiren was administered; kidneys were harvested 7 days afterward. Original magnification ×400. C: extensive labeling for [3H]-aliskiren is seen over glomeruli (G) and DNS, but PT are not labeled over background. Note labeling over extensive length of AE (arrows). [3H]-aliskiren was administered for 7 days by sc minipump, and kidneys harvested thereafter. Original magnification ×200. D: labeling over thick ascending limbs of Henle and/or collecting ducts in renal medulla. [3H]-aliskiren was administered for 7 days by sc minipump, and kidneys were harvested 3 days afterward. Original magnification ×200. E: labeling over glomerulus (G) and AE (long arrows) from its point of origin (short arrow). Note label over AE cells close to glomerulus (JG cells; *). [3H]-aliskiren was administered for 7 days by sc minipump, and kidneys harvested after a 7-day washout. Original magnification ×400. F: labeling over vascular walls of intrarenal artery (white asterisk) and branch (*). A single iv dose of [3H]-aliskiren was administered, and kidneys were harvested 3 days afterward. Original magnification ×400.

mesangium (Fig. 4B). Notably, label was frequently observed over cells of the afferent arteriole near its entrance to the glomerulus, presumably in juxtaglomerular (JG) cells (Fig. 4E). Indeed, silver grains were sometimes observed over lengths of afferent arterioles that extended considerable distances from their glomeruli (Fig. 4, C and E), as well as over the walls of larger arteries (Fig. 4F). Thus vascular localization of radiolabel was not restricted to afferent arterioles. Labeling was not identified in efferent arterioles. The results of semiquantitative grading of specific radioactive labeling in various renal compartments are summarized in Fig. 5. In the cortical renal microvasculature, glomeruli were generally most in-

Fig. 5. Renal distribution of aliskiren by light microscopy autoradiography (LMAR). Wistar rats received [3H]-aliskiren as noted, and kidneys were harvested at times indicated. The presence of silver grains (reflecting [3H]-aliskiren) in renal compartments was assessed in renal cryosections previously subjected to autoradiographic processing. Sections were graded semiquantitatively for the extent of autoradiographic grains: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, or 5 = striking; n = 4/group.
tensely labeled, followed by arterioles > capillaries. With increasing time after bolus (iv) administration of [3H]-aliskiren, and after 7 days washout in the minipump group, intensity of labeling diminished. In the renal medulla in rats receiving iv [3H]-aliskiren, labeling in the medulla increased and then decreased with time after the bolus injection. Label for aliskiren persisted in all renal structures, including the JG cells, 7 days after dosing stopped (Fig. 5). There was no evidence of movement between renal compartments, including the tubulointerstitium.

Overall, the intensity of labeling was strongest 2 h after iv administration of [3H]-aliskiren. Thus, after this short period, labeling was slightly stronger even compared with kidneys from rats that received labeled drug for 7 days via minipumps (Fig. 5). It is likely that in the 2-h group, the presence of [3H]-aliskiren in lumens of glomeruli and vessels at least partially accounted for this observation. After the 7-day washout period for both routes of administration, label was still observed in the lumens of arteries, indicating persistence of the drug in the circulation.

Aliskiren is only minimally metabolized, and thus the levels of plasma radioactivity are assumed to reflect plasma aliskiren concentrations. In rats treated with aliskiren by single iv injection, plasma radioactivity levels decreased with time after injection (Fig. 6). In rats treated with [3H]-aliskiren via minipumps, plasma radioactivity rose between days 3 and 7 and declined thereafter. Regardless of the route of administration, plasma radioactivity did not reach undetectable levels even after the 7-day washout period.

Renal Immunohistochemical Localization of Renin in Rats Treated with Aliskiren (Study 3)

Results from IHC staining for renin in Wistar rats treated with aliskiren revealed staining that was highly specific for the afferent arterioles. Differences in staining intensity or extent were not detected between vehicle- and aliskiren-treated rats. Glomeruli did not stain for renin. Tubular staining was seen infrequently and was usually confined to the subcapsular region. Because this staining pattern was rare and irregular, it was considered to be an artifact.

DISCUSSION

Three new findings emerged from this work. First, we show that aliskiren remained in the kidneys of dTGR even after a 3-wk washout period, indicating a long renal residence of the drug. Second, autoradiographical evidence indicated that aliskiren localizes and is retained in renin-producing renal structures, including the afferent arteriole and JG cells. Third, prolonged suppression of renal expression of profibrotic genes suggests that renoprotective effects observed with aliskiren in models of renal injury may persist even after treatment is stopped. The aforementioned findings were made within the range of clinically relevant plasma aliskiren levels (24).

The observation that the renin inhibitor remikiren showed high renal partitioning in rodents and primates (20) prompted the suggestion that the kidney acts as a “reservoir” for renin inhibitors (20). The current investigation confirms this concept as well as previous results (7) showing that aliskiren partitions to the kidneys of rats beyond a simple equilibrium with the plasma. Here, we extend our previous findings by showing that aliskiren was detected in the kidneys of dTGR at levels 100-fold greater than the IC50 for human renin (0.6 nM) even 3 wk after stopping of treatment; at this time, blood levels of the drug were not detectable. To our knowledge, this is the first report of such persistent localization of a renin inhibitor in the kidney. In contrast, enalaprilat was not detected in the plasma or kidney at the end of the washout period.

The current study also identified the likely renal compartments in which aliskiren is retained after stopping treatment as well as new intrarenal structural targets of aliskiren. In a previous study in normotensive Wistar rats (7), LMAR revealed images suggestive of afferent arteriolar localization of aliskiren. Here, we present conclusive morphological evidence in normotensive rats that aliskiren localizes in the afferent arteriole near its entrance to the glomerulus (i.e., in the region of the JG cells), indicating that aliskiren can enter the JG cells in vivo. Afferent arterioles were defined by the thicker vascular wall and longer, straighter length compared with efferent arterioles. It is noteworthy that such a localization pattern was seen even 7 days after a single iv dose, at a time when circulating radioactivity (i.e., aliskiren) was very low (e.g., 28 ng/ml). Based on these results, we expect a similar localization pattern to have manifested in the current study with dTGR. We therefore believe that some of the renal aliskiren detected after washout in these dTGR resided in JG cells.

In the present study, we did not observe striking functional benefits on BP and UAE that could be attributed to the persistence of aliskiren in kidneys of dTGR. One possible explanation for this is that with low/undetectable plasma aliskiren levels, systemic ANG II formation may have resumed in widespread extrarenal sources of human renin in dTGR (3). Such sources may not be accessible to aliskiren, and/or the drug may not accumulate there. Therefore, in these dTGR the rises in BP and UAE during the washout may reflect diminishing plasma levels of aliskiren.

In contrast to our results in dTGR, Rakusan et al. (19) reported in mRen-2 rats persistent antihypertensive and antialbuminuric effects of aliskiren after treatment was stopped. The differences in these two studies may reflect, in part, differences in the quantity and primary sources of transgenic renin in these models (e.g., dTGR: kidney (3), mRen-2: adrenal (13)) and

Fig. 6. Plasma aliskiren levels in Wistar rats after treatment with [3H]-aliskiren by single iv dose or sc minipumps continuously for 7 days. A single iv dose of aliskiren was administered; blood was sampled 3 min, 3 days, or 7 days after injection. For minipump infusion, blood was sampled after 3 or 7 days of continuous infusion, or after 3 or 7 days washout that followed 7 days of continuous infusion. Aliskiren levels were computed based on plasma radioactive counts.
therefore differences in their responses to renin inhibition. It is possible that due to the highly stimulated RAAS in dTGR, under certain circumstance BP and UAE may not be totally dependent on renal renin activity. Thus this model may not be optimally informative for this type of experimental question. Regardless, the previously cited results (19) probably reflect the preclinical analog to findings in humans (14, 15) in whom BP and albuminuria are reduced by aliskiren treatment and remain low after therapy is stopped. We believe that both observations are explained by the renal retention of aliskiren as described in the current work.

After stopping aliskiren and enalapril treatments in dTGR, UAE was reestablished relatively slowly, being temporally dissociated from the fairly rapid rise in BP. In aliskiren-treated dTGR, it seems unlikely that this dissociation is due to the persistence of the inhibitor in these kidneys since enalapril-treated rats showed a similar functional profile, but that drug was not detected in the kidneys. Rather, it is likely that in both drug-treated “stopped” groups, structural renal protection was consequent to the ameliorated hypertension during the treatment phase (17). The lag in the appearance of albuminuria may reflect the time necessary for injurious effects of reestablished hypertension to become manifested. We therefore speculate that administration of RAAS blockade early in the life of dTGR (i.e., during the development of hypertension) may have prevented damaging rises in intraglomerular pressure, thereby preventing early glomerular damage and stabilizing renal structures against injury from hypertension that followed the cessation of treatments.

However, other evidence from our study suggests that the long renal residence time of aliskiren has the potential to contribute to renoprotection. In dTGR, gene and protein expression of two profibrotic factors, TGF-β and collagen IV, was strongly suppressed in the aliskiren-continued group, while remaining partially suppressed vs. untreated dTGR 3 wk after treatment with aliskiren was stopped. These findings are consistent with the demonstrated reduction of collagen IV protein expression in dTGR continuously treated with aliskiren (17). In the current study, the observed suppression of profibrotic gene and protein expression is unlikely to be due merely to a benefit from the antihypertensive effect of aliskiren during the treatment phase, because in the enalapril-stopped group BP was comparably lowered but collagen IV gene expression was significantly higher than in aliskiren-stopped dTGR. Indeed, suppression of collagen IV gene expression was associated with a prolonged renal presence of aliskiren, while enalaprilat was not detected in kidneys in which collagen IV mRNA levels resembled those in untreated dTGR. Thus the reduction of collagen IV gene expression in aliskiren-stopped dTGR appears to be a BP-independent effect.

This study provides the first in vivo evidence that aliskiren localizes to, and persists in afferent arteriolar and JG cells, where renin is made. This finding may have important implications for the mechanism of action of this drug. We propose that at least some of the radiolabel observed in afferent arterioles reflects aliskiren that is bound to renin in these renin-producing cells. In such a position, aliskiren may inhibit renin even before its secretion. In vitro evidence for such a mechanism was reported (10) using a human mast cell model of renin secretion. If such a mechanism operates in vivo, renin may be released as an already inhibited enzyme and delivered in this form to local (renal) and distal (extrarenal) tissue sites. This would confer efficiency to aliskiren-induced tissue RAAS blockade by precluding the need for the inhibitor to diffuse (after extravasation) through the interstitium toward locations of ANG II production. Additional evidence supporting the ability of aliskiren to access and possibly inhibit intracellular renin stems from in vitro work in podocytes (6), cardiomyocytes (22), and mesangial cells (16). Thus the persistence of aliskiren in JG cells may lead to prolonged release of “already inhibited renin” and contribute to the antihypertensive and renoprotective effects observed in humans (14, 15) and rats (19) after treatment was stopped.

Interestingly, in Wistar rats treated with [3H]-aliskiren the localization of autoradiographic grains in the afferent arteriole was sometimes observed at a considerable distance from the glomerulus. This pattern resembles that of renin staining reported in normotensive rats in which renin-producing cells were recruited following RAAS blockade (8) and further suggests the potential for aliskiren binding to intracellular renin at this site. This observation is also consistent with the ex vivo specific binding at the JG apparatus of the radiolabeled renin inhibitor H77 on cryostat sections of dog kidneys (23).

The absence of immunohistochemically detectable renin in glomeruli of Wistar rats treated with unlabeled aliskiren suggests that, in Wistar rats administered [3H]-aliskiren, the presence of autoradiographic grains in glomeruli reflected trafficking of aliskiren through the glomerular tuft and not binding of the drug to glomerular renin. While this interpretation does not exclude the possibility that aliskiren could bind to renin that may be present in podocytes (5), mesangial cells (1, 21), or trafficking through the mesangial matrix, the data indicate that for aliskiren to accumulate in glomeruli, the presence of renin is not required. This interpretation is consistent with recent findings in mice (11) showing that renal accumulation of aliskiren was not affected by AT1 receptor deletion (renal overexpression of renin) or Ren1c gene deletion (no renal renin expression). Aliskiren may enter renin-devoid glomerular cells and partition to an intracellular compartment where it may escape degradation. Thus intracellular binding of aliskiren to renin may not be required for aliskiren localization but may be necessary for intracellular retention of the inhibitor.

In rats injected with [3H]-aliskiren, plasma levels of radioactivity revealed the presence of very low levels of the drug at the end of the washout period, indicating that high (i.e., steady state) levels of circulating aliskiren are not necessary to maintain renal localization of the drug.

Another new finding in this study is the evidence for aliskiren localization in the distal nephron, a location that may suggest a new mechanism of action for this drug. It has been proposed that angiotensinogen derived from proximal tubules is secreted into the tubular lumen and delivered to connecting tubules and collecting ducts where it is cleaved by renin secreted from these distal nephron components, with subsequent ANG II formation (18). These events have been postulated to contribute to ANG II-mediated hypertension (18). In this context, aliskiren in DNS may suppress ANG II formation and therefore blood pressure, possibly for extended periods in high-renin, high-ANG II conditions. We note that in our study, we could not use immunohistochemistry on the autoradiographic renal sections to identify markers of the specific distal nephron compartments in which [3H]-aliskiren localized. This
procedure would have risked washing away the tritium label during the staining process. Therefore, we are not certain whether [3H]-aliskiren localized in the same DNS structures described previously (18) to contain renin.

The localization of [3H]-aliskiren in the distal nephron is relevant also to the report that the collecting duct is a primary source of prorenin in diabetes (9). Since aliskiren can inhibit the enzymatic activity of prorenin (2, 7), DNS-derived prorenin should also be blocked before its secretion, and this may prevent conversion of prorenin to (active) renin at distal tissue sites.

Several questions that merit further exploration arose from this work. First, the reason for, and the physiological relevance of, aliskiren’s localization in a widespread medullary pattern is unknown. Conceivably, this localization pattern resulted merely from the concentrating function of the renal medulla. Whether aliskiren has an effect on the concentrating function of the thick ascending limb of Henle remains to be explored. Second, our study could not determine the route and mechanism by which aliskiren enters DNS and ascending thick limbs of Henle. A role for renal tubular transporters for uptake of aliskiren into these cells seems unlikely since no evidence exists for such a mechanism. It is possible that cellular uptake is mediated by bulk-phase endocytosis of aliskiren into tubular (and perhaps glomerular) cells. Small amounts of aliskiren appear in the urine of healthy individuals (24), and the molecule is small enough to be filtered by the glomeruli. Thus a pathway from the tubular lumen to intracellular sites is conceivable, but secretion from the peritubular capillaries is also possible. In this context, it is noteworthy that aliskiren does not seem to enter proximal tubular cells.

In summary, we provide evidence that aliskiren, in contrast to an ACE inhibitor, partitions to and is retained in kidneys of dTGR, where it has the potential to exert BP-independent antifibrotic effects even after treatment is stopped. Autoradiographic evidence for localization of aliskiren in JG cells suggests that this DRI may bind to renin in these renin-producing cells, thus potentially inhibiting renin even before its secretion. This may explain the persistent antihypertensive and antialbuminuric effects seen in humans after cessation of treatment. Moreover, the presence of aliskiren in the distal nephron suggests the possibility of renin inhibition at this site and may reveal a new mechanism of action for this drug. Taken together, these findings may have clinical relevance for the potential renoprotective effects of aliskiren.

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DISCLOSURES
D. Feldman, L. Jin, H. Xuan, and W. Zhou are current full-time employees of the Novartis Institutes for Biomedical Research. E. Persohn and H. Schuetz are former (retired) full-time employees of the Novartis Institutes for Biomedical Research. D. Mueller and F. Luft have served on advisory boards for, and received research grants from, the Novartis Institutes for Biomedical Research.

AUTHOR CONTRIBUTIONS

REFERENCES


