Toxicology Reports 3 (2016) 153–159

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# Nrf2 activators as potential modulators of injury in human kidney cells

Amandla Atilano-Roque[a], Xia Wen[b], Lauren M. Aleksunes[b], Melanie S. Joy[a,c,*]

[a] Department of Pharmaceutical Sciences, Skaggs School of Pharmacy & Pharmaceutical Sciences, University of Colorado, 12850 E. Montview Blvd, Aurora, CO 80045, United States

[b] Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers University, 170 Frelinghuysen Rd, Piscataway, NJ 08854, United States

[c] Division of Renal Diseases and Hypertension, School of Medicine, University of Colorado, Anschutz Medical Campus, 12605 E. 16th Ave, Aurora, CO 80045, United States

ARTICLE INFO

*Article history:*
Received 2 November 2015
Received in revised form 7 January 2016
Accepted 7 January 2016
Available online 12 January 2016

*Keywords:*
Nrf2
Kidney
Nephrotoxicity
Cisplatin
Sulforaphane
Oltipraz
Oleanolic acid

ABSTRACT

Cisplatin is a chemotherapeutic agent used in the treatment of solid tumors, with clinical use often complicated by kidney toxicity. Nuclear factor (erythroid-derived-2)-like 2 (Nrf2) is a transcription factor involved in kidney protectant effects. The purpose of this study was to determine whether the Nrf2 activators oltipraz, sulforaphane, and oleanolic acid could protect human kidney cells against cisplatin-induced injury and to compare the protective effects between three Nrf2 activators. Human proximal tubule cells (hPTC) and human embryonic kidney 293 cells (HEK293) were exposed to cisplatin doses in the absence and presence of Nrf2 activators. Pre- and delayed-cisplatin and Nrf2 activator exposures were also assessed. Cell viability was enhanced with Nrf2 activator exposures, with differences detected between pre- and delayed-treatments. Both sulforaphane and oltipraz increased the expression of anti-oxidant genes *GCLC* and *NQO1*. These findings suggest potential human kidney protective benefits of Nrf2 activators with planned exposures to cisplatin.



## 1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum) is one of the most commonly used chemotherapy drugs for the treatment of solid tumors. Cisplatin and other related platinum-based therapeutics are effective against lung, head and neck, prostate, ovarian, and bladder cancers [1–3]. However, acute kidney injury can develop after a single dose. Existing clinical studies report reductions in estimated glomerular filtration rates, increases in urinary albumin excretion, and elevations in serum creatinine within 10 days following a cisplatin dose in 8–40% of patients [4–7]. Kidney disease manifestations can also include electrolyte wasting and persistent hypomagnesemia [8]. Nephrotoxicity may limit cisplatin's clinical use and resultant treatment efficacy. Current therapies, including fluid administration have variable efficacy in preventing kidney damage. Thus, interventions that can prevent or ameliorate kidney injury in human kidney cells exposed to cisplatin are warranted.

Several mechanisms contribute to the onset and pathogenesis of cisplatin-induced kidney injury, including vascular injury, inflammation, ischemia, oxidative stress, and tubular cell death [3,6,9–11]. Although cisplatin nephrotoxicity involves many different mechanisms, tubular cell death plays an important role in its progression. Studies using cultured renal tubular cells exposed to cisplatin demonstrated apoptotic and necrotic cell death [12]. These results were confirmed in animals, where both necrosis and apoptosis were induced in renal tubules following cisplatin administration [13–15]. Another study found that cisplatin administration in rats increases oxidative stress resulting in down-regulation of tight junction proteins and potentiation of proximal tubule damage [16]. Reducing exposure of tubule cells to cisplatin is an approach to limit kidney toxicity.

Prior research demonstrated enhanced kidney injury in Nrf2-null mice [17,18] suggesting a protective effect from this transcription factor. Under non-stressed conditions, Nrf2 is sequestered in the cytosol by kelch-like ECH associating protein 1 (Keap1) [19]. However, when oxidative stress, electrophilic stress, or the presence of Nrf2 activators becomes prevalent, Nrf2 and Keap1 dissociate resulting in accumulation of free Nrf2 in the cytosol and an increase in Nrf2 translocation into the nucleus [20]. Once in the

* Corresponding author at: The University of Colorado, Skaggs School of Pharmacy and Pharmaceutical Sciences, Department of Pharmaceutical Sciences, Mail Stop C238, Room V20-4108, 12850 E. Montview Blvd, Aurora, CO 80045, United States.
*E-mail address:* Melanie.Joy@ucdenver.edu (M.S. Joy).

http://dx.doi.org/10.1016/j.toxrep.2016.01.006

nucleus, Nrf2 heterodimerizes and binds to antioxidant response elements (ARE) which leads to the transcription of various cytoprotective and antioxidant genes [21]. Activation of transcription factors such as Nrf2 that regulate uptake and efflux transporters localized to kidney proximal tubule is a strategy to modulate cisplatin exposures [6,17]. As Nrf2 can regulate proteins involved in the metabolism and excretion of organic chemicals [22], treatment with Nrf2 activating compounds, including oltipraz, sulforaphane, and oleanolic acid, would be a plausible approach to limit exposure of human kidney cells to cisplatin. For the current study, we aimed to explore the therapeutic potential of known Nrf2 activators to modulate cisplatin-induced human kidney cell injury. Furthermore, we sought to determine optimal exposure regimens for Nrf2 activating compounds for nephrotoxicity prophylaxis and favorable effects on Nrf2 antioxidant genes with exposure to cisplatin.

## 2. Materials and methods

### 2.1. Cell culture & reagents

Human embryonic kidney 293 (HEK293, ATCC, Rockville, MD) and human proximal tubule epithelial (hPTC, ScienCell, Carlsbad, CA) cells were used for *in vitro* studies. HEK293 cells were routinely grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin per product information (Life Technologies, Grand Island, NY). Epithelial cell medium (EpiCM, ScienCell) supplemented with 2% fetal bovine serum, 1% epithelial cell growth supplement, and 1% Penicillin/Streptomycin was used to culture hPTC cells. Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% $CO_2$. Cisplatin (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) to 100 mM. Nrf2 activators (sulforaphane, oltipraz, and oleanolic acid) were purchased (Sigma Chemical Co.) and dissolved in DMSO. Cells were treated with Nrf2 activators either before or after cisplatin exposure. Unless specified otherwise, the doses of Nrf2 activators (sulforaphane, oltipraz, and oleanolic acid) were 5, 12, and 5 μM, respectively. RNA extraction kits were purchased from Life Technologies. PCR reagents and Taqman gene expression assays were obtained from Life Technologies.

### 2.2. Cell viability assay

HEK293 and hPTCs were plated in a 96-well configuration. Cells were incubated overnight at 37 °C in a humidified incubator with an atmosphere of 5% $CO_2$ prior to treatment. The cell treatments consisted of 1) 0.1% vehicle control (DMSO), 2) cisplatin at 0, 50, 80 μM doses ($LC_{50}$ 23.4–60 μM), 3) Nrf2 activator [sulforaphane (5 μM), oleanolic acid (5 μM), or oltipraz (12 μM)], or 4) cisplatin (at the above doses) combined with a Nrf2 activator at specified doses. Cells were incubated with Nrf2 activators for 12, 24, or 48 h beginning either 3 h prior to cisplatin or 3 h after initiation of cisplatin exposure. After the specified incubation time, MTT reagent (2 mg/mL) (Sigma Aldrich) in DMEM or EpiCM containing no FBS was added and cells were incubated for 4 h at 37 °C. MTT reagent was removed after the 4 h incubation period, MTT solubility solution (2% HCl, 25% $H_2O$, 73% 2-propanol) was added and cell viability was analyzed at 550 nm by VersaMax microreader plate (Molecular Devices, Sunnyvale, CA).

### 2.3. Gene expression

Gene expression was only evaluated in hPTCs to enable a closer *in vitro* approximation to humans. The expression of *NFE2L2*/NRF2 and detoxifying enzymes *GCLC* and *NQO1* were evaluated in hPTCs that received Nrf2 activators before and after treatment with cisplatin. For these studies cisplatin doses of low, moderate, and high were chosen. The cell treatments consisted of 1) 0.1% vehicle control (DMSO), 2) cisplatin at 0, 5, 25 and 80 μM doses, 3) Nrf2 activator [sulforaphane (5 μM), oleanolic acid (5 μM), or oltipraz (12 μM)], or 4) cisplatin (5, 25, 80 μM) combined with a Nrf2 activator at the specified doses. Cells were harvested for gene expression studies at 12, 24, or 48 h post treatment. Cells were collected and lysed and total mRNA prepared from cell lysates using Ambion RNA Extraction Kit (Life Technologies). cDNA was generated using Taqman reverse transcription reagents (Applied Biosystems) and an Applied Biosystems 2720 thermal cycler. Commercial gene expression assays: *GCLC* (Hs00155249), *NQO1* (Hs02512143), *NFE2L2* (Hs00975961), and housekeeping gene *GAPDH* (Hs02758991) were used (Applied Biosystems). Real-time PCR was performed using the 7500 Real Time PCR system. Generated data was analyzed by relative quantitation using the comparative $C_T$ method ($2^{-\Delta\Delta CT}$).

### 2.4. Statistical analysis

The data were expressed as mean ± SEM. Graphs were created using Graph Pad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Analysis was performed using GraphPad InStat 3.0. Data were analyzed using the one-way analysis of variance with Student–Newman–Keuls *post hoc* test for groups of 3 or more. Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Cisplatin toxicity: exposure of hPTC and HEK293 cells to cisplatin

We analyzed the viability of hPTCs and HEK293 cells following exposure to cisplatin (50 and 80 μM) for 12, 24, or 48 h. Cisplatin doses of 50 μM and above resulted in significantly lower hPTC and HEK293 cell viability. hPTCs and HEK293 cells treated with cisplatin alone demonstrated decreased survival over time, with hPTCs demonstrating more sensitivity to cisplatin (Fig. 1A & B). After 12, 24, and 48 h incubation with cisplatin, only 59%, 57% and 5% of hPTCs treated with 50 μM cisplatin were viable compared to vehicle controls, respectively (Fig. 1A). HEK293 cells incubated with cisplatin (50 μM) exhibited 75% viability during the first 12 h of exposure (Fig. 1B). After 24 and 48 h incubation with cisplatin (50 μM), cell viability was 57% and 18%, respectively (Fig. 1B). Cell viability was further decreased in hPTCs and HEK293 cells treated with 80 μM cisplatin (Fig. 1A & B).

### 3.2. Cisplatin toxicity: pre- and delayed-treatment with NRF2 activators

hPTCs cells were treated before (pre-treatment) or after (delayed treatment) cisplatin (50 and 80 μM) with Nrf2 activators for 12, 24, and 48 h, respectively. Preliminary cell viability experiments determined the optimal Nrf2 activator doses for sulforaphane, oleanolic acid, and oltipraz to be 5 μM, 5 μM, and 12 μM, respectively. hPTCs treated with 80 μM cisplatin had decreased cell viability compared to vehicle controls (Fig. 2A). hPTCs pre-treated with oltipraz or oleanolic acid had greater cell viability relative to cisplatin 80 μM alone treated cells (Fig. 2A). Sulforaphane demonstrated less impressive results than oltipraz and oleanolic acid when compared to cisplatin 80 μM alone and vehicle control treated cells. However, pre-treatment with sulforaphane displayed greater viability at 24 h and 48 h relative to cisplatin alone treated cells. Delayed-treatment with oltipraz and oleanolic acid at 12 and 24 h demonstrated higher cell viability as compared to cisplatin 80 μM alone treated cells (Fig. 2B). Delayed-treatment with