Title Page

Title of the manuscript:

Benzbromarone mitigates cisplatin nephrotoxicity through enhanced peroxisome proliferator-activated receptor-alpha (PPAR-α) expression

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Abstract

Aim: Despite the great efficacy reported for cisplatin as a widely used chemotherapeutic agent, its clinical use is limited by the challenge of facing its serious side effect; nephrotoxicity. In this study, the effect of the benzbromarone on peroxisome proliferator-activated receptor-alpha (PPAR-α) was investigated against cisplatin nephrotoxicity.

Main methods: Rats were administered benzbromarone (10 mg/kg/day; p.o.) for 14 days, and cisplatin (6.5 mg/kg; i.p.) as a single dose on the 10^{th} day. Blood and kidney tissue samples were collected for determination of kidney function, biochemical and molecular markers, as well as histopathological investigation.

Key findings: Benzbromarone improved kidney function, that was evidenced by reduced serum creatinine and blood urea nitrogen to nearly the half, compared to the group administered cisplatin alone. The protein expression of PPAR-α was enhanced with benzbromarone treatment, along with a considerable suppression of oxidative stress as benzbromarone reduced mRNA expression of NADPH oxidase, while increased the anti-oxidant HO-1 protein expression through enhancing Nrf2. Besides, it displayed a marked anti-inflammatory effect via suppression of p38 MAPK/NF-κB p65 signaling pathway and its downstream targets. Moreover, benzbromarone retarded apoptosis through reducing the pro-apoptotic (Bax) and enhancing the anti-apoptotic (Bcl-2) protein expressions. The protective effects of benzbromarone were also confirmed by histopathological results.

Significance: Our data confirm the relation between PPAR-α, and the deleterious effects induced by cisplatin. It can also be suggested that enhancing PPAR-α expression by benzbromarone is a promising therapeutic approach that overcomes cisplatin nephrotoxicity through regulation of different signaling pathways: Nrf2/HO-1, p38 MAPK/NF-κB p65, and Bax/Bcl-2.

Keywords: Cisplatin nephrotoxicity; Benzbromarone; PPAR-α expression; Nrf2/HO-1; p38 MAPK/NF-κB p65; Bax/Bcl-2

Abbreviations:

PPAR-α, Peroxisome proliferator-activated receptor-alpha; BUN, Blood urea nitrogen; ROS, Reactive oxygen species; NADPH, Nicotinamide adenine dinucleotide phosphate; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor erythroid 2-related factor 2; HO-1, Heme oxygenase-1; GSH, Reduced glutathione; NO, Nitric oxide; NO_x, Total nitrate/nitrite; MDA, Malondialdehyde; p38 MAPK, p38 Mitogen-activated protein kinase; NF-κB p65, Nuclear factor kappa-light-chain-enhancer of activated B cells p65; TNF-α, Tumor necrosis factor-alpha; IL-6, Interleukin-6; MCP-1, Mnocyte chemotactic protein-1; CMC, Carboxymethyl cellulose; H & E, Hematoxylin and eosin.

1. Introduction

Cisplatin is considered as one of the most applicable chemotherapeutic agents [1], which participates a crucial role in struggling against a diversity of neoplastic diseases such as non-small cell lung cancer, ovarian, and breast cancers [2]. However, studies demonstrated that cisplatin causes dose-dependent nephrotoxicity and tubular dysfunction [3, 4]. For many years, scientists made a great effort for developing other platinum (Pt) derivatives including carboplatin and oxaliplatin associated with fewer side effects, but they offer much less therapeutic spectrum. Therefore, the research is still standing for getting new adjunct treatments that can mitigate cisplatin nephrotoxicity [5].

The molecular mechanisms of nephrotoxicity induced by cisplatin are complicated and multifactorial including various processes, such as reactive oxygen species (ROS) production, inflammatory response, as well as activation of apoptotic cascade leading to cell death [6, 7]. Several studies cleared that accumulation of cisplatin in the mitochondria of renal cells results in ROS production via phosphorylation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, while a significant decline in the antioxidant nuclear factor erythroid-2 related factor 2 (Nrf2), and consequently heme oxygenase-1 (HO-1) [8, 9]. Meanwhile, the decrease in renal anti-oxidant defenses sequentially triggers apoptosis by elevation of the pro-apoptotic protein Bax, together with a decline in the anti-apoptotic protein Bcl-2, that has been tightly related to the deleterious effects of cisplatin [7].

Additionally, inflammation is considered as a prime event in the pathogenesis of cisplatin nephrotoxicity, that leads to an increase of various inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) [2, 7].

Benzbromarone is a benzofuran derivative, which is a potent uricosuric agent that has been widely used for more than 25 years for prophylaxis of acute gouty attacks [10, 11]. It has been classified as a peroxisome proliferator-activated receptor-alpha (PPAR-α) agonist [12], that accounts for its potential nephroprotective effect. PPAR-α is a subtype of PPAR nuclear receptors, which is involved in the modulation of different cellular functions including lipid metabolism, glucose homeostasis, and inflammation [13]. Furthermore, renal PPAR-α critically participates in defense against cisplatin-induced oxidative stress, inflammatory response, as well as initiation of apoptotic cascade [14-16]. Benzbromarone has been reported to exhibit anti-oxidant effect [17, 18], together with anti-inflammatory and anti-apoptotic responses [19-21]. Therefore, benzbromarone was predicted to be a novel and promising therapeutic agent to prevent cisplatin-induced nephrotoxicity.

2. Materials and methods

2.1. Animals

Male Wistar rats (130-160 g) were purchased from the Animal House of the Holding Company for Biologics and Vaccines-VACSERA (Giza, Egypt). Animals were subjected to controlled conditions including: temperature at 23 ± 2 ºC, normal photoperiod (12-h light/12-h dark cycles), while humidity was 60 ± 10%. The animals were maintained on standard pellet diet and water throughout the experimental work. All animal experiments were according to the National Institutes of Health guide for the care and use of laboratory animals, and were also approved by Institutional Animal Care and Use Committee, Beni-Suef University, Egypt (BSU-IACUC 018-45).

2.2. Drugs and chemicals

Cisplatin was purchased from Mylan (Rockford, USA). Benzbromarone was obtained from Marcyrl Pharmaceutical Industries (El-Obour City, Egypt) as a gift. Carboxymethyl cellulose (CMC) and all other chemical reagents used in this study were of highest analytical grade commercially available.

2.3. Experimental design

Rats were randomly allocated into four groups, each consisting of eight animals, and treated as follows:

- Group I (Normal): received vehicle (0.5% CMC) p.o. for 14 consecutive days and on the 10th day received a single i.p. injection of isotonic saline.
- Group II (Cisplatin): received vehicle (0.5% CMC) p.o. for 14 consecutive days and on the 10th day received a single i.p. injection of cisplatin (6.5 mg/kg) [22].
- Group III (Benzbromarone+Cisplatin): received benzbromarone (10 mg/kg/day; p.o.) [23, 24] for 14 consecutive days in combination with a single i.p. injection of cisplatin (6.5 mg/kg) on the 10th day.
- Group IV (Benzbromarone): received benzbromarone (10 mg/kg/day; p.o.) for 14 consecutive days and on the 10th day received a single i.p. injection of isotonic saline.

On the 14th day, blood and kidney samples were collected for biochemical and molecular investigations. Serum was separated at 4000 ×g at 4˚C, then stored at -20˚C till estimation of creatinine and blood urea nitrogen (BUN) levels. Kidney samples were rapidly snap-frozen in liquid nitrogen, and then stored at -80˚C till their use. To prepare the homogenate of kidney tissue, a section of singular kidney was weighed to get one gram tissue that was homogenized in ice-cooled PBS with added protease inhibitor. The homogenate was then centrifuged for 10 minutes at 4000 ×g at 4˚C to separate the supernatant, which was utilized for measurement of GSH, malondialdehyde (MDA), total nitrate/nitrite

(NOx), HO-1, TNF-α, and IL-6 levels. The remaining portion of the kidney was left at -80˚C until PCR and western blot analysis. The latter kidney was drenched in 10% formol saline for histopathological investigation.

*2.4. **Estimation of kidney function***

Serum creatinine and BUN levels were estimated utilizing commercial quantitative diagnostic kits of Diamond Diagnostics (Egypt) according to the manufacturer's instructions [25, 26].

2.5. Assessment of oxidative stress biomarkers in renal tissue homogenates

Glutathione content was measured colorimetrically upon reaction with Ellman's reagent, as mentioned before [27, 28], Total nitrate/nitrite (NOx) was determined according to the procedure defined by Miranda et al. [29]. The lipid peroxidation product; MDA was measured depending on the color intensity resulting from reaction with thiobarbituric acid, as previously determined [28, 30]. Moreover, kidney HO-1 content was determined using BioVision's ELISA kit (USA).

2.6. Assessment of kidney inflammatory biomarkers

Interleukin-6 and TNF-α contents were measured in kidney tissue homogenates using ELISA kits of RayBio (USA), and Sigma-Aldrich (USA), respectively, according to-manufacturer's instructions.

2.7. Quantitative real-time PCR (qRT-PCR)

Total RNA was separated by Thermo Fisher Scientific kit. (GeneJET, Germany). Bioline kit (SensiFAST™ SYBR® Hi-ROX One-Step Kit, UK) was used to assess the quantitative real time-PCR (qRT-PCR). The sequence of primers for all the studied target genes and the reference housekeeping gene are shown in table 4. The thermal profile cycling of qRT-PCR was 45˚C/15 minutes one cycle for reverse transcription; cDNA synthesis. This was followed by the amplification step, which consisted of 40 cycles of denaturation at 95˚C/15 seconds, annealing at 60˚C/30 seconds and extension at 72˚C/30 seconds.

Then, the data were expressed in Cycle threshold (Ct). The PCR data sheet included Ct values of assessed genes (NADPH-oxidase, Keap1, p38 MAPK, NF-κB p65, MCP-1), and the reference gene (β-actin). The relative quantitation (RQ) was made for each target gene according to the calculation of delta-delta Ct (ΔΔCt).

2.8. Western blotting analysis

Preparation of kidney tissue lysate was carried out using RIPA lysis buffer (PL005, Bio Basic Inc, Marhham Ontario L3R 8T4, Canada). The buffer was supplemented with protease/phosphatase inhibitor cocktail to maintain protein integrity. TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE), which was provided by Bio-Rad Laboratories Inc (USA), was used to separate equivalent volumes of the extracted protein from all studied groups. Incubation with 5% non-fat dry milk, Tris-HCl, 0.1% Tween 20 at room temperature for 1 h, was performed to block the membranes, followed by incubation with PPAR-α, Nrf2, HO-1, Bax, Bcl-2, and β-actin primary antibodies (Santa Cruz Biotechnology, USA) at 4℃ overnight. Afterwards, the membranes were incubated with the appropriate secondary antibodies for 2 h at room temperature, followed by washing twice in 1x TBS-T. The immunoblots densitometric analysis was done to quantify the amounts of PPAR-α, Nrf2, HO-1, Bax, and Bcl-2 against β-actin, utilizing image analysis software on the ChemiDoc^{TM} MP imaging system (version 3) provided by Bio-Rad (Hercules, CA).

2.9. Histopathological examination

Kidney samples from different studied groups, were fixed in 10% buffered formalin solution in normal saline for 24 h. Afterward, the paraffin blocks were dehydrated in ethanol of graded concentrations (50-100%), and cleared in xylene. Paraffin sections, about 4-5 µm, were prepared and stained with Hematoxylin and Eosin (H & E) dye using standard techniques for photomicroscopic observations [31]. To evaluate the severity degree of the observed histopathological lesions, four point numerical scoring system was used, where 0 indicated no change, while 1, 2, and 3 indicated mild,

moderate, and severe changes, respectively) (Table5). The degree of severity was determined by percentage according to Arsad et al. [32] as follows: mild changes (<30%), moderate changes (30–50%), and severe changes (>50%).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc, USA). Data were expressed as a mean ± standard error of the mean (SEM). To identify statistical significance, one-way Analysis of Variance (ANOVA) test was applied in order to detect statistical difference, followed by Tukey-Kramer post hoc test. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1. Benzbromarone ameliorates the elevation in serum creatinine and BUN levels induced by cisplatin

In the present study, cisplatin induced evidenced renal nephrotoxicity, as indicated by significant elevation of serum creatinine and BUN, in comparison with the normal control group (Fig. 1). Contrariwise, pretreatment with benzbromarone afforded a significant refinement of renal functions, in comparison with cisplatin group. That was indicated by pronounced decrease in creatinine and BUN levels to about (50%) and (51%), respectively.

3.2. Benzbromarone counteracts oxidative stress induced by cisplatin and enhances anti-oxidant defenses in kidney

Intoxication with cisplatin was accompanied by oxidative stress in rats' kidneys. This was evidenced by an elevation of MDA, and a decline in GSH and NOx contents, as compared to normal rats. On the other hand, pretreatment with benzbromarone showed a great protective effect against cisplatin-induced renal oxidative stress as demonstrated by prominent decrease in MDA content to about 35% and restoring GSH and NOx contents to about 2.6 and 4.4 fold, respectively. Moreover, pretreatment with

benzbromarone resulted in an increase of HO-1 level to about 4.2 fold, compared to cisplatin control group, thus confirming the anti-oxidant effect afforded by benzbromarone (Table 1).

3.3. Benzbromarone alleviates the inflammatory events of nephrotoxicity induced by cisplatin

The acute inflammatory response evoked by cisplatin is regarded as an initial step in the pathogenesis of nephrotoxicity. That was indicated by the increase of TNF-α, which is an essential cytokine implicated in cisplatin-induced acute kidney injury, that promotes the release of other pro-inflammatory cytokines like an elevation of IL-6 (Table 2). Pretreatment with benzbromarone significantly reduced renal TNF-α and IL-6 contents to about 30% and 35%, respectively, thus exerting a reasonable anti-inflammatory effect.

3.4. Effects of benzbromarone on relative mRNA expressions of NADPH oxidase, Keap1, p38 MAPK, NF-κB p65, and MCP-1 in cisplatin treated rats

Cisplatin promotes cell death through inflammatory cascade and apoptosis. Besides, it triggers excessive production of ROS by increasing the expression of the superoxide-generating enzyme; NADPH oxidase. Therefore, mRNA expression of the inhibitory Keap1 which controls the release and nuclear translocation of the anti-oxidant Nrf2 was measured. We further demonstrated the involvement of p38 MAPK, since cisplatin-mediated ROS production activates p38 MAPK, which subsequently contributes to the initiation of the inflammatory events. That was investigated through assessing mRNA expressions of p38 MAPK, NF-κB p65, and MCP-1. According to the results of our study, the administration of benzbromarone significantly diminished relative mRNA expressions of NADPH oxidase, Keap1, p38 MAPK, NF-κB p65, and MCP-1 to about 71%, 55%, 53%, 49%, and 33%, respectively (Table 3).

3.5. Western blot demonstrating effects of benzbromarone on renal protein expressions of PPAR-α, Nrf2, HO-1, Bax, and Bcl-2 in cisplatin treated rats

According to the results of western blot analysis (Fig. 2A), the protein expression of renal PPAR-α was reduced by cisplatin administration. Benzbromarone guarded against cisplatin nephrotoxicity through up-regulation of PPAR-α expression (3 fold) (Fig. 2B), which contributes to alleviation of oxidative insult, inflammatory response, and apoptosis.

The present study showed that administration of cisplatin caused a marked decrease of renal anti-oxidant defense as indicated by down-regulation of nuclear Nrf2 expression, as well as the anti-oxidant protein HO-1 expression which is substantial for the elimination and inactivation of ROS. Benzbromarone pretreatment guarded against oxidative stress, that was manifested by up-regulation of Nrf2 (3.1 fold) (Fig. 2C), and HO-1 (3.6 fold) expressions (Fig. 2D). These results confirm that benzbromarone exhibits a considerable anti-oxidant effect.

Cisplatin triggered cell death that was demonstrated by enhanced pro-apoptotic (Bax) and reduced anti-apoptotic (Bcl-2) protein expressions. These events were notably mitigated by benzbromarone which suppressed Bax (39%) (Fig. 2E) expression, but elevated Bcl-2 expression (5.3 fold) (Fig. 2F). That confirms the ability of benzbromarone to attenuate cisplatin-induced apoptosis.

3.6. Benzbromarone reduces cisplatin-evoked histopathologic modulations in renal tissues

Sections of kidney tissues of normal control rats showed renal glomeruli and tubules with normal histological structure (Fig. 3A). Similarly, the examination of kidney tissues of normal rats treated with benzbromarone (Fig. 3B) revealed a normal appearance of renal tubules and glomeruli along with high integrity of the tissue. Otherwise, the renal tissues of cisplatin administered rats showed severe histopathological alterations: congestion of inter-tubular blood vessels and glomerular capillaries, and the renal tubular epithelial lining showed diffuse swelling, vacuolar degeneration, and necrosis; where some of these cells appeared with pyknotic nuclei (Fig. 3C1). The hyaline cast was detected in the lumen of

some tubules, with necrosis, apoptosis, and desquamation of tubular epithelium, as well as pockets of inter-tubular hemorrhage (Fig. 3C2). The renal glomeruli showed parietal layer thickening of the Bowman's capsule, mesangial necrosis and hyalinization (Fig. 3C3). Renal medulla showed marked vacuolation and tubular lining necrosis, existence of hyaline cast in the lumen of some tubules and hyalinization of some tubules (Fig. 3C4). In regard to benzbromarone treated group, the examination of which showed a good protective effect: moderate vacuolar degeneration was found in the renal tubular epithelial lining, together with scattered necrotic cells that some of which showed pyknotic nuclei, as well as some regenerated cells (Fig. 3D1), and scarce apoptotic bodies (Fig. 3D2).

4. Discussion

As the number of deaths from cancer increases every year, therefore, the demand for cancer treatments became essential [1]. Cisplatin remains one of the most effective antitumor drugs until now. Despite its profound efficacy, the therapeutic use of cisplatin is frequently limited by nephrotoxicity which is considered as a chief cause for serious morbidity and mortality among patients [2, 33]

Benzbromarone is clinically used for the prophylaxis and treatment of acute gout attacks via the suppression of uric acid resorption by inhibition of urate transporter- 1(URAT1) [12], having a great effectiveness in patients with renal insufficiency rather than other uricosuric agents [34]. Benzbromarone has also shown a reputation as a renoprotective agent [20]. Furthermore, many studies confer the renoprotective effect of PPAR-α agonists against cisplatin nephrotoxicity [14, 15]. That presents a promising therapeutic way for benzbromarone to overcome nephrotoxicity induced by cisplatin via the concerted modulation of PPAR-α expression, resulting in anti-oxidant together with anti-inflammatory and anti-apoptotic responses**.**

Multiple lines of evidence advocated that cisplatin accumulation in the mitochondria of renal tubular cells renders the mitochondria more targeted than nuclear DNA. That leads to difference between renal cells in their sensitivity to cisplatin according to the mitochondrial density, with the renal proximal

tubules most affected due to their highest mitochondrial density within the kidney [7]. In these proximal tubular cells, fatty acids are considered as the main fountain of energy. Moreover, it has been reported that cisplatin causes disruption of energy production in the mitochondria of the proximal tubular cells by inhibiting the oxidation of fatty acids through reduced expression of PPAR-α [7, 35, 36].

Peroxisome proliferator-activated receptors-alpha are well-known as nuclear sensor receptors [37], that act mainly as transcriptional factors implicated in the regulation of numerous biological processes such as lipid and glucose metabolism, and energy consumption [38]. Besides, they have been emerged as important regulators of inflammatory signaling, particularly in the metabolic diseases [39]. Moreover, PPAR-α is most highly expressed in tissues where the chance of oxidative stress is higher such as liver, kidney, heart and skeletal muscles [40]. It has been confirmed by previous studies that cisplatin accumulation in the mitochondria is associated with down-regulation of PPAR-α expression and deletion of PPAR-α gene leads to retrogradation in acute kidney injury induced by cisplatin [7, 14]. On the other hand, our study consistent with previous reports [13-15, 41] suggests that treatment with a PPAR-α agonist such as benzbromarone could afford a great renoprotective effect, resulting in mitigation of the deleterious effects of cisplatin or any insult that precipitate acute kidney injury.

Previous studies highlighted the protective effects of PPAR-α agonists through their anti-oxidant properties. That was demonstrated by the reduced activity of renal NADPH oxidase and augmented HO-1 expression by means of activating Nrf2/Keap1 pathway, thus affording a considerable suppression of ROS production along with nitric oxide synthase (NOS) activation and enhancement of NO production [42-44]. Moreover, the PPAR-α agonists confer a remarkable anti-inflammatory effect, which was manifested by inhibiting the phosphorylation of MAPK and reducing its activity, in addition to inhibiting cisplatin-induced phosphorylation of IκBα with subsequently diminished expression of renal NF-κB and its downstream targets [39, 45]. Furthermore, modulation of the apoptotic pathway by down-regulation of the pro-apoptotic Bax protein, while up-regulating the anti-apoptotic Bcl-2 protein has also been reported as a protective mechanism of PPAR-α agonists [46, 47].

Till now, the mechanisms underlying nephrotoxicity induced by cisplatin have not been fully explained. Supportive evidence confirms that oxidative stress strongly contributes to cisplatin cytotoxicity via activation of NADPH oxidase. This is considered as the major biochemical pathway and cytosolic source for generation of ROS, which are harmful in excess; interfere with important cellular functions and cause damage to several cellular structures [2, 33]. Meanwhile, these ROS result in promoting lipid peroxidation, that was demonstrated by increased MDA level together with a decline in GSH content. In line with previous studies, cisplatin evoked a significant decline in renal NOx, which is indicative of NO generated by NOS, that may be through inhibiting the dephosphorylation of NOS, and so its activation [48]. Moreover, the increase in free radicals especially superoxide anion, which interacts with NO leading to its abstraction from the system, decreased vasodilation and glomerular filtration rate, with subsequent nephrotoxicity [49]. According to previous studies, cisplatin cytotoxicity is associated with down-regulation of Nrf2 expression which is a transcriptional factor essential for the regulation and activation of anti-oxidant proteins such as HO-1 [2, 9]. Similarly, our investigation revealed that Nrf2 and HO-1 expressions, which are implicated in neutralization of ROS in cisplatin-induced renal injury, were significantly reduced compared to normal control. On the other hand, mRNA expression of the inhibitory protein Keap1 was increased, that limits the release and nuclear translocation of Nrf2 [50]. Thus, enhancement of Nrf2 expression is regarded as an important molecular target of cytoprotective agents [51].

Importantly, pretreatment with the PPAR-α agonist benzbromarone suppressed lipid peroxides generation and enhanced cellular GSH and NOx production. In harmony with other studies [17, 18], these outcomes imply that the anti-oxidant properties of benzbromarone are strictly linked to the alleviation of renal damage induced by cisplatin. A likely explication for the mitigation of renal oxidative stress was the capability of the benzbromarone to down-regulate NADPH oxidase expression [18], which was significantly elevated in cisplatin-treated rats. In parallel, suppression of the redox-sensitive cascade of NF-κB, can give a share in the anti-oxidant action of benzbromarone [19, 21]. In the present study, the

alleviation of oxidative stress by benzbromarone was also demonstrated by enhanced protein expression of Nrf2 and the antioxidant HO-1, along with suppressed mRNA expression of the inhibitory Keap1. These findings emphasize that of previous studies [52, 53], concluding that PPAR-α agonists could afford renoprotective effect through the enhancement of anti-oxidant defense mechanisms**.**

It has been reported that cisplatin triggers an acute inflammatory response, which is regarded as a primary event that promotes cisplatin nephrotoxicity [6]. Interestingly, ROS, which are implicated in renal injury, participate in activation of various downstream proteins such as p38 MAPK that exhibits a critical role in promoting pro-inflammatory cytokines release and mediating cell apoptosis [7, 54]. Our investigation revealed that the inflammatory response, evoked by cisplatin administration, was indicated by increased mRNA expression of p38 MAPK, which is responsible for activation and nuclear translocation of NF-κβ. This results in production of pro-inflammatory cytokines such as TNF-α, which leads to the activation of other pro-inflammatory cytokines like IL-6 and MCP-1 [2, 6, 7]. Along the same line of evidence in previous studies, our results revealed a great anti-inflammatory action of benzbromarone indicated by suppression of the pro-inflammatory signaling pathway p38 MAPK/NF-κB p65 and its down-stream targets; TNF-α, IL-6 and MCP-1 [19, 21]. Moreover, benzbromarone modulated the balance between pro- and anti-apoptotic signals towards the anti-apoptotic cascade. That was evidenced by down-regulation of Bax protein expression, but up-regulation of Bcl-2 expression in renal tissues, which is definitely related to its renoprotective effect against cisplatin nephrotoxicity. These results are consistent with that of Jia et al. [21], who demonstrated that benzbromarone up-regulates Bcl-2 and consequently attenuates apoptosis in pancreatic β-cells by suppressing NF-κB signaling.

Li et al. [55] proved experimentally that increased PPAR-α expression in transgenic mice protects against acute kidney injury induced by cisplatin or ischemia/reperfusion. Moreover, using PPAR-α knock-out mice demonstrated a marked degree of oxidative stress, inflammation, and apoptosis than wild-type mice [56]. These findings illustrate the existence of an intrinsic anti-oxidant, anti-inflammatory, and anti-apoptotic mechanisms mediated directly by PPAR-α expression. In accordance

with these previous experiments, the nephroprotective effect of benzbromarone could be tightly linked to the enhanced expression of PPAR-α, rendering these receptors as important targets to overcome cisplatin nephrotoxicity.

5. Conclusion

In conclusion, this study elucidated for the first time that benzbromarone can attenuate the nephrotoxicity caused by cisplatin through enhanced PPAR-α expression, resulting in anti-oxidant effect via activation of Nrf2/HO-1 signaling pathway. Additionally, benzbromarone resulted in an anti-inflammatory response, through suppression of p38 MAPK/NF-κB p65 pathway and its downstream targets (TNF-α, IL-6, MCP-1), and rescued renal tissue from apoptotic cell death by reducing Bax/Bcl-2 ratio (Fig. 4). Therefore, prophylactic treatment with benzbromarone can provide a novel therapeutic strategy in attempting to control cisplatin nephrotoxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Benzbromarone improves kidney functions deteriorated by cisplatin in rats.
A) Serum creatinine levels, **B)** BUN levels. Rats received the treatment suspended in CMC for 14 days plus a cisplatin single dose or saline on 10^{th} day. Values are represented as the mean ± SEM (n = 6-8). One-way ANOVA was used for statistical analysis, followed by Tukey–Kramer post hoc test. [a] statistically significant from normal control at $P < 0.05$, [b] statistically significant from cisplatin at $P < 0.05$.

Fig. 2. Benzbromarone enhances renal protein expressions of PPAR-α, Nrf2, HO-1, Bcl-2, and suppresses that of Bax in cisplatin treated rats
A) Western blotting. **B-F)** Graphs representing changes in relative protein expressions (against β-actin) of: **B)** PPAR-α, **C)** Nrf2, **D)** HO-1, **E)** Bax, **F)** Bcl-2. Data are represented as the mean ± SEM (n = 3). One-way ANOVA was used for statistical analysis, followed by Tukey–Kramer post hoc test. [a] statistically significant from normal control at $P < 0.05$, [b] statistically significant from cisplatin at $P < 0.05$.

Fig. 3. Benzbromarone ameliorates renal histopathological injury induced by cisplatin in rats. **A.** Kidney of normal control rat showing normal histological structure of glomeruli (G) and tubules (T) (H&E, X200). **B.** Kidney of benzbromarone group showing normal appearance of tubules (T) and glomeruli (G) (H&E, X200). **C.** Kidneys of cisplatin administered rats showing, **C1:** congested interstitial vessels (C), diffuse swelling, vacuolar degeneration (short arrow), apoptosis (long arrow), and necrosis (dashed arrow) of the tubular epithelial linings, some of which appearing with pyknotic nuclei (arrow head) (H&E, X400), **C2:** congestion (C), pockets of inter-tubular hemorrhage (P), hyaline cast (H) in the lumen of some tubules, vacuolar degeneration, necrosis, apoptosis and desquamation of tubular epithelium (arrow) (H&E, X400), **C3:** Thickening of the parietal layer (arrow) of the Bowman's capsule, mesangeal necrosis (dashed arrow), as well as hyaline droplets (arrow head) among the degenerated tubular epithelium (H&E, X400). **C4:** renal medulla with marked vacuolation and necrosis of the tubular lining (arrow), presence of hyaline cast (dashed arrow) in the lumen of some tubules and hyalinization of some tubules (H) (H&E, X200). **D.** Kidneys of cisplatin administered rats that were treated with benzbromarone showing, **D1:** moderate vacuolar degeneration of the renal tubular epithelial lining, scattered necrotic cells (arrow) and some regenerated cells (H&E, X400). **D2:** scarce apoptotic bodies (arrow) and few pyknotic nuclei (dashed arrow) of the degenerated cells (H&E, X400).

Fig. 4. Overview of the molecular pathways of the protective effect of benzbromarone against cisplatin-induced nephrotoxicity in rats

Table legends

Table 1 Benzbromarone retards oxidative stress and enhances anti-oxidant defenses in kidneys of cisplatin treated rats

Groups	GSH (µM)	MDA (µM)	NO_x (µM)	HO-1 (ng/g tissue)
Normal	4.72 ± 0.31	1.70 ± 0.14	17.35 ± 0.35	2.61 ± 0.26
Cisplatin	1.37 ± 0.048[a]	4.69 ± 0.38[a]	4.35 ± 0.29[a]	0.73 ± 0.06[a]
Benzbromarone +Cisplatin	3.50 ± 0.31[b]	1.66 ± 0.16[b]	19.15 ± 1.86[b]	3.06 ± 0.09[b]
Benzbromarone	4.18 ± 0.38[b]	1.68 ± 0.15[b]	15.62 ± 0.86[b]	2.39 ± 0.18[b]

Rats received the treatment suspended in CMC for 14 days plus a cisplatin single dose or saline on 10th day. Values are represented as the mean ± SEM (n = 6). One-way ANOVA was for statistical analysis, followed by Tukey–Kramer post hoc test. [a] Statistically significant from normal control at $P < 0.05$, [b] Statistically significant from cisplatin at $P < 0.05$.

Table 2 Benzbromarone mitigates inflammatory biomarkers evoked by cisplatin in rats' kidneys
Rats received the treatment suspended in CMC for 14 days plus a cisplatin single dose or saline on 10th day. Values are represented as the mean ± SEM (n = 6). One-way ANOVA was used for statistical analysis, followed by Tukey–Kramer post hoc test. [a] statistically significant from normal control at $P < 0.05$, [b] statistically significant from cisplatin at $P < 0.05$.

Groups	TNF-α (pg/g tissue)	IL-6 (pg/g tissue)
Normal	58.73 ± 2.45	92.08 ± 6.62
Cisplatin	413.3 ± 26.12[a]	473.6 ± 37.11[a]
Benzbromarone+Cisplatin	123.4 ± 1.99[a,b]	168.0 ± 6.22[a,b]
Benzbromarone	59.10 ± 5.23[b]	87.58 ± 8.06[b]

Table 3 Benzbromarone alleviates relative mRNA expressions of cisplatin-induced NADPH oxidase, Keap1, p38 MAPK, NF-κB p65, and MCP-1 in renal tissues

Quantitative real time-PCR was used for determination of mRNA expressions, then relative quantitation (RQ) was made for each target gene to the reference gene (β-actin). Data are represented as the mean ± SEM (n = 3-6). One-way ANOVA was used for statistical analysis, followed by Tukey–Kramer post hoc test. [a] statistically significant from normal control at $P < 0.05$, [b] statistically significant from cisplatin at $P < 0.05$.

Groups	NADPH oxidase	Keap-1	p38 MAPK	NF-κB p65	MCP-1
Normal	1.49 ± 0.03	1.61 ± 0.07	1.61 ± 0.08	0.79 ± 0.03	0.51 ± 0.04
Cisplatin	4.24 ± 0.03[a]	3.41 ± 0.18[a]	4.72 ± 0.15[a]	2.82 ± 0.12[a]	2.98 ± 0.07[a]
Benzbromarone +Cisplatin	3.01 ± 0.18[a,b]	1.87 ± 0.03[b]	2.48 ± 0.16[a,b]	1.39 ±0.06[a,b]	0.98 ± 0.09[a,b]
Benzbromarone	1.44 ± 0.03[b]	1.54 ± .04[b]	1.68 ± 0.08[b]	0.77 ± 0.03[b]	0.49 ± 0.03[b]

Table 4 Primer sequence for all the studied target genes

Gene symbol	Primer sequence from 5′- 3′
NADPH oxidase	F: TTGTGGCACACTTGTTCAACCTGG
	R: TCACACGCATACAAGACCACAGGA
Keap1	F: TCAGCTAGAGGCGTACTGGA
	R: TTCGGTTACCATCCTGCGAG
P38 MAPK	F: GTGCCTATGAGCACCAAGTCAG
	R: TTCATGCTCTGGTTAGGGTGAG
NF-κB p65	F: GTCTCAAACCAAACAGCCTCAC
	R: CAGTGTCTTCCTCGACATGGAT
MCP-1	F: CTCACCTGCTGCTACTCATTC
	R: GCTTGAGGTGGTTGTGGAAAA
β-actin	F: ATGGATGACGATATCGCTGC
	R: CTTCTGACCCATACCCACCA

Table 5 Scoring of the main histopathological findings

Groups	Normal		Cisplatin		Benzbromarone + Cisplatin		Benzbromarone	
	No.	NS.	No.	NS.	No.	NS.	No.	NS.
Lesions								
1. Vacuolar degeneration of the tubular epithelium.	0	0	3	3[a]	3	1.3[b]	1	0.3[a]
2. Tubular epithelial necrosis.	0	0	3	3[a]	3	1.6[a,b]	1	0.3[b]
3. Hyaline droplets.	0	0	3	2.5[a]	2	1.3[a,b]	0	
4. Hyaline cast.	0	0	3	3[a]	2	1[b]	0	
5. Congestion of glomerular capillaries.	0	0	3	3[a]	2	0.6[b]	1	0.3[b]
6. Glomerular changes.	0	0	3	2.6[a]	3	1[b]	0	
7. Congestion of the inter-tubular blood capillaries.	0	0	3	2.5[a]	3	1[b]	1	0.3[b]
8. Inter-tubular pockets of hemorrhage.	0	0	3	2.3[a]	2	0.6[b]	0	

No: Animals' number that showed the lesion, NS: Numerical score.
Data are represented as the mean (n = 3). [a] statistically significant from normal control at $P < 0.05$, [b] statistically significant from cisplatin at $P < 0.05$.