Probenecid prevents acute tubular necrosis in a mouse model of aristolochic acid nephropathy

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Experimental aristolochic acid nephropathy is characterized by early tubulointerstitial injury followed by fibrosis, reproducing chronic lesions seen in humans. *In vitro*, probenecid inhibits aristolochic acid entry through organic anion transporters, reduces specific aristolochic acid-DNA adduct formation, and preserves cellular viability. To test this *in vivo*, we used a mouse model of aristolochic acid nephropathy displaying severe tubulointerstitial injuries consisting of proximal tubular epithelial cell necrosis associated to transient acute kidney injury followed by mononuclear cell infiltration, tubular atrophy, and interstitial fibrosis. Treatment with probenecid prevented increased plasma creatinine and tubulointerstitial injuries, and reduced both the extent and the severity of ultrastructural lesions induced by aristolochic acid, such as the loss of brush border, mitochondrial edema, and the disappearance of mitochondrial crests. Further, the number of proliferating cell nuclear antigen–positive cells and total aristolochic acid-DNA adducts were significantly reduced in mice receiving aristolochic acid plus probenecid compared with mice treated with aristolochic acid alone. Thus, we establish the nephroprotective effect of probenecid, an inhibitor of organic acid transporters, *in vivo* toward acute proximal tubular epithelial cell toxicity in a mouse model of aristolochic acid nephropathy.

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Human aristolochic acid nephropathy (AAN) is a tubulointerstitial (TI) nephritis reported after intake of herbal remedies containing aristolochic acid (AA).[1,2] It is histologically characterized by a typical corticomedullary gradient of interstitial fibrosis and the progressive atrophy of proximal tubules, resulting in the rapid deterioration of renal function to the end stage.[3,4] AA intoxication also leads to the formation of specific AA-DNA adducts, which are premutagenic lesions involved in the development of AAN-associated urothelial cancer, and their long-term presence in renal tissue is used as a biomarker of AA exposure.[5,6]

AA-induced TI nephritis was experimentally reproduced in rabbits, mice, and rats.[7–10] A biphasic evolution of TI lesions was identified in our Wistar rat model.[11,12] In the early, so-called *acute phase*, transient tubular necrosis located in the S3 segment (proximal tubular epithelial cells (PTECs)) and mononuclear cell infiltration are observed; later, in the so-called *chronic phase*, tubular atrophy and interstitial fibrosis are clearly the prominent features. In this step-by-step model, inflammatory cells were proposed as the physiopathological link between both phases.[11] *In vitro* data early confirmed that PTECs were the target of AA,[13] suggesting the presence of specific molecular mechanisms responsible for the accumulation of AA in PTECs. The excretion of numerous organic anions (OAs), including endogenous metabolites, through PTECs is actually achieved via unidirectional transcellular transport, involving the uptake of OAs from the blood across the basolateral membrane and their extrusion across the apical membrane into the tubular lumen. OA transporters (OATs) have a key role in this process. At least 11 isoforms of OATs have been identified; a majority of them was found in the kidney. OATs are exchangers linked to two other transporters, the sodium dicarboxylate cotransporter and the sodium-potassium ATPase. OAs are taken up by OAT 1 and/or 3 in the basolateral membrane of the proximal tubule. This uptake is

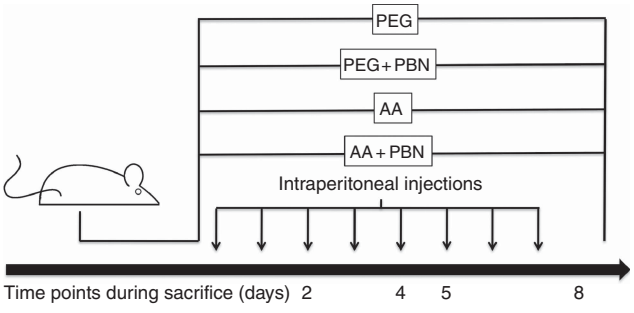

Figure 1 | Schematic representation of experimental protocols performed in the mouse model of aristolochic acid nephropathy (AAN). C57BL/6 male mice ($n = 96$) were randomized in four groups of 24 mice each. Aristolochic acid (AA) (5 mg/kg body weight) was injected once a day and probenecid (PBN) (150 mg/kg body weight) twice a day. After 2, 4, 5, and 8 days of injection, six mice/group were killed and blood sample and kidneys were harvested for further analysis.

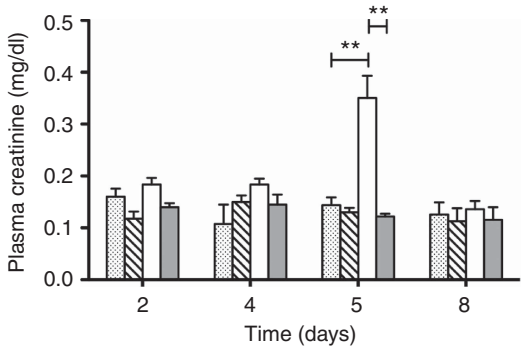

Figure 2 | Evolution of plasma creatinine levels. Plasma creatinine from aristolochic acid (AA) (white columns), AA + probenecid (PBN) (gray columns)-treated mice as compared with polyethylene glycol (PEG) + PBN (dashed columns), and PEG (dotted columns) controls from days 2 to 8. Results are presented as the mean ± s.e.m.; $n = 6$ mice per group (**$P < 0.01$).

processed in parallel to the countertransport of α-ketoglutarate. The drug then crosses the cell and is excreted in the lumen of the tubule.[14,15] The activity of OATs has been associated with proximal tubular injury due to the accumulation of toxics, such as uremic toxins, drugs, and mercuric species.[14–17] In human embryonic kidney cells (HEK293) and *Xenopus laevis* oocytes, three human isoforms (OAT1, OAT3, or OAT4) were reported to have a role in intracellular accumulation of AA.[18,19] Moreover, probenecid (PBN) blocked AA entry by inhibition of human OATs, reducing the formation of AA-DNA adduct,[19] and preserved cell viability.[18]

We investigated this last aspect *in vivo* in a mouse model of AAN. We hypothesized that PBN, by reducing AA entry through OATs, could protect PTECs against lesions, preventing AA-DNA adduct formation, and thus preserve cell viability.

RESULTS

Ninety-six mice C57BL/6 were randomly assigned to four groups of 24 animals each. According to group, mice were injected with AA, AA + PBN, or solvent (polyethylene glycol (PEG)) + PBN. The control group was injected with PEG (Figure 1). AA (5 mg/kg body weight) or PEG was injected once a day and PBN (150 mg/kg body weight) twice a day. These dosing regimens of PBN have been shown to inhibit OAT.[20]

Plasma creatinine (PCr) level, TI lesions, DNA-repair processes (proliferating cell nuclear antigen tissue expression), and AA-DNA adduct formation were quantified in each group after 2, 4, 5, and 8 days of AA injections.

PBN prevents AA-induced acute kidney injury

A transient acute kidney injury, as reflected by a significant increase in PCr levels, was observed in mice receiving AA after 5 days of injections as compared with control animals (PCr (mg/dl), median (min–max): 0.353 (0.222–0.504) vs 0.135 (0.112–0.211); $P < 0.0022$). The addition of PBN prevents PCr increase in AA animals (PCr (mg/dl), median (min–max): 0.125 (0.105–0.139) vs 0.353 (0.222–0.504); $P < 0.0022$). No significant change in PCr levels was measured in the PEG + PBN group as compared with controls (Figure 2).

PBN significantly reduces AA-induced TI injury

As demonstrated in Figures 3 and 4a–h, the renal parenchyma from PEG and PEG + PBN groups remained normal in optical microscopy analyses at all studied time points of the protocol. In contrast, early histological lesions were present in the AA group (Figures 3 and 4i–l). As early as day 2, a swelling of PTECs was found in the medullary rays (Figure 4i). In the same areas, prominent PTEC necrosis was observed at days 4 and 5 (Figures 3 and 4j–k). After 8 days of AA treatment, tubular atrophy was clearly widespread as reflected by dilatation and flattening of PTECs, as well as tubular basement membrane thickening. In the surrounding interstitial areas, mononuclear cell infiltration was observed at day 4 and progressively extended to day 8. At that time point, this inflammatory process was associated with extracellular matrix deposition. In mice receiving AA + PBN, swelling and necrosis of PTECs was limited to few tubules located in the medullary rays only at day 4 without any interstitial inflammatory cell infiltration (Figures 3 and 4n). Moreover, proximal tubules, as well as the surrounding interstitial areas, appeared normal under optical microscopy analysis at days 5 and 8 (Figures 3 and 4o–p). Throughout the protocol, no abnormality was detected within the glomeruli from all groups under optical microscopy analysis.

As compared with controls, the semiquantitative score of TI injury obtained in AA-treated mice revealed tubular necrosis from day 4 to 8, with an evident peak at day 5 (Figure 5a), lymphocytic infiltration from day 5 (Figure 5b), and marked tubular atrophy at day 5 accompanied by progressive interstitial fibrosis (Figure 5c and d, respectively). In the AA + PBN group, a significant reduction of all the semiquantitative scores was found: of tubular necrosis on days 5 ($P < 0.0013$) and 8 ($P < 0.0025$), of lymphocytic infiltrate ($P < 0.0013$), of tubular atrophy ($P < 0.0018$) (day 8),