INTRODUCTION

Cisplatin is a highly effective chemotherapeutic agent that is used to treat a wide variety of malignancies (1). However, various side effects, including nephrotoxicity, may limit its clinical use. The adverse renal effects are ranging from isolated renal tubular defects to severe acute renal failure (2). Different proposed mechanisms of cisplatin-induced nephrotoxicity include direct toxicity to renal tubular epithelial cells (3), apoptosis (4), oxidative stress and inflammation (5). Moreover, recent studies including ours (6, 7), highlighted the participation of lipoxygenase/leukotrienes (LOX/LTs) pathway in mediating cisplatin-induced renal insults.

LOX (5-, 12-, and 15-LOX) enzymes are a group of lipid metabolizing enzymes. With the help of 5-LOX activating protein (FLAP), 5-LOX can generate leukotriene A4 (LTA4), which is hydrolyzed by LTA4 hydrolase to form, the non cysteinyl LT (non Cys-LTs), would differentially contribute to cisplatin-induced acute renal damage in rats. Male Sprague-Dawley rats were treated with a single intraperitoneal dose of cisplatin (5 mg/kg) alone or combined with montelukast (Cys-LT receptor blocker, 10 mg/kg/day, orally), or ONO-4057 (leukotriene B4 receptor blocker, 300 mg/kg/day, orally). Both drugs were administered for one week; starting 4 days before cisplatin injection. Cisplatin nephrotoxicity was evidenced by alteration in renal indices of functional (blood urea nitrogen, and serum creatinine), oxidative (increased thiobarbituric acid reactive substances, nitrite/nitrate level and decreased total superoxide dismutase activity), inflammatory (increased tumor necrosis factor-α and myeloperoxidase), anti-inflammatory (interleukin-10), apoptotic (caspase-3), fibrotic (transforming growth factor-β1), and histopathological profiles. These changes were accompanied by increased renal levels of Cys (leukotriene D4, 3.9-folds) and non Cys-LTs (leukotriene B4, 1.3-folds). Simultaneous administration of montelukast, but not ONO-4057, to cisplatin-treated rats reversed the nephrotoxic manifestations, even though in the presence of elevated renal leukotriene B4. These data suggest, for the first time, the predominant role of Cys-LTs in mediating cisplatin-induced acute renal damage in rats.

Key words: cisplatin, nephrotoxicity, montelukast, leukotriene D4, leukotriene B4, leukotriene B4 receptor antagonist
considerable up regulation in the mRNA and protein expression levels of 5-LOX, FLAP, 12-LOX, LTA₄, hydrolase, LTC₄, synthase, LTB₄, receptor, and Cys-LTR 1 and 2. In addition to increased renal and serum levels of LTB₄, and other inflammatory mediators (6). Thus, the current study aimed to test the hypothesis whether these products, namely LTD₄ and LTB₄, would differentially contribute to cisplatin induced acute renal damage in rats. To achieve this goal, pharmacologic antagonist studies (using montelukast; Cys-LTR blocker, and ONO-4057; LTB₄ receptor blocker) were undertaken as well as indices of renal function, morphology, inflammation, apoptosis, and oxidative stress were evaluated.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (Laboratory Animal Colony, Helwan, Cairo, Egypt) weighing 180 – 200 g were used in this study. Animals were housed individually in stainless steel cages with wood shaving bedding and kept on a light-dark cycle of equal duration (on 0600, off 1800), under constant environmental conditions (temperature; 25 ± 2°C and humidity; 50 – 55%). Rats were fed with a commercially available rat normal pellet diet (carbohydrates 35%, proteins 25%, lipids 7%, and vitamins 3%) and water ad libitum. All efforts were made to minimize animal suffering. Experiments were performed under institutional animal care and use guidelines.

The research experimental protocol was approved by the Institutional Animal Care and Use Committee (CU-IACUC), Cairo University, Egypt (Approval No. CU/IIF/28/18).

Chemicals and Drugs

ONO-4057 supplied by Ono Pharmaceutical Co. (Osaka, Japan). Cisplatin (Onotec Pharma production, Germany), montelukast (European Egyptian Pharmaceutical Industries, Egypt), and thiopental sodium (Biochemie GmbH, Vienna, Austria). Western blot chemicals/buffers/SDS polyacrylamide gel, (Bio-Rad® Hercules, CA, USA), Cu-Zn superoxide dismutase; SOD 1 (rat-polyclonal antibody, product 10011387, Cayman Chemical, Michigan 48108 USA), polyvinylidene difluoride membranes, β-actin (Thermo-scientific, Rockford, Illinois, USA), were purchased from commercial agents. Drugs were dissolved/dispersed in saline, pH 7.4, immediately before use.

Experimental Protocols

A total of four groups of rats (n = 8 each) were used in the current study

(a) Control: Vehicle, given physiological saline (1 ml/kg/day, orally for one week);
(b) Cisplatin, given a single dose of cisplatin; 5 mg/kg, intraperitoneally; i.p. (21);
(c) Cisplatin + montelukast (Cys-LTR antagonist); 10 mg/kg/day, orally (22);
(d) Cisplatin + ONO-4057 (LTB₄ receptor antagonist); 300 mg/kg/day, orally (23).

Montelukast and ONO-4057 were administered for one week; starting 4 days before cisplatin injection. Animals were checked twice a day after cisplatin administration and any changes in total body weight were recorded. Seventy two hours after the cisplatin injection (24), overnight-fasted rats were anaesthetized with thiopental sodium (50 mg/kg, i.p.) (25), and blood was collected from the orbital plexus and separated at 1200 × g for 10 min, and the aspirated serum was divided into aliquots and stored at –80°C till biochemical analyses of blood urea nitrogen and creatinine.

Rats were then sacrificed with an overdose of thiopental, abdomen was opened, internal viscera pulled aside, then the right kidney was quickly removed, weighed and homogenized in ice-cold PBS (pH = 7.4) to give 40% homogenate. Homogenate was divided into small aliquots and stored at –80°C for the measurement of total protein content, renal oxidative (thiobarbituric acid reactive substances (TBARS) and nitrite/nitrate (NOx)), anti/or inflammatory (interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α)), fibrotic (transforming growth factor β1 (TGF-β1)) parameters, and renal levels of Cys and non Cys-LTs (LTD₄, LTB₄). One aliquot was freshly analyzed for the kinetic determination of myeloperoxidase, total SOD, and caspase-3 enzymatic activities. The left kidney was kept for histopathological studies and fixed in 10% formaldehyde for 18 h at 4°C then embedded in paraffin blocks.

Biochemical analyses

1. Enzyme-linked immunosorbent assays

ELISA technique was used for the determination of TNF-α, TGF-β1, IL-10, LTD₄, and LTB₄ in renal tissues homogenate of all studied groups as instructed by the manufacturers. Antigen of purpose in samples/ standards was bound to wells by an immobilized antibody then biotinylated anti-rat specific antibody, followed by HRP conjugated streptavidin. The color develops with the addition of TMB substrate solution, and finally the reaction was terminated by the addition of stop solution where the change in color from blue to yellow was measured at 450 nm (with a wavelength correction set to 540 or 570 nm).

2. Assessment of renal function

Serum creatinine: colorimetric determination was done according to the improved Jaffe method; a red colored complex is formed with picrate and measured at 450 nm (QuantiChrom™ kit cat.# DICT500, BioAssay Systems, Hayward, CA, USA).

Blood urea nitrogen: direct colorimetric determination was done according to the improved Jung method; a blue colored complex is formed with p-phenylendiamine, and N-(1-naphthyl) ethylenediamine and measured at 520 nm (QuantiChrom™ kit cat.# DIYR 500, BioAssay Systems, Hayward, CA, USA). Blood urea nitrogen was computed from urea levels (Blood urea nitrogen = serum urea in mg/dl × 2.14).

3. Determination of renal leukotriene D₄ and leukotriene B₄

LTD₄ level was determined by ELISA (Rat LTD₄ Kit cat.#CSB-EQ027312RA, CUSABIO, Baltimore, USA). LTB₄ level was determined by ELISA (Rat LTB₄ Kit cat.#CSB-E08035r, CUSABIO, Baltimore, USA).

4. Determination of kidney anti/or inflammatory mediators

Myeloperoxidase activity: quantitative fluorimetric determination of renal myeloperoxidase peroxidation was done using (EnzyFluo™ myeloperoxidase Assay Kit cat.#EMPO-100, BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA). The kinetic reaction of peroxidase enzymes with hydrogen peroxide oxidized a dye reagent to a highly fluorescent product. The fluorescence intensity was measured at λex/em = 530/585 nm, which is proportional to the total peroxidation activity in the sample. In parallel the reaction was done with the
addition of a myeloperoxidase inhibitor (2.5 mM 4-aminobenzhydrazide) to suppress the enzyme activity in order to subtract other peroxidase activities that may be present in the samples.

TNF-α was determined by ELISA (Rat TNF-α Kit cat.#CSB-E08857r, RayBio®, Ray Biotech Inc., Georgia, USA).

IL-10 was determined by ELISA (Quantikine® ELISA Rat IL-10 Immunoassay Kit cat.#R1000, R&D Systems Europe, Ltd.).

5. Determination of renal transforming growth factor-β

TGF-β1 was determined by ELISA (Rat TGF-β1 Kit cat.#CSB- E04727r, CUSABIO, Baltimore, USA).

6. Determination of kidney apoptotic activity

Caspase-3 activity: the absolute value for caspase-3 activity was determined by comparison to the signal given by the p-nitroaniline calibrator (Caspase-3 colorimetric detection kit cat.#ADI-907-013, Enzo Life Sciences, CH-4415 Lausen, Switzerland), measured at λ 405 nm, according to manufacturer instructions.

7. Determination of kidney oxidative stress

Lipid peroxidation level: TBARS was assayed spectrophotometrically using the modified TBARS assay, according to manufacturer instructions, in which the developed reddish color was measured at λ 532 nm (OxiSelect™ TBARS Assay Kit cat.# STA-330, Cell Biolabs, Inc., San Diego, USA).

Nitrite/nitrate level: NOx metabolites were determined spectrophotometrically using the modified Griess assay of Guevara et al. (26). The assay involved the enzymatic conversion of nitrate to nitrite, by nitrate reductase, followed by colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm (Nitric Oxide “NO₂/NO₃” Assay Kit cat.#917-010, Assay Designs, Inc., Ann Arbor, USA).

Total superoxide dismutase activity: total SOD activity was detected by kinetic measurement, according to manufacturer instructions. The assay relied on the ability of the enzyme to inhibit the phenazinemethosulphate-mediated reduction of nitroblue tetrazolium dye (Superoxide dismutase colorimetric method, biodiagnostic, Dokki, Giza, Egypt. www.biodiagnostic.com).

8. Determination of renal total protein content

Total protein was measured according to Bradford; based on binding of protein with brilliant coomassie blue which changes reddish color of dye to deep blue and measured colorimetrically.

---

Fig. 1. Photomicrographs (<400) showing inflammatory cells infiltration scoring scale.

Fig. 2. Blood urea nitrogen and serum creatinine obtained from Sprague-Dawley rats treated with vehicle, single dose of cisplatin (5 mg/kg, i.p.), cisplatin + montelukast (10 mg/kg/day, orally) or cisplatin + ONO-4057 (300 mg/kg/day, orally). Each drug was given for one week; starting 4 days before cisplatin injection. Values are means ± S.E.M. of 8 observations. *, + and # denote significant difference (P < 0.05) versus control, cisplatin and cisplatin + montelukast values, respectively.
Renal histopathological studies

Renal histopathological changes were assessed in kidney sections using the haematoxylin and eosin (H&E) stain. Masson’s trichrome staining was used for detection of renal fibrosis. The inflammatory cells infiltration score was assessed semiquantitatively as described in previous studies including ours (22, 27, 28) by examining 10 random sections (×400) from each kidney and scoring the inflammatory cells infiltration as absent (0), minimal (1) when covered 25% of examined fields, moderate (2) when covered 50% of examined fields and severe (3) when covered 75% of examined fields. Histology, scoring and conclusion were blindly evaluated to prevent influence on interpretation (Fig. 1).

Statistical analysis

Data were expressed as means ± SEM. Normal distribution was checked using column statistics (modified Kolmogorov-Smirnov test). Because data were normally distributed and included one independent variable (drug treatments) and multiple comparisons (more than two experimental groups), statistical significance was tested with the one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc-test. The analysis was performed using Graph Pad Prism, software release 3.02 (La Jolla, CA, USA). Probability levels P < 0.05 were considered significant.
Kidneys obtained from rats treated with cisplatin showed vacular degeneration of renal tubular epithelium, cystic dilatation of renal tubules, focal interstitial inflammatory cells infiltration (Fig. 3A), in addition to a significant increase in the inflammatory cells infiltration score compared with control rats (Fig. 3C). Staining with the Masson’s trichrome demonstrated interstitial fibrosis in kidneys of cisplatin-treated rats (Fig. 3B). Renal tissues of cisplatin + ONO-4057-treated rats showed focal interstitial inflammatory cells infiltration with vacular degeneration of renal tubular epithelium (Fig. 3A) while those of cisplatin + montelukast-treated rats revealed normal renal parenchyma with no histopathological changes. Furthermore, the significant increase in the inflammatory cells infiltration score induced by cisplatin was dramatically reduced in rats treated concomitantly with montelukast but not ONO-4057 (Fig. 3C).

The effects of cisplatin, cisplatin + montelukast and cisplatin + ONO-4057 on the levels of renal cys-LT (LTD₄) and LTB₄ are shown in Fig. 4. Acute cisplatin administration caused significant increases in renal levels of LTD₄ (3.9-folds) and LTB₄ (1.3-folds) compared with control rats. The co-administration of montelukast, but not ONO-4057, abolished the remarkable increase in renal LTD₄. On the other hand, ONO-4057, but not montelukast, restored the significant increase in renal LTB₄ to control values.

As shown in Figs. 5 and 6, cisplatin caused significant increases in renal contents of TNF-α, TGF-β1, TBARS, NOx, myeloperoxidase and caspase-3 activities and decrease in renal anti-inflammatory IL-10 and total SOD activity. The inflammatory, fibrotic, oxidative, and apoptotic effects of cisplatin were significantly reduced in rats treated concomitantly with the selective Cys-LTR antagonist montelukast in contrast to no effect for the LTB₄ receptor blocker ONO-4057 (Figs. 5 and 6).

DISCUSSION

Previous studies, including ours, demonstrated that activation of LOX pathway and its products play a role in mediating cisplatin nephrotoxicity (6, 7). Thus, here we investigated, for the first time, whether these products, namely Cys and non Cys-LTs, would differentially contribute to cisplatin-induced acute renal damage in rats. In line with previous studies (21, 29, 30), our results demonstrated the nephrotoxic effect of cisplatin, manifested as significant increase in the levels of serum creatinine and blood urea nitrogen and further confirmed histopathologically. Moreover, cisplatin-induced renal detrimental actions were associated with significant increases in renal LTD₄ and LTB₄ (the increase in LTD₄ is approximately 2.6-folds greater than that in LTB₄), inflammatory (TNF-α, myeloperoxidase), apoptotic (caspase 3), fibrotic (TGF-β1), and oxidative stress markers. The selective blockade of Cys-LTR with montelukast reversed the cisplatin-induced nephrotoxicity and restored all parameters near to control values (except renal LTB₄), in contrast to no effect for the LTB₄ receptor blocker ONO-4057 (despite that fact that, ONO-4057 restored the increased renal LTB₄ level to control values).

Several mechanisms were suggested to explain cisplatin-induced renal failure, including cisplatin accumulation in renal cells, generation of inflammatory cytokines, apoptosis, oxidative stress (29, 31) and recently increased LOX pathway products (6, 7). The Cys-LTs, play a functional role in mediating kidney functions, including glomerular filtration rate regulation and renal vasoconstriction (14, 32). Moreover, both LTB₄ and Cys-LTs are considered to be pro-inflammatory in many kidney disorders as glomerulonephritis (32). Similarly, Noiri et al. documented the

RESULTS

Acute cisplatin administration (5 mg/kg, i.p.) resulted in a typical form of acute renal failure, manifested as significant increases in the levels of blood urea nitrogen and serum creatinine compared with control group (Fig. 2). Furthermore, the final body weights were significantly decreased in cisplatin-treated rats (data not shown). These effects of cisplatin were significantly reduced after concurrent administration of montelukast (Cys-LTR blocker) but not ONO-4057 (LTB₄ receptor blocker) (Fig. 2).
important role of LTB₄ in mediating acute renal ischemic-reperfusion injury, via activating neutrophils and promoting their binding to endothelium by inducing cell-adhesion molecules (33). However, according to the results of the current study, cisplatin administration seemed to shift the LOX pathway towards more Cys-LTs production (3-folds increase). This may be attributed to the elevation in renal TNF-α level, associating cisplatin administration, known to stimulate the arachidonate cascade towards Cys-LTs production in vivo (34). Moreover, other studies suggested that increased levels of interleukins during inflammation, may coordinately increase Cys-LTs generation in vivo probably via the up-regulation of LTC₄ synthase, shifting the LOX pathway towards Cys-LTs production (15, 35). Indeed, the role of elevated TNF-α/other inflammatory interleukins had been documented in mediating cisplatin acute renal damage. Being a pro-inflammatory cytokine, TNF-α is involved in the early stages of the inflammatory response initiating a cascade of other mediators such as IL-6, IL-8 and IL-10. Furthermore, TNF-α may activate the apoptotic pathways in a variety of cells mediating further cellular damage (36).

The conclusion that Cys-LTs predominately mediate cisplatin-induced acute renal damage was further supported by the fact that selective blockade of Cys-LTR with montelukast reversed the cisplatin-induced nephrotoxicity, even in the presence of elevated renal LTB₄. In spite the fact that, previous studies demonstrated the protective role of montelukast against cisplatin-induced renal damage (37-39). The current study, for the first time, shed light upon the differential contribution of the LOX pathway products, Cys and non Cys-LTs, to cisplatin-induced nephrotoxicity, using montelukast as a pharmacologic tool rather than a protective modality, correlating its effect to restoring the renal LTD₄ level. Furthermore, the current study differs from previous ones employing montelukast in many aspects. For instance, Beytur et al. attributed the beneficial effect of montelukast to its antioxidant and anti-inflammatory properties (37). Beytur et al. suggested that the antioxidant mechanism may be due to direct elimination of free oxygen radicals or direct increase in the antioxidant enzymatic activities (37). One limitation to this study was that no antioxidant enzymes were measured. Our results supported this suggestion.
and further confirmed the antioxidant ability of montelukast, being able to reverse the cisplatin-induced reduction in total SOD activity. Restored SOD activity will subsequently prevent the inactivation of NO, decreasing renal peroxynitrite and TBARS. Moreover, Beytur et al. reported that montelukast exerted only therapeutic effects against cisplatin-induced acute renal damage without any protective effect (37). However, here we reported the renoprotective effect of montelukast against cisplatin-induced acute renal damage. This discrepancy may be attributed to the difference in the dosing schedule, where they used two treated groups in which cisplatin was either given after the 10-days treatment of montelukast or montelukast administration was started 3 days after the cisplatin dose (37).

Suddek, (38) also demonstrated the renoprotective effect of montelukast against cisplatin-induced nephrotoxicity. Suddek attributed this action again to the antioxidant (restoring TBARS, SOD activity and reduced glutathione) and anti-inflammatory effects of montelukast (38), an assumption that was not supported by measuring renal levels of Cys-LTs or any other inflammatory markers. On the contrary, the current study related the protective effect of the Cys-LTR blocker montelukast to its ability to decrease the renal Cys-LTs-mediated inflammation, manifested as reduced renal levels of TNF-α and myeloperoxidase and increased anti-inflammatory IL-10.

Most recently, Gad et al. (39) conducted a deeper mechanistic investigation about the ameliorative effect of montelukast against cisplatin-induced nephrotoxicity attributing this effect to its anti-inflammatory and anti-apoptotic actions (39). According to Gad et al., the selective blockade of Cys-LTR reduced the vasoconstrictor effect of LTD₄/LTC₄, halting the activation of nuclear factor-κB (39, 40) known to upregulate several genes promoting NO, TNF-α, and the early tubular injury mediator monocyte chemoattractant protein-1 (MCP-1) generation (39, 41). MCP-1 is known to further stimulate leukocyte infiltration and more TNF-α production. Thus, decreased MCP-1 and TNF-α production would eventually lead to decreased caspase-3 activity with subsequent apoptosis (39). In line with Gad et al. (39), the current results further confirmed the anti-inflammatory and anti-apoptotic effects of montelukast. However, here we confirmed for the first time the causal

Fig. 6. Renal thiobarbituric acid reactive substances (TBARS, Panel A), nitrite/nitrate (NOx, Panel B), total superoxide dismutase (SOD, Panel C), and caspase-3 activities (Panel D) in kidney homogenates obtained from Sprague-Dawley rats treated with vehicle, single dose of cisplatin (5 mg/kg, i.p.), cisplatin + montelukast (10 mg/kg/day, orally) or cisplatin + ONO-4057 (300 mg/kg/day, orally). Each drug was given for one week; starting 4 days before cisplatin injection. Values are means ± S.E.M. of 8 observations. *, † and # denote significant difference (P < 0.05) versus control, cisplatin and cisplatin + montelukast values, respectively.
relationship between the anti-inflammatory/anti-apoptotic effects of montelukast and the selective blockade of Cys-LTR/subsequent decrease in renal level of LTD4. Moreover, the notion that the selective blockade of the LTB4 receptors with ONO-4057 failed to reverse the cisplatin-induced renal detrimental effects, in spite of decreasing the renal level of LTB4 to control values, further confirms the conclusion that Cys-LTs pathway predominantly mediates cisplatin-induced acute renal damage in rats.

Acknowledgements: Special thanks to Ono Pharmaceutical Co. (Osaka, Japan) for generously supplying us with ONO-4057, and to Dr. Kawkab A. Ahmed, Department of Pathology, Faculty of Veterinary Medicine, Cairo University, for assistance in the histopathology.

Conflict of interests: None declared.

REFERENCES

16. Sharma JN, Mohammed LA. The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets? *Inflammopharmacology* 2006; 14: 10-16.


Received: September 4, 2018
Accepted: October 30, 2018

Author’s address: Dr. Mai M. Helmy, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Alazarita, 21521, Alexandria, Egypt.
E-mail address: meeshoo7@hotmail.com