Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects

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1Mario Negri Institute for Pharmacological Research, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso, Bergamo, Italy; 2Mario Negri Institute for Pharmacological Research, Centro Aldo e Cele Daccò, Ranica, Bergamo, Italy; and 3Unit of Nephrology and Dialysis, Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo, Italy

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Zoja C, Corna D, Nava V, Locatelli M, Abbate M, Gaspari F, Carrara F, Sangalli F, Remuzzi G, Benigni A. Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects. Am J Physiol Renal Physiol 304: F808–F819, 2013. First published November 7, 2012; doi:10.1152/ajprenal.00376.2012. —Bardoxolone methyl is an antioxidant inflammation modulator acting through induction of Keap1-Nrf2 pathway. Results from a recent phase IIb clinical trial reported that bardoxolone methyl was associated with improvement in the estimated glomerular filtration rate in patients with advanced chronic kidney disease and Type 2 diabetes. However, increases in albuminuria, serum transaminase, and frequency of adverse events were noted. We studied the effect of 3-mo treatment with RTA 405, a synthetic triterpenoid analog of bardoxolone methyl in Zucker diabetic fatty rats with overt Type 2 diabetes. Rats were treated from 3 mo of age with vehicle, RTA 405, ramipril, or RTA 405 plus ramipril. RTA 405 caused severe changes in food intake and diuresis with decline in body weight, worsening of dyslipidemia, and increase in blood pressure. Early elevation in serum transaminase was followed by liver injury. RTA 405 worsened proteinuria, glomerulosclerosis, and tubular damage. Ramipril was renoprotective, but when given with RTA 405 it was not able to limit its worsening effects. These data could be due to degradation products in the drug substance used, as disclosed by the company once the study was concluded. To overcome such a drawback, the company offered to test db404, a variant of RTA 405, in Zucker diabetic fatty rats. The db404 did not display beneficial effects on proteinuria, glomerulosclerosis, and interstitial inflammation. Rather, kidneys from three rats receiving db404 showed the presence of a granulomatous and inflammatory process reminiscent of a pseudotumor. Altogether these data raise serious concerns on the use of bardoxolone analogs in Type 2 diabetic nephropathy.

Type 2 diabetic nephropathy; Zucker diabetic fatty rats; RTA 405; db404; liver toxicity; renal injury

Diabetes mellitus is a public health concern and projections for the future are alarming. According to the World Health Organization, diabetes affects over 170 million people worldwide, and this will rise to more than 300 millions by 2030 (30). About one-third of diabetic patients have progressive deterioration of renal function and ultimately require renal replacement therapy by dialysis or transplantation (21). Diabetic nephropathy is associated with a dramatic excess of cardiovascular morbidity and diabetic patients with renal involvement eventually die of cardiovascular disease threefold more than diabetic patients without signs of renal failure (18). In patients with early renal involvement, lowering blood pressure and the levels of urinary albumin by renin-angiotensin system (RAS) inhibitors reduces the risk of end-stage kidney disease (ESKD), as well as that of myocardial infarction, heart failure, and stroke. Effectiveness of RAS inhibitors crucially depends on the time at which the treatment is started, to the extent that imperfect renoprotection was observed when therapy was given in the advanced phase of the disease (4, 14, 23). In this context, 10% of patients with overt proteinuria and worsening kidney function continue to progress to ESKD or die prematurely of myocardial infarction or stroke every year despite RAS inhibitor therapy. Novel intervention strategies targeting different pathogenic pathways in addition to angiotensin II are therefore worth exploring for diabetic patients who remain at high risk of poor renal and cardiovascular outcomes.

Bardoxolone methyl [2-cyano-3,12-dioxoleana-1,9(11)dien-28-oic acid-methyl ester (CDDO-Me)] is a synthetic triterpenoid belonging to the antioxidant inflammation modulator class of compounds, the most potent inducers of the Keap1-Nrf2 pathway, which plays important role in maintaining kidney function and structure (6, 12, 15, 27, 32). Bardoxolone methyl directly interacts with Keap 1, allowing Nrf2 to translocate to the nucleus where it upregulates antioxidant and cytoprotective genes (6, 27, 32). Structure and activity profile of bardoxolone methyl resemble those of the cyclopentenone prostanoids, the endogenous activators of Nrf2, that favor the resolution of inflammation (13). Similarly to cyclopentenone prostanoids, bardoxolone methyl has anti-inflammatory activity by inhibiting IKKβ/NF-κB signaling pathway (1). Preclinical studies have shown that bardoxolone methyl ameliorated murine ischemic acute kidney injury and increased the expression of the renal-protective genes Nrf2, PPARγ, and HO-1 (31). Treatment with the bardoxolone methyl analog RTA 405 attenuated blood pressure increase and endothelial dysfunction in a 5/6 nephrectomy model of pressure overload (25). In a mouse model of protein overload proteinuria early administration of RTA 405 limited interstitial inflammation and fibrosis and reduced oxidative stress in the kidney (34). RTA 405 was used as a tool molecule since bardoxolone methyl undergoes rodent-specific metabolism to toxic moieties.

In a recent phase 2, double-blind, randomized, placebo-controlled trial study treatment with bardoxolone methyl increased in a dose-dependent manner the estimated glomerular filtration rate (eGFR) in patients with advanced chronic kidney disease (CKD) and Type 2 diabetes at 24 wk (19). The improvement in eGFR persisted at 52 wk, suggesting that bardoxolone methyl may have promise for the treatment of CKD. However, criticism has emerged that the effect on the
eGFR could have been mediated by a potentially deleterious increase in intraglomerular pressure, which could also explain the increase in albuminuria observed in patients receiving bardoxolone methyl (16, 22, 29). Also raising concern was the increased frequency of adverse events including muscle spasm, hypomagnesemia, elevations in alanine aminotransferase levels and gastrointestinal effects.

In the present study we took advantage of a Type 2 diabetes model, the Zucker diabetic fatty (ZDF) rats, to evaluate the effect of the bardoxolone methyl analog RTA 405 given alone or in combination with angiotensin converting enzyme (ACE) inhibitor on proteinuria and renal disease progression. Treatments started at the age of 3 mo when animals are already proteinuric and lasted up to 6 mo. In additional experiments, a variant of bardoxolone methyl and RTA 405, the novel synthetic triterpenoid derivative dihydro-CDDO-trifluoroethyl amide (dh404) (11), was also tested in this model.

MATERIALS AND METHODS


Fig. 1. Body weight (A), food intake (B), and diuresis (C) evaluated in control lean rats and Zucker diabetic fatty (ZDF) rats receiving vehicle, RTA 405 (50 and 100 mg/kg), ramipril (1 mg/kg), and their combination from 3 to 6 mo of age. Values are means ± SE. *P < 0.05. **P < 0.01 vs. age-matched lean rats; *P < 0.05; **P < 0.01 vs. vehicle.
Committee of “Mario Negri” Institute, Milan, Italy. Two-month-old male ZDF rats (ZDF/Gmi-fa/fa) and aged-matched nondiabetic lean rats (ZDF/Gmi-fa/+/) (Charles River Laboratories Italia, Calco, Italy) were kept on a 12-h light-dark cycle with free access to water. ZDF rats were maintained on Purina 5008 rat chow (protein 26.8 kcal%, carbohydrate 56.4 kcal%, fat 16.7 kcal%) to accelerate onset of diabetes. At 3 mo of age ZDF rats were randomized to receive the following daily until month 6 (n = 8 each group): group 1, vehicle (sesame oil by gavage); groups 2, 3, RTA 405 [2-cyano-3,12-dioxooleana-1,9(11)di-en-28-oic acid-ethyl amide (CDDO-EA); Reata Pharmaceuticals, Irving, TX] at doses of 50 and 100 mg/kg by gavage; group 4, ramipril (1 mg/kg in the drinking water); groups 5 and 6, RTA 405 (50 and 100 mg/kg) plus ramipril. RTA 405 is a synthetic oleanane triterpenoid, analog to bardoxolone methyl (CDDO-ME) but yielding an ethyl amide instead of a methyl ester at the C17 position (15, 27). The dose of RTA 405 was chosen on the basis of previous experiments (Reata Pharmaceuticals, data on file). Seven lean rats were used as controls.

In additional experiments, to test the effect of a variant of bardoxolone methyl and RTA 405, the novel synthetic triterpenoid derivative dihydro-CDDO-trifluoroethyl amide (dh404) (11), the groups of ZDF rats were daily treated from 3 mo with the following: vehicle (sesame oil by gavage) (n = 11 rats); dh404 at the doses of 5 and 25 mg/kg by gavage (n = 14 rats/each dose), ramipril (1 mg/kg in the drinking water) (n = 8 rats). After 1–1.5 mo of treatment with vehicle or dh404, n = 4–5 rats/each group underwent renal hemodynamic studies. The remaining rats were euthanized at 6 mo. Lean rats (n = 11) were used as controls.

Biochemical parameters and SBP. Blood glucose levels were assessed with reflectance meter (OneTouch UltraEasy, LifeScan, Milan, Italy). Serum cholesterol, triglycerides, and blood urea nitrogen (BUN) were measured by a Cobas Mira autoanalyzer (Roche Diagnostic Systems, Basel, Switzerland). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by Reelforston test (Roche Diagnostic, Indianapolis, IN). Serum magnesium was measured with an autoanalyzer (CX5, Beckman Instruments). Urinary excretion of proteins was measured in 24-h urine samples by the Coomassie method with a Cobas Mira autoanalyzer (Roche Diagnostic Systems). Urinary albumin excretion was measured using Reflotron test (Roche Diagnostic, Indianapolis, IN). Serum creatinine, serum p-aminohippurate (PAH) clearances, as previously described (5).

Statistical analysis. Results are means ± SE. Data were analyzed by nonparametric Mann-Whitney test or Kruskal-Wallis test for multiple comparisons, as appropriate. Statistical significance level was defined as P < 0.05.

RESULTS

Animal survival. By the end of the 6-mo period the following mortality was recorded among ZDF rats: one rat in each of the groups receiving vehicle, 100 mg/kg RTA 405, or ramipril; two rats in the groups treated with 50 mg/kg RTA 405 alone, or combined with ramipril; three rats in the group given 100 mg/kg RTA 405 plus ramipril. All lean rats were alive.

Body weight, food and water intake, diuresis. ZDF rats given vehicle gained weight along the study and were heavier than lean rats (Fig. 1A). Diabetic rats receiving RTA 405 at both doses, either given alone or combined with ramipril, grew significantly less than animals on vehicle. At 6 mo a 30% reduction in body weight was reached in response to 100 mg/kg RTA 405 compared with vehicle. Ramipril-treated rats weighed less than ZDF rats on vehicle at 4 and 5 mo; thereafter the difference did not achieve statistical significance (Fig. 1A).

Food intake of ZDF rats receiving vehicle was significantly (P < 0.05) higher than that of corresponding lean rats (Fig. 1B). Following RTA 405 administration, diabetic rats showed a sharp decrease in food consumption.

As shown in Fig. 1C, diuresis significantly increased in ZDF rats given vehicle with respect to lean rats. A decrease in urine volume was noted in all the groups receiving RTA 405, particularly at the highest dose, compared with diabetic rats on vehicle.

SBP. ZDF rats given vehicle developed mild hypertension (Fig. 2). A significant increase in SBP was observed at month 4 in rats receiving RTA 405 at the dose of 50 mg/kg alone or in combination with ramipril compared with vehicle. At month 6 statistical significance was not achieved. A trend to increase was observed in rats receiving the highest dose of RTA 405 alone or with ACE inhibitor. Ramipril reduced SBP at levels significantly lower than those of vehicle-treated rats.

Laboratory tests. Blood glucose levels of all ZDF rat groups were significantly (P < 0.01) higher than those of lean rats. In rats treated with RTA 405 blood glucose levels were lower than in the vehicle-treated group, possibly reflecting the decrease in food intake observed in these animals (Fig. 3A).

![Fig. 2. Time course of systolic blood pressure (SBP) in control lean rats and ZDF rats treated with vehicle, RTA 405 (50 and 100 mg/kg), ramipril (1 mg/kg), or their combination from 3 to 6 mo of age. Values are means ± SE.](http://www.ajprenal.org)
As shown in Fig. 3B, ZDF rats given vehicle were hypercholesterolemic compared with lean rats. A further increase in serum cholesterol levels was observed in all groups of rats receiving RTA 405 starting after 1 mo of treatment. Ramipril-treated rats had serum cholesterol levels significantly lower than those of ZDF rats on vehicle at months 4 and 5; at a later point a trend was observed but no statistical difference was achieved.

All diabetic rats independently of treatments showed higher serum triglyceride levels than lean rats (Fig. 3C). Notably, in the group of rats receiving RTA 405 at the dose of 50 mg/kg levels peaked at 4 and 5 mo.

A transient increase in both serum AST and ALT levels was recorded after 1 mo of treatment in ZDF rats receiving RTA 405 alone or in combination with ramipril compared with ZDF rats given vehicle (Fig. 4, A and B). After 3 mo, transaminase levels in the groups receiving RTA 405 were not different from those measured in the vehicle-treated rats.

Serum magnesium concentrations evaluated at the end of the study were not different among the experimental groups (data not shown).

Liver histology. Liver weight was enhanced after RTA 405 administered alone or together with ACE inhibitor, with values exceeding 60 g in rats receiving the highest dose of RTA 405 (vs. values ranging from 16 to 24 g in ramipril- or vehicle-treated rats) (Fig. 5).

Light microscopy analysis of liver obtained from ZDF rats given vehicle showed cytoplasmic vacuolization of hepatocytes.
cytes, with loss of staining with eosin dye presumably because of fat accumulation as also shown previously (10) (Fig. 6). Livers of ZDF rats receiving ramipril also had vacuolization of hepatocytes, which, however, appeared mild in the majority of animals. In contrast, livers of ZDF rats receiving RTA 405 at both doses exhibited severe and diffuse vacuolization, degeneration, and marked swelling of hepatocytes that were worst at 50 mg/kg. Livers of ZDF rats given RTA 405 at the dose of 50 mg/kg plus ramipril showed moderate degenerative changes. Livers of rats given RTA 405 at the dose of 100 mg/kg plus ramipril had severe and diffuse hepatocyte vacuolization, swelling, and degeneration. No abnormalities were present in the livers of control lean rats.

Renal parameters. The time course of urinary protein excretion is shown in Fig. 7. At month 3, before treatment, ZDF rats had proteinuria levels significantly ($P < 0.01$) higher than those of lean rats. In diabetic rats given vehicle, proteinuria progressively increased averaging 321 ± 56 mg/day at the end of the study. Proteinuria was exacerbated by RTA 405 therapy at both doses ($P < 0.01$ vs. vehicle) with levels reaching even more than 1,000 mg/day in some animals. Ramipril alone exerted an important antiproteinuric effect resulting in 48–57% reduction of proteinuria levels vs. vehicle ($P < 0.05$). Ramipril was not able to limit RTA 405-induced proteinuria, so that, in the groups of ZDF rats receiving the combined therapy, proteinuria values were significantly ($P < 0.01$) higher than in vehicle rats during the whole study.

Serum BUN was mildly increased in ZDF rats on vehicle at 6 mo compared with lean rats (29 ± 4 vs. 19 ± 1 mg/dl). No differences among treated ZDF rat groups were observed (not shown).

Renal structural changes. At 6 mo of age ZDF rats given vehicle developed glomerulosclerosis affecting on average 11 ± 3% of glomeruli (Fig. 8A). Glomerular damage worsened after RTA 405 in a way that did not appear to be dose dependent. At the dose of 50 mg/kg the percentage of glomeruli with sclerotic changes averaged 43 ± 5% in rats receiving the drug alone and 33 ± 4% in those with the combined therapy. In the two groups of ZDF rats treated with 100 mg/kg the percentages of sclerotic glomeruli averaged 24 ± 4 and 29 ± 3%. Ramipril given alone decreased the incidence of glomerulosclerosis to 8 ± 1%. Tubular damage, assessed as dilatation and atrophy, was more severe, and the number of tubular casts was higher in diabetic rats exposed to 50 mg/kg RTA 405 compared with rats given vehicle. No difference was observed in the tubular damage scores between vehicle-treated rats and those that received 100 mg/kg of RTA 405. Tubular casts were numerically increased in the two groups of rats receiving the drug but statistical significance was not achieved (Fig. 8, B and C). Representative images of renal morphology are shown in Fig. 9.

Interstitial accumulation of ED-1-positive monocytes/macrophages was increased in ZDF rats on vehicle with respect to lean (9.0 ± 1.3 vs. 2.2 ± 0.2 cells/HPF, $P < 0.01$). RTA 405 at the dose of 50 mg/kg did not display anti-inflammatory effect (8.3 ± 1.09 cells/HPF), whereas at the dose of 100 mg/kg reduced cell infiltrate by 34% (5.6 ± 0.79 cells/HPF). Ramipril limited inflammation to a significant extent if given alone (2.4 ± 0.08 cells/HPF, $P < 0.01$ vs. vehicle). Combined therapies resulted in 43 and 49% reduction of monocyte/macrophages accumulation compared with vehicle.

Analysis by LC-MS/MS of RTA405 preparation. When we submitted the present results to the drug company that sponsored the study, they performed a post hoc analysis of retained drug samples and found that impurities/degradation products were present in the material tested, i.e., lot 1 (no. 0115-53-1) used for the bulk of the study and lot 2 (no. 0115-56-1) used for the last 2 wk of the study. Results of Reata Pharma impurity analysis by LC-MS/MS in lots 1 and 2 of RTA 405, compared...
with a reference control lot, are shown in Fig. 10. Both unknown impurities and structurally identified drug-related peaks were found in the test lots of RTA 405. Lot 1 exhibited structurally unidentified impurities defined as unknown 1 and unknown 3 with intensities 8.76- and 18.6-fold greater than in the reference lot. Lot 2 exhibited substantially higher amounts of structurally unidentified impurities, unknown 2 and unknown 4, which were 302- and 272-fold greater than in the reference lot. Regarding structurally identified degradation products, the company found in both lots the presence of RTA 401, a key intermediate during the synthesis of RTA 405, and of its 1,2-dihydro derivative, DH RTA 401. RTA 401 levels were comparable in lots 1 and 2, being 1.68-fold and 1.61-fold higher than the reference lot, respectively. The 1,2-DH RTA 401 levels were 7.49-fold higher in lot 1 and 3.29-fold higher in lot 2 compared with the reference lot. At this point, one could have concluded that the RTA 405 adverse effects we have documented above are simply the result of an artifact related to drug contamination by RTA 401 and 1,2-DH RTA 401. However, it should be considered that the percentages of RTA 401 and 1,2-DH RTA 401 were very low, accounting for 0.164 and 0.00384%, respectively, in lot 1, and 0.158 and 0.00168% in lot 2.

Effect of bardoxolone analog dh404 on systemic and renal parameters. To establish the cause of the adverse effects, i.e., RTA 405 per se or impurities/contaminating degradation products, our intention was to perform a new experiment with a “clean” RTA 405 preparation. Because it was not available by the company, they instead provided us with another bardoxolone analog, dh404 (11), claiming that the fluorine substituent on dh404 (11) provides protection against the possible formation of rodent-specific metabolites. Table 1 reports laboratory parameters and SBP at 6 mo in ZDF rats treated with dh404. A significant reduction in body weight was observed with the highest dose of dh404. No significant changes have been recorded in blood glucose and serum lipid levels. SBP was comparable in ZDF and lean rats and was not affected by dh404. Instead, ramipril significantly reduced SBP with respect to vehicle. As shown in Fig. 11, proteinuria increased in vehicle-treated rats during time; dh404 did not protect animals from the development of proteinuria, but rather numerically increased its

Fig. 6. Liver morphology (hematoxylin and eosin staining) evaluated at 6 mo in ZDF rats treated with vehicle, RTA 405 (50 and 100 mg/kg), ramipril (1 mg/kg), or their combination, and control lean rats. Hepatocyte vacuolization, swelling, and degeneration are shown in representative ZDF rats after RTA 405 treatment (magnification ×200).
values when administered at the dose of 25 mg/kg. As expected, ramipril had an antiproteinuric effect. Consistent with the effect of dh404 on proteinuria, glomerulosclerosis and tubular damage tended to increase, while limited by ramipril. Of note, interstitial inflammation, evaluated as accumulation of ED1-positive cells, which was increased in ZDF rats vs. lean, was not ameliorated or even was worsened by dh404 therapy. Kidneys from three rats receiving dh404 (1 rat at the low and 2 at the highest dose) showed the presence of large lesions with clear cell features that was reminiscent of a renal cell pseudotumor. The absence of atypical nuclei and the presence of neutrophils and macrophages was suggestive of a granulomatous and inflammatory lesion (Fig. 12).

Fig. 7. Time course of urinary protein excretion in control lean rats and ZDF rats treated with vehicle, RTA 405 (50 and 100 mg/kg), ramipril (1 mg/kg), or their combination from 3 to 6 mo of age. Values are means ± SE. *P < 0.01 vs. lean rats; *P < 0.05; **P < 0.01 vs. vehicle.

Renal hemodynamics in ZDF rats treated with dh404. Table 2 reports data of renal function evaluated as iohexol and PAH clearances in ZDF rats after 1–1.5 mo of treatment with dh404. GFR and RPF were reduced in ZDF rats given vehicle with respect to lean rats. A trend toward increase in GFR was observed in ZDF rats given 5 mg/kg dh404. RPF was not affected by the therapy. Because of the increase in GFR induced by the low dose of dh404, filtration fraction was increased in this group of animals with respect to rats on vehicle.

DISCUSSION

Bardoxolone methyl and related synthetic triterpenoid analogs showed promise as therapeutic agents for prevention and treatment of tissue injury caused by inflammatory and oxidative stress (15, 27). Bardoxolone methyl, unless administered acutely at low doses, is not well tolerated in rodents because of species-specific CYP metabolism. Other molecules, including the triterpenoid CDDO-ethyl amide or RTA 405 (15), with similar target-based activity and same site relative to bardoxolone methyl, but devoid of the major adverse rodent metabolism, have been extensively explored in the literature as tool compounds for the class. Bardoxolone methyl and related analogs demonstrated efficacy in models of acute kidney injury including ischemia-reperfusion (31) and cisplatin-induced nephrotoxicity (2). These molecules also effectively improved glucose control, lowered plasma triglyceride and free fatty acid levels, reduced hepatic lipid accumulation and inflammation in both high-fat diet-induced and genetically induced mouse models of obesity and diabetes without apparent signs of toxicity (24, 26).

In the present study we used ZDF rats, a substrain of Zucker obese rats with mutation in leptin receptor gene that are selectively inbred for hyperglycemia (28). ZDF rats progress to frank diabetes because of failure to compensate adequately for insulin resistance. They are characterized by altered metabolic profile, hyperlipidemia, progressive renal disease, and cardiac abnormalities (3, 8, 33). Animals received two dosages of RTA 405 alone or in combination with ramipril initiating at a phase
of overt disease. Unexpectedly, we found that RTA 405 caused an increase in mortality and adverse changes in the physical status of ZDF rats, as early as 1 mo after starting the treatment. Acute reductions in food intake and diuresis were noted, with subsequent decline in body weights. Serum cholesterol and triglyceride levels were elevated. Increases in blood pressure were also recorded after 1 mo of dosing. Additionally, early elevation in serum ALT and AST further suggested acute toxicity of the molecule. Some potentially similar trends were reported in the recent BEAM clinical trial in CKD with Type 2 diabetes (19). Transient increases in ALT levels were found in 120 of 170 (71%) bardoxolone methyl-treated patients, which peaked 2–4 wk after the initiation of treatment or an increase in the dose and generally resolved while the patients continued to receive the drug. A total of 18 patients (11%) had ALT elevation of more than three times the upper limit of the normal range. However, signs and symptoms of hepatic injury were not described in these patients with ALT elevation. Notably, no changes in serum bilirubin were observed. Transaminase elevations have been observed upon genetic or pharmacological induction of Nrf2, the biological target of bardoxolone methyl (17). It has been shown that Nrf2 transcriptionally upregulates both the ALT and AST genes and that Nrf2-mediated transaminase induction is associated with increased hepatic glutathione levels and hepatoprotection in animal models of liver injury (17). In our study, the increase in transaminase levels in ZDF rats after Nrf2 activation by RTA 405 was instead followed by liver injury, as documented by severe and diffuse hepatocyte vacuolization, swelling, and degeneration.

Another finding of the present study was the detrimental effect of RTA 405 treatment on proteinuria, which increased even more than threefold over the values measured in ZDF rats that received vehicle. Actually, in patients receiving bardoxolone methyl, transient increase in albuminuria that followed a pattern similar to the increases in eGFR has been observed (19). Increase in proteinuria in response to bardoxolone methyl has been suggested to occur because of the increase in GFR with resultant decreased protein residence time in proximal tubules because of reduced expression of megalin and cubilin, which has been observed in vitro and in vivo (7, 20). However, in rats the increase in proteinuria translated in severe glomerular and tubular damage, as assessed after 3 mo of RTA 405 treatment. Consistent with previous observations (33), ZDF rats well responded to ramipril therapy, showing a reduction in proteinuria and renal damage with respect to rats receiving vehicle. However, when given in combination with RTA 405 the ACE inhibitor was not able to limit the worsening effects of the
molecule on both proteinuria and renal structure. The reasons for the adverse effects experienced by ZDF rats undergoing RTA 405 administration are not clear. It is not possible to exclude a pattern of toxicity specifically due to an adverse metabolism of RTA 405 in the present ZDF rat colony, similar to acute toxicity described in rodents treated with bardoxolone methyl, because of rodent-specific CYP P450-mediated transformation to toxic metabolites (Reata, data on file). However, a post hoc analysis of retained drug samples performed by Reata Pharma revealed the presence of both unknown impurities and structurally identified drug-related peaks, i.e., RTA 401, a key intermediate during the synthesis of RTA 405, and its 1,2-dihydro derivative, DH RTA 401. At this point, one could have concluded that the RTA 405 adverse effects found in ZDF rats were simply the result of an artifact related to drug contamination by RTA 401 and 1,2-DH RTA 401. However, since the percentages of RTA 401 and 1,2-DH RTA 401 were very low, it turns out that the toxicity (at least for rodents) of RTA 401 and 1,2-DH RTA 401 is extremely high, which could represent a very critical issue as for the control of quality in the production of various bardoxolone methyl analogs. Further experiments with a “clean” batch of RTA 405 appeared therefore necessary to establish whether the negative profile that accompanied RTA 405 treatment in ZDF rats was indeed attributable to the impurities/degradation products.

![Fig. 10. LC-MS chromatogram of 3 different lots of RTA 405.](image)

Table 1. **Laboratory parameters and systolic blood pressure in ZDF rats treated with dh404**

<table>
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<tr>
<th>Treatment</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
<th>Serum Cholesterol, mg/dl</th>
<th>Serum Triglycerides, mg/dl</th>
<th>SBP, mmHg</th>
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<tr>
<td>Vehicle</td>
<td>548 ± 26</td>
<td>477 ± 53</td>
<td>411 ± 54</td>
<td>1,628 ± 394</td>
<td>138 ± 0.9</td>
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<tr>
<td>dh404 (5 mg/kg)</td>
<td>517 ± 21</td>
<td>518 ± 53</td>
<td>366 ± 18</td>
<td>1,111 ± 231</td>
<td>139 ± 1.5</td>
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<tr>
<td>dh404 (25 mg/kg)</td>
<td>441 ± 13†</td>
<td>537 ± 26</td>
<td>400 ± 39</td>
<td>1,020 ± 226</td>
<td>141 ± 2.6</td>
</tr>
<tr>
<td>ACEi</td>
<td>579 ± 20</td>
<td>461 ± 46</td>
<td>341 ± 29</td>
<td>782 ± 39</td>
<td>120 ± 2.9*</td>
</tr>
<tr>
<td>Lean</td>
<td>407 ± 7</td>
<td>134 ± 6</td>
<td>172 ± 12</td>
<td>158 ± 23</td>
<td>141 ± 1.4</td>
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</table>

Data are means ± SE. ZDF rats, Zucker diabetic fatty rats; SBP, systolic blood pressure; ACEi, angiotensin-converting enzyme inhibitor. *P < 0.05, †P < 0.01 vs. vehicle.
present in the material tested or because of the molecule per se. However, the company was not available to provide RTA 405 in a “purified” status, because the molecule was out of production. Instead they allowed us to test a variant of bardoxolone methyl and RTA 405, the synthetic triterpenoid derivative dihydro-CDDO-trifluoroethyl amide (dh404)(11) that is better tolerated by rodents. After 3 mo of treatment dh404 did not display beneficial effects on renal disease of ZDF rats, but it rather caused a trend toward increase of proteinuria, glomerulosclerosis, tubular casts, and interstitial inflammation.

Measurement of GFR and RPF (9) in dh404-treated ZDF rats helped reproduce, at least with the low dose, the increase in estimated GFR noted in patients who received bardoxolone methyl (19). Although micropuncture studies could not be performed in ZDF rats, it is conceivable that the increase in GFR could be the result of potentially deleterious increase in intraglomerular pressure as recently hypothesized (16, 29). Another worrying result is that the kidney from 3 of 20 ZDF rats treated with dh404 for 3 mo showed the presence of a granulomatous and inflammatory process that was suspicious of a pseudotumor, raising serious concerns on the use of bardoxolone analogs in severe Type 2 diabetic nephropathy.

In summary, the ZDF rats represented an useful model to reproduce some drawbacks of bardoxolone therapy in humans, even using analogs of the molecule. The detrimental changes observed in ZDF rats in response to both RTA 405 and dh404 strongly suggest that a note of caution should be taken either in interpreting the results of the recent BEAM clinical trial (19) or in the setting of new clinical trials with such molecules where careful and appropriate safety follow-up is required.

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Fig. 11. Proteinuria (A), glomerulosclerosis (B), tubular casts (C), and interstitial accumulation of ED1-positive monocytes/macrophages in lean rats and ZDF rats treated with vehicle, dh404 (5 and 25 mg/kg), or ramipril (1 mg/kg) from 3 to 6 mo of age. Values are means ± SE. *P < 0.05, **P < 0.01 vs. lean rats; *P < 0.05; **P < 0.01 vs. vehicle.

Fig. 12. A: macroscopic view of a portion of kidney taken from a ZDF rat given dh404 (25 mg/kg). Light micrographs showing massive accumulation of clear cells and inflammatory cells (B, hematoxylin-eosin stain, magnification ×200) containing abundant ED-1 positive macrophages (C, magnification ×100).
Table 2. Effect of dh404 on renal hemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>GFR, ml/min/100 g</th>
<th>RPF, ml/min/100 g</th>
<th>FF, %</th>
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<tbody>
<tr>
<td>ZDF</td>
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<td></td>
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<tr>
<td>vehicle</td>
<td>0.48 ± 0.09</td>
<td>1.15 ± 0.27</td>
<td>42.9 ± 3.17</td>
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<td>dh404 (5 mg/kg)</td>
<td>0.58 ± 0.06</td>
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<td>49.6 ± 1.31</td>
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<td>dh404 (25 mg/kg)</td>
<td>0.48 ± 0.07</td>
<td>1.01 ± 0.14</td>
<td>47.6 ± 2.3</td>
</tr>
<tr>
<td>Lean</td>
<td>0.65 ± 0.07</td>
<td>1.45 ± 0.13</td>
<td>44.5 ± 2.5</td>
</tr>
</tbody>
</table>

Data are means ± SE. GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction.

NOTE ADDED IN PROOF

While the present study was under revision, Reata decided to terminate the phase 3 BEACON trial of bardoxolone methyl in patients with stage 4 chronic kidney disease and type 2 diabetes because of safety concerns due to excess serious adverse events and mortality in the bardoxolone methyl arm. (From a Company Statement by Reata).

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DISCLOSURES

Giuseppe Remuzzi is member of the Reata Bardoxolone Methyl CKD Steering Committee. He does not accept any personal remuneration, and compensation is paid to his institution for research and educational activities outside the Nephrology division.

AUTHOR CONTRIBUTIONS


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