# RETINOIC ACID RECEPTOR-BETA PREVENTS CISPLATIN-INDUCED PROXIMAL TUBULAR CELL DEATH

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**Abbreviations**: ATRA: *All*-trans-retinoic acid; BG: bromocresol green; BrdU: 5'-Br-2'-deoxyuridine; DIDS: 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; $iPGE_2$: intracellular prostaglandin $E_2$; PGT: prostaglandin uptake transporter; PI: propidium iodide; PTC: proximal tubular HK-2 cells; RAR-β: retinoic acid receptor-β; VDAC1: voltage-dependent anion channel 1.

Keywords: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid;, cisplatin, prostaglandin $E_2$; human adenocarcinoma cells; proximal tubular cells ; retinoic acid receptor-β.

SUMMARY

Cisplatin's toxicity in renal tubular epithelial cells limits the therapeutic efficacy of this antineoplastic drug. In cultured human proximal tubular HK-2 cells (PTC) a prostaglandin uptake transporter (PGT)-dependent increase in intracellular prostaglandin $E_2$ ($iPGE_2$) mediates cisplatin's toxicity (i.e. increased cell death and loss of cell proliferation) so that it is prevented by PGT inhibitors. Here we found in cisplatin-treated PTC that 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a PGT inhibitor, prevented cisplatin's toxicity but not the increase in $iPGE_2$. Because expression of retinoic acid receptor-β (RAR-β) is dependent on $iPGE_2$ and because RAR-β is a regulator of cell survival and proliferation, we hypothesized that RAR-β might mediate the protective effect of DIDS against cisplatin's toxicity in PTC. Our results confirmed this hypothesis because: i) protection of PTC by DIDS was abolished by RAR-β antagonist LE-135; ii) DIDS increased the expression of RAR-β in PTC and prevented its decrease in cisplatin-treated PTC but not in cisplatin-treated human cervical adenocarcinoma HeLa cells in which DIDS failed to prevent cisplatin's toxicity; iii) while RAR-β expression decreased in cisplatin-treated PTC, RAR-β over-expression prevented cisplatin´s toxicity. RAR-β agonist CH55 or RAR pan-agonist all-trans retinoic acid did not prevent cisplatin´s toxicity, which suggests that RAR-β does not protect PTC through activation of gene transcription. In conclusion, RAR-β might be a new player in cisplatin-induced proximal tubular injury and the preservation of its expression in proximal tubules through treatment with DIDS might represent a novel strategy in the prevention of cisplatin's nephrotoxicity without compromising cisplatin's chemotherapeutic effect on cancer cells.

## INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II) has a significant antitumor effect in various solid tumors including prostate, ovarian, non-small cell lung, head and neck, testicular and uterine cervical carcinoma, yet its clinical application is limited because its nephrotoxicity (25–40% of treated patients) [1].Cisplatin can induce acute kidney injury, an urgent condition with a high mortality, in which renal proximal tubular injury and cell death play a relevant role. Apoptotic cell death is induced by a low concentration of cisplatin, while necrosis is induced by a higher concentration [2].

We have previously found in cultured human proximal tubular HK-2 cells (PTC) that intracellular prostaglandin $E_2$ ($iPGE_2$) increases following treatment with cisplatin and that this event is crucial for the induction of apoptosis and the inhibition of cell proliferation [3]. Given that newly synthesized $PGE_2$ is quickly released to the extracellular medium, any increase in $iPGE_2$ requires the return of extracellular $PGE_2$ to the inside the cell. This task is mainly accomplished by the prostaglandin uptake transporter (PGT) [4] and, consequently, its inhibition prevents cisplatin-induced apoptosis and loss of cell proliferation in PTC [3]. Importantly, the tumoricidal effect of cisplatin on human cervical adenocarcinoma HeLa cells is not affected by inhibition of PGT [3]. Thus, treatment with PGT inhibitors might represent a novel strategy in the prevention of cisplatin's nephrotoxicity.

In the present work we analyzed the preventive effect of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of PGT [5], on cisplatin's toxicity in PTC. Our results indicated that DIDS prevented the increase in cell death and the loss of cell proliferation, but not the increase in $iPGE_2$, induced by cisplatin. Therefore, we explored an alternative mechanism that could explain the protective effect of DIDS. We focused our attention in transcription factor retinoic acid receptor-β (RAR-β) -one of the three subtypes of retinoic acid receptors- because its expression is regulated by $iPGE_2$ in PTC [6] and because RAR-β has been previously shown to affect cell survival in several contexts such as cancer [7], axonal regeneration in corticospinal neurons after corticospinal tract injury [8] or oxidation-induced motor neuron death [9]. Our results revealed that cisplatin inhibited the expression of RAR-β in PTC, which was prevented by DIDS, and that RAR-β -most likely through a mechanism that does not involve transcriptional regulation- not only mediates the preventive effect of DIDS against cisplatin's toxicity but that over-expression of RAR-β itself is enough to protect PTC. Importantly, DIDS did not inhibit the antineoplastic effect of cisplatin on human adenocarcinoma HeLa cells, probably because it was unable to prevent the loss of RAR-β expression in cisplatin-treated HeLa cells. This suggests that the preventive effect of DIDS on cisplatin-induced PTC injury might be cell-specific so that DIDS would not interfere with the cytotoxic effect of cisplatin on cancer cells.

In conclusion, our results strengthen the idea that RAR-β might be a therapeutic target against cisplatin-induced acute kidney injury and underscore the potential of the pharmacological preservation of the expression of RAR-β in proximal tubules -through treatment with DIDS or other drugs- as a novel therapeutic strategy for the prevention of cisplatin's nephrotoxicity.

## MATERIALS AND METHODS

### *Reagents and antibodies*

*All*-trans-retinoic acid (ATRA), cisplatin, bromocresol green (BG), hypoxia-inducible factor 1α inhibitor YC-1, 5'-bromo-2'-deoxyuridine (BrdU), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), MTT reactive (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), antibody anti-β-actin, anti-mouse IgG and anti-rabbit IgG peroxidase conjugated from Sigma Aldrich (St. Louis, MO); antibodies anti-$PGE_2$, and anti-RAR-β from Abcam (Cambridge, UK); antibody anti-BrdU and annexin-V–FITC/Propidium iodide (PI) apoptosis detection kit from BD Biosciences (Palo Alto, CA); antibody anti-cleaved caspase-3 was from Cell Signaling

Technology (Leiden, The Netherlands); Pierce BCA-200 Protein Assay Kit was from ThermoFisher. Grand Island, NY, USA. ProLong® with DAPI, antibodies anti-mouse-Alexa-Fluor® 488 and anti-rabbit-Alexa-Fluor® 488 and 568 were from Invitrogen (Carlsbad, CA). RAR-β agonist CH55 and RAR-β antagonist LE-135 were a generous gift from Prof. Hiroyuki Kagechika (Tokyo Medical and Dental University, Japan); Lipofectamine 2000 reagent from Thermo Fisher Scientific (Waltham, MA); CANFAST Transfection Reagent kit (Canvax Biotech S.L., Córdoba, España).

*Cell culture*

Human proximal tubular epithelial HK-2 cells (PTC) and human cervical adenocarcinoma HeLa cells were purchased from American Type Culture Collection (Rockville, MD). PTC were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10.000 units/ml)/streptomycin (10 mg/ml)/amphotherycin B (25 µg/ml) and 1% Insulin (10 mg/l), Transferrine (5,5 mg/l) and Selenium (5 µg/l) (Sigma, St. Louis, MO). HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin/amphotherycin B. The culture was performed in a humidified 5% $CO_2$ environment at 37°C. In all the experiments, cells were plated at 70-90% confluence.

*Immunofluorescence analysis of $iPGE_2$ and cleaved caspase-3*

Cells were plated in coverslips and pretreated with DIDS (100 or 200 µM) for 1 h and then treated with 25 µM cisplatin for 24 h. Before the treatments, PTC and HeLa cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0,1% Triton X-100 (Sigma Aldrich, San Louis, MO) for 10 min and blocked for 1 h with 4% bovine serum albumin (BSA) (ChemCruz®, Santa Cruz, San Diego, CA) at room temperature. Afterwards, cells were incubated overnight at 4°C with anti-cleaved caspase-3 (1:50 dilution) or anti-$PGE_2$ (1:100) antibodies, then cells were incubated with α-rabbit-Alexa-Fluor® 568 or α-rabbit-Alexa-Fluor® 488 for 1 h in the dark at room temperature. Finally, the coverslips were washed and mounted with ProLong with DAPI. Detection was done by Zeiss LSM70 inverted confocal though the Confocal and Optical Microscopy Service (SMOC) of the Centro de Biología Molecular Severo Ochoa (CBMSO, Madrid, Spain). The percentage of cleaved caspase-3 positive cells was determined through manual count by examining, in a blind manner, five fields in each experimental condition, whereas $iPGE_2$-dependent immunofluorescence intensity was quantified after digital capture using Image-J software.

*Scanning electron microscopy*

Cells were grown on 12 $mm^2$ cover glass and treated with cisplatin or DIDS and cisplatin. Cells were fixed in 1.5% glutaraldehyde in phosphate-buffered saline for 30 min and, after washing twice with PBS, the samples were dehydrated in graded ethanol solutions for 5 min each (50%, 70%, 80%, 95%, and 100%) then completely dehydrated by immersing them in a solution 100% for 15 min followed by air–drying. Dried samples were further processed with gold coating, viewed with Hitachi S-3000N scanning electron microscope from SiDi service (Universidad Autónoma de Madrid).

*Protein isolation and Western blot analysis*

Cells were split into six-well plates at a density of 1,5 x $10^5$ cells/well and incubated for 24 h before starting the experiments. Once finished the treatments, cells were washed twice with ice-cold phosphate-buffered saline, harvested by scraping into phosphate-buffered saline and then pelleted by centrifugation at 500 x g, for 5 min, at 4°C. Afterwards immunoblotting was performed essentially as described previously [10]. Briefly, cells were homogenized in a solution containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and protease inhibitors and were kept on ice for 30 min and then, were pelleted by centrifugation at 5000 x g, for 5 min. Proteins from cell lysates were quantified by measuring their protein content using the BCA Protein Assay Kit. Then, protein were denatured by denaturing loading buffer (50 mM Tris (pH 6.8), 50% glycerol, 0.125% bromophenol blue, 15%

SDS and 25% 2.5 M β-mercaptoethanol) and by heating. Then, approximately 35 µg of protein were resolved by 8-15% SDS–PAGE, and blotted onto a 0.4% nitrocellulose membrane or 0.2% PVDF membrane (Bio-Rad Laboratories, CA) by semidry transfer (60 min) in 48 mM Tris (pH 9.2), 39 mM glycine, 0.12 mM SDS, and 20% methanol. Membranes were blocked for 1 h with 5% powdered skimmed milk/0,1% Tween 20 in phosphate-buffered saline and then incubated overnight at 4°C with primary antibodies: anti-cleaved caspase-3 (1:750, rabbit), anti-RAR-β (1:1000, rabbit) or anti-β-actin antibody (1:5000, mouse) as loading control. After incubation with the antibodies, membranes were washed 3 times, during 10 minutes with phosphate-buffered saline. Finally, membranes were incubated for 1 h at room temperature with the corresponding secondary antiserum (1:4000), washed with phosphate-buffered saline (3 times, for 10 minutes), and the signals were detected with enhanced chemiluminescence reagent. Quantification of band densities was performed using Quantitive One Program (Bio-Rad, Alcobendas, Spain)

*MTT assay*

MTT assay was used to determine cell viability/toxicity through colorimetric changes. This assay measures the conversion of MTT reactive to insoluble formazan by dehydrogenase enzymes of the integral mitochondria of living cells. Cells were cultured in 24-well plates ($4 \times 10^4$ cells/well) before being treated as indicated in the results section. Afterwards, cells were incubated with 0.1 mg MTT/ml during the last 2 h of incubation (at 37°C) and the number of viable cells was evaluated by measuring the conversion of the tetrazolium salt MTT to formazan crystals (violet color). Once finished the incubation, medium was removed, and the precipitates were solubilized by 500 µl of dimethyl sulfoxide (DMSO). The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring absorbance at 570 nm using a Synergy®HT ELISA plate reader. The absorbance results were interpolated to a calibration curve (optical density vs number of cells) to obtain number of cells in each assay.

*Flow cytometric analysis of Annexin V/ Propidium iodide (PI)*

Apoptotic and necrotic PTC and HeLa cells were detected using an annexin-V–FITC/Propidium iodide (PI) apoptosis detection kit on a flow cytometer as previously described [11]. Harvested cells were washed in cold phosphate-buffered saline, pelleted by centrifugation and resuspended in 100 µL of binding buffer. Cells were then incubated for 15 min at room temperature in darkness with 5 µl of FITC-conjugated Annexin V and PI was added to the final concentration of 1 mg/ml before the analysis to distinguish cells that had lost membrane integrity. Finally, cell death was analyzed by flow cytometer (FACSCalibur, Becton Dickinson, USA). Live cells showed no staining, early apoptotic cells were positive to annexin V staining, late apoptotic cells showed both PI and annexin V staining and necrotic cells were positive to PI.

*Trypan blue exclusion test of cell viability*

Cisplatin-induced HeLa injury was also quantitatively assessed by counting HeLa stained with trypan blue manually with a hemocytometer. Trypan blue dye is a “vital stain” allowing discrimination between viable cells, which exclude the dye, and cells with damaged membrane, that are positive for trypan blue staining and therefore they are usually considered to be non-viable, dead cells. Harvested cells were washed in phosphate-buffered saline, pelleted by centrifugation and the cells were resuspended in a suitable volume of phosphate buffered saline. Following addition of an equal volume of 0.8% trypan blue, trypan blue positive cells versus total cells were counted in six random fields per well and the percentage of death cells was calculated.

*Cell proliferation assay with 5’-Br-2’-deoxyuridine (BrdU)*

DNA synthesis was assessed by BrdU uptake. Cells were placed in 24-well plates ($5 \times 10^4$ cells/well) and were maintained in medium for 24 h before being treated as indicated in the results section. Cells were pulsed with 10 µM BrdU during the last 2 h of incubation. Afterwards,

the cells were fixed with 4% paraformaldehyde for 15 min. DNA was partially denatured by incubation with 2 M HCl, for 20 min, at room temperature, and the effect were neutralized by incubation with 0.1 M $Na_2B_4O_7$ for 2 min. Cells were permeabilized and blocked with 2% BSA/0.1% Triton X-100, for 10 min. Subsequently, cells were incubated overnight at 4°C with anti-BrdU monoclonal antibody (1:50) and then incubated with α-mouse-Alexa-Fluor® 488 (1:400) for 1 h in darkness. The cell nuclei were contrasted with DAPI. Detection was performed by fluorescent microscopy Olympus BX63. To estimate DNA synthesis, the percentage of BrdU-positive nuclei was determined through manual count by examining in a blind manner five fields in each experimental condition.

*RNA isolation and RT-PCR*

Total cell RNA was isolated with TriReagent from Sigma (St. Louis, MO) according the instructions of the manufacturer. 1 µg of total RNA was reverse-transcribed using 200 U high retrotranscriptase in the enzyme buffer supplemented with 10 µM Oligo(dT) primer, 0,2 mM of deoxyribonucleotides (dNTPs) and 1,5 mM $MgSO_4$ (Biotools B&M Labs S.A.). Two microliters of the RT reaction were amplified by PCR with specific primers of RAR-β: sense 5'- GGT TTC ACT GGC TTG ACC AT-3', an antisense 5'- AAG GCC GTC TGA GAA AGT CA-3'. PCR conditions were: 95°C for 2 min followed by 34 cycles of 95°C 1 min, 57°C 1, 72°C 1 min, and at the end of the cycles 10 min 72°C. The signals were normalized by β-actin gene expression level with specific primers: sense 5'-AGA AGG ATT CTT ATG TGG GC-3', and antisense 5'-CAT GTC GTC CCA GTT GGT GAC-3'. The PCR products were separated by electrophoresis and visualized in 1,5% agarose gels.

*Cell transfection*

Cells were plated in 6 well or 24 well plates (1,5 x $10^5$ cells/well and 4 x $10^4$ cells/well respectively) 24 h before the transfection. Cells were transfected by CANFAST Transfection Reagent kit with a mammalian pSG5 expression vector containing the cDNA of the wild-type human RAR-β gene [12], which was generously donated by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université de Strasbourg/Collège de France, Strasbourg, France). Cells were transfected according the manual instructions and RAR-β expression was evaluated by Western blot analysis.

For HIF-1α inhibition we used HIF-1α siRNA sc-44225 (Santa Cruz Biotechnologies) containing 3 sequences against 3 different HIF-1α exons, and scramble siRNA AM4637 (Applied Biosystems) as a control. PTC cells at 70% of confluence were transfected with HIF-1α siRNA or scramble siRNA using Lipofectamine 2000 reagent. 24 h after transfection, cells were used for the experiments.

*Statistical analysis*

The results are expressed as the mean ± SD. They were subjected to one-way analysis of variance (ANOVA) following by the Bonferroni's test for multiple comparisons. The level of significance was set at $P < 0.05$. Each experiment was repeated at least three times.

## RESULTS

*Treatment with DIDS prevents cisplatin-induced PTC death and inhibition of PTC proliferation but not cisplatin-induced increase in $iPGE_2$*

We have previously demonstrated that inhibition of the prostaglandin uptake transporter PGT in cultured human proximal tubular HK-2 cells, which blunts the increase in intracellular prostaglandin $E_2$ ($iPGE_2$) triggered by cisplatin, prevents the apoptotic cell death induced by this chemotherapeutic agent [3]. Therefore, we postulated that PGT inhibitor DIDS would also prevent cisplatin-induced PTC death. To confirm this prediction, we assessed apoptotic cell death, cell viability and total cell death -through caspase-3 activation detection, MTT reduction

assay and flow cytometry determination of cells positive for both annexin V and IP, respectively- in cisplatin-treated cells which were pre-treated or not with DIDS. Our results confirmed our postulate, since DIDS prevented the activation of caspase-3 (Fig. 1 a), the loss of cell viability (Fig. 1 b), the increase in total cell death (Fig. 1 c) and the morphological apoptotic changes (Fig. 1 d) induced by cisplatin.

Cisplatin reduces the proliferation of PTC [13, 14], which is prevented by inhibition of PGT with bromocresol green, bromosulfophthalein or transfection with siRNA [3]. Therefore, we also postulated that PGT inhibitor DIDS would prevent cisplatin-induced inhibition of PTC proliferation, which was confirmed by the observation that pre-treatment with DIDS resulted in full prevention of the loss of cell viability induced by cisplatin, as assessed through BrdU incorporation in PTC (Fig. 1 e). Thus, the protective effect of DIDS becomes apparent in the increased ability of cells to proliferate after cisplatin exposure.

As indicated in the Introduction, it is assumed that $PGE_2$ is released to the extracellular medium immediately after being synthesized. Therefore, the increase in $iPGE_2$ in PTC exposed to cisplatin would be the consequence of the return of $PGE_2$ to the inside the cell through its inward transport by PGT. This is why inhibition of PGT prevents cisplatin-induced loss in cell survival and cell proliferation in PTC [3]. Accordingly, we expected that the beneficial effects of DIDS in cisplatin-treated PTC were due to its inhibitory effect on PGT. In order to verify this postulate, we first asked whether DIDS actually inhibited PGT in PTC. To this end, we studied by immunofluorescence the effect of pre-incubation with DIDS on the increase in $iPGE_2$ in PTC after addition of $PGE_2$ to the culture medium. As shown in Fig. 1 f (left panel), shortly after adding $PGE_2$, there was an increased in the content of $PGE_2$ and this change was fully prevented by DIDS, which indicated its inhibitory effect on PGT. Therefore, we next sought to confirm that DIDS actually prevented the increase in $iPGE_2$ induced by cisplatin in PTC cells but, to our astonishment, the content in $PGE_2$ of cisplatin-treated PTC cells remained in high values even when they were pre-treated with DIDS (Fig. 1 f, right panel; compare with the inhibitory effect of another PGT inhibitor (bromocresol green, BG). Note also that DIDS by its own determined a slight but statistically significant increase in $iPGE_2$). This unexpected result ruled out that the preventive effect of DIDS on cisplatin-induced loss in cell survival and cell proliferation in PTC cells was due to inhibition of the increase in $iPGE_2$ triggered by cisplatin.

*RAR-β protects against cisplatin-induced PTC death and mediates the preventive effect of DIDS on cisplatin's toxicity*

We have just shown in Fig. 1 that the PGT inhibitor DIDS prevents the toxic effect of cisplatin on PTC while keeping high $iPGE_2$ levels. In this connection, our previous work has displayed that $iPGE_2$ regulates the expression of transcription factors RAR-β and hypoxia-inducible factor-1α (HIF-1α) in PTC [10]. Since both transcription factors may affect cell survival, we hypothesized that they might mediate the protective effect of DIDS against cisplatin in PTC. We addressed this issue through assessing the effect of pre-incubation with RAR-β inhibitor LE-135 [15] or HIF-1α inhibitor YC-1 [16] on the prevention by DIDS on cisplatin's toxicity. Our results (Fig. 2 a, left) indicated that LE-135, but not YC-1, blunted the protective effect of DIDS, as indicated by flow cytometry of annexin V/PI staining. Further studies showed that the beneficial actions of DIDS on cell viability (Fig. 2 a, center) and caspase-3 activation (Fig. 2 a, right) in cisplatin-treated cells were also prevented by LE-135 but not by YC-1 or siRNA HIF-1α, which also supported the role of RAR-β in the protective effect of DIDS on cisplatin's toxicity. Additional evidence on this role was provided by the fact that LE-135 also prevented the protective effect of DIDS on cisplatin-induced inhibition of cell proliferation (Fig. 2 b).

Given that an increased content in $iPGE_2$ results in enhanced expression of RAR-β in PTC [10], we postulated that DIDS up-regulates RAR-β and that this is a critical event for prevention of cisplatin-induced PTC death (as suggested by the results shown in Fig. 2a). In order to explore this possibility, we first studied the effect of DIDS on the expression of RAR-β in control PTC as well as in cisplatin-treated PTC. Our experiments confirmed that, in both instances, treatment with DIDS increased the expression of RAR-β and that transcriptional mechanisms contributed

to this increase (Fig. 2 c, left). Interestingly, PTC exposed to cisplatin exhibited lower expression of RAR-β than control cells (Fig. 2 c, right).

The results shown in Figs. 2 a to c suggested that RAR-β itself might prevent cisplatin´s toxicity in PTC. We explored this possibility through overexpressing RAR-β in PTC by transient transfection with a mammalian pSG5 expression vector containing the cDNA of the wild-type human RAR-β gene. Then cells were exposed to cisplatin for 12 hours and caspase-3 activation and cell death were respectively assessed by Western blot analysis/immunofluorescence and flow cytometry of annexin V/PI staining. As shown in Fig. 2 d, overexpression of RAR-β prevented the increase in caspase-3 and in cell death induced by cisplatin, which confirmed the protective role of RAR-β against the toxicity of cisplatin in PTC.

Retinoic acid receptors (RARs) regulate gene transcription in a ligand-dependent manner mainly by binding as heterodimers with RXRs to retinoic acid response elements upstream of target genes, thus eliciting changes in their expression [17]. The protective effect of RAR-β in our experiments was evident in the absence of treatment with RAR-β ligands and therefore it was likely independent of RAR-β-dependent activation of gene transcription. In order to explore this possibility, though in a preliminary manner, we assessed the effect of the activators of RAR-β-dependent transcription CH55, a RAR-β agonist [18] or all-trans retinoic acid, a RAR pan-agonist [17], on cisplatin's toxicity. We found that pre-treatment with them, under the same conditions as in pre-treatment with DIDS, did not result in prevention of cisplatin-induced cell death and loss of cell proliferation (Fig 2 e). This result suggests that RAR-β-dependent gene transcription is not likely responsible for the protective effect of DIDS or RAR-β over-expression against cisplatin´s toxicity.

Taken together, the results shown in Fig. 2 indicate that RAR-β, most likely through a mechanism that does not involve transcriptional regulation, plays a critical protective role against cisplatin-induced PTC death.

*Cell type specificity of the protection by DIDS: human cervical adenocarcinoma HeLa cells are not protected by DIDS against cisplatin*

Our results indicate that treatment with DIDS, through up-regulation of RAR-β expression, is effective against the cytotoxic effect of cisplatin on PTC and, therefore, that over-expression of RAR-β might be a new and useful therapeutic approach to prevent cisplatin's nephrotoxicity. However, there is the possibility that DIDS also protects cancer cells, which would lead to a loss of the tumoricidal effect of cisplatin. We addressed this issue in HeLa cells and found that the effect cisplatin's toxicity was unaffected by treatment with DIDS (Fig. 3 a to c). There is a sharp contrast, though, between our results and those published in a previous work in which DIDS protected HeLa cells against cisplatin [19]. We speculate that this discrepancy might be due to the use by Ben-Hail & Shosnan-Barmatz of dimethyl sulfoxide (DMSO) to dissolve cisplatin because DMSO is frequently used in research to dissolve cisplatin [20] and, unlike dimethylformamide (DMF) (in which we dissolved cisplatin), DMSO reduces dramatically the toxic effect of cisplatin in several cancer cell lines, including parental human cervical carcinoma cell line KB-3-1 (a subline of HeLa) [20]. Therefore, if DIDS had a very mild effect against cisplatin's cytotoxicity in HeLa cells, it would be theoretically possible that its protective effect were only evident when HeLa cells were treated with cisplatin deactivated by DMSO but not when HeLa cells were treated with full active cisplatin (i.e. cisplatin solved in DMF). We tested this hypothesis in HeLa cells which were treated with cisplatin dissolved in either DMSO or DMF after being preincubated with DIDS. Our results confirmed our hypothesis because DIDS only protected against cisplatin deactivated in DMSO (Fig. 3 d)

We have previously shown that the increase in $iPGE_2$ mediates cisplatin's toxicity in PTC [3] and we have shown here that DIDS prevents cisplatin's toxicity in PTC even though it is unable to prevent the increase in $iPGE_2$ induced by cisplatin (Fig. 1 e). In addition, we have shown in Fig. 2 that RAR-β mediates the preventive effect of DIDS on cisplatin's toxicity. Therefore, we

hypothesized that differences between PTC and HeLa cells regarding the response to DIDS, in terms of $iPGE_2$ and RAR-β, might explain why DIDS protects against cisplatin's toxicity in PTC but not in HeLa cells. Accordingly, we studied the changes in $PGE_2$ content and RAR-β expression in HeLa in our experimental setting and found important differences with respect the ones found in PTC. In the first place, as shown in Fig. 3 e, neither treatment with cisplatin nor with DIDS and cisplatin resulted in changes in $iPGE_2$ in HeLa cells (although DIDS by its own determined a slight but statistically significant increase in $iPGE_2$). In contrast, $iPGE_2$ did increase in both instances in PTC (Fig. 1 f). In the second place, although RAR-β expression was inhibited by treatment with cisplatin in HeLa cells (Fig. 3 f, left), DIDS was unable to increase of RAR-β expression as well as to prevent cisplatin-induced inhibition RAR-β expression (Fig. 3 f, right). This is in sharp contrast with the stimulating effect of DIDS on RAR-β expression in cisplatin-treated PTC (Fig. 2 c, left). This difference between both cell lines is significant, given the critical role of the increased expression of RAR-β in the protective effect of DIDS against cisplatin's toxicity in PTC. In fact, transfection with a mammalian pSG5 expression vector containing the cDNA of the wild-type human RAR-β gene did not protect HeLa cells against cisplatin (Fig. 3 g)

In summary, the results shown in Fig. 3 suggest that the preventive effect of DIDS on cisplatin-induced proximal tubular cell injury might be cell-specific so that DIDS would not interfere with the cytotoxic effect of cisplatin on cancer cells.

## DISCUSSION

In the present work we have found in human proximal tubular HK-2 cells (PTC) that RAR-β plays a key role in the prevention of cisplatin's toxicity. In effect, while RAR-β expression decreased in cisplatin-treated PTC, manoeuvres leading to increase RAR-β (i.e. treatment with DIDS or transfection with a RAR-β plasmid construct) resulted in prevention of cisplatin's toxicity but not in human cervical adenocarcinoma HeLa cells. Therefore, preservation of the expression of RAR-β in proximal tubules through treatment with DIDS might represent a novel strategy in the prevention of cisplatin's nephrotoxicity without compromising cisplatin's chemotherapeutic effect on cancer cells.

$PGE_2$ is released to the extracellular medium immediately after being synthesized and this is why the increase in $iPGE_2$ in cisplatin-treated PTC is due the return of newly synthesized $PGE_2$ to the inside the cell through its inward transport by PGT [3]). We confirmed that DIDS actually inhibits the transport of PGE2 into PTC (Fig. 1 f, left) but, unexpectedly, it did not prevent cisplatin-induced increase in iPGE2. This is a relevant issue because cisplatin-induced cell death is prevented by inhibition of the increase in iPGE2 induced by this chemotherapeutic agent [3]. $PGE_2$ is transported out of cells by multiple drug resistance-associated protein 4 (MRP4) [21], which is capable of pumping several xenobiotic and endogenous organic anionic compounds out of the cell [22]. On the other hand DIDS is a well-known inhibitor of several organic anion transporters [23]. Therefore, one may hypothesize that DIDS inhibits the transport of PGE2 out of the cells so that, when synthesis of PGE2 increases in cisplatin-treated PTC, the prostanoid will accumulate inside PTC (instead of being actively exported into the extracellular space) whenever MRP4 has been previously inhibited by DIDS. The observation that intracellular PGE2 content in both HK-2 cells and HeLa cells increases upon treatment with DIDS (Figs. 1 f right and 3 f right) agrees well with an inhibitory effect of DIDS on the MRP4-dependent transport of PGE2 to the outside the cells. However, specific experiments should confirm this hypothesis.

Retinoic acid receptors (RARs) regulate gene transcription in a ligand-dependent manner mainly by binding as heterodimers with RXRs to retinoic acid response elements (RAREs) upstream of target genes, thus eliciting changes in their expression [24]. However, it is unlikely that RAR-β-dependent gene transcription was involved in the protective effect of DIDS or RAR-β over-expression against cisplatin´s toxicity because activation of RAR-β-dependent gene transcription by CH55, a RAR-β specific agonist or all-trans retinoic acid, a RARs pan-agonist,

did not protect PTC against cisplatin. These results are in good agreement with previous reports showing that all-trans retinoic acid, the physiological agonist of RARs, does not protect porcine LLC-PK1 PTC against cisplatin [25] and that, in fact, treatment with all-trans retinoic acid potentiates cisplatin-induced kidney injury in rats [26]. If RAR-β-dependent gene transcription is not likely involved in the protective effect of DIDS or RAR-β over-expression against cisplatin´s toxicity, an alternative non-genomic mechanism should be proposed. Today there is mounting evidence that RARs have a wider spectrum of biological activities, through nonconventional, non-genomic mechanisms [24]. For instance, it has been found that RARα regulates synthetic events in anucleate human platelets [27]. Another example is the role of RAR-γ as a tumor promoter in hepatocellular carcinoma through controlling the balance between AKT and p53 [28, 29]. In the case of RAR-β, it has been described that it has anti-AP-1 activity in the absence of ligand [30]. Interestingly, inhibition of AP-1 protects non-cancer cells against cisplatin cytotoxicity [31]. Another non-genomic mechanism that may explain the protective effect of RAR-β against cisplatin's toxicity involves voltage-dependent anion channel 1 (VDAC1). VDAC1 is located in the outer mitochondrial membrane and it is considered a key protein that acts as a gatekeeper for mitochondria-mediated cell survival and death signalling pathways [32]. This channel is formed by a large and dynamic complex of proteins, its opening is voltage-dependent and it is more known for being involved in release of mitochondrial proteins such as cytochrome C or apoptosis-inducing factor during apoptosis. Regarding to our experimental context, VDAC1 has been suggested to play a critical role in cisplatin-induced apoptosis in human proximal tubular HK-2 cells and rat proximal tubular NRK-52E cells by the release into the cytosol of apoptosis-inducing factor through VDAC1 oligomerization [33]. Interestingly, besides its classical nuclear location, RAR-β has been also found in mitochondria [34] which opens the possibility that the inhibitory effect of DIDS on VDAC-1 (and thereby in cisplatin-induced apoptosis) might be dependent on interaction between RAR-β and VDAC1. Experimental evidence for this hypothesis comes from the effect of other mitochondrially localized nuclear receptors on apoptosis, which extends outside the nucleus the known nuclear role of these receptors [35]. Because DIDS has been previously shown to inhibit VDAC1 [19], it is also theoretically possible that this inhibitory effect may be mediated by interaction between RAR-β and VDAC1. Clearly, further experiments are required to confirm that inhibition of AP-1 by RAR-β and/or interaction between VDAC1 and RAR-β are involved in the protective effect of RAR-β against cisplatin's toxicity in PTC.

Most studies on the role of RAR-β in cell survival have been performed in cancer cells and their results are in sharp contrast with ours. Thus, esophageal cancer cells stably transfected with RAR-β expression vector had decreased cell growth and colony formation and increased apoptosis [36]. In a similar way, upregulation of RAR-β in cholangiocarcinoma cells was shown to increase the expression of proapoptotic genes bax, bak and bim, in addition to caspase-3 activity, and decrease the expression of antiapoptotic genes bcl-2, bcl-xL and mcl-1. As a result, cholangiocarcinoma cells were more susceptible to caspase-dependent apoptosis induced by cisplatin and other chemotherapeutic agents [37]. Furthermore, many studies have demonstrated that loss of RAR-β expression is relatively frequent and progressive in premalignant and malignant tissues and cells, including breast, pancreas, prostate, lung, cervix, head and neck and esophagus [7]. In consequence, the silencing of the RAR-β gene trough methylation of its promoter has a great potential as a prognostic and diagnostic biomarker in several types of cancer [7]. This is why, for instance, demethylation of RAR-β by lidocaine sensitizes the cytotoxicity of cisplatin in breast cancer cells [38]. It is likely that the reason of the differences between the role of RAR-β expression in cancer cells and PTC relays in the fact that the actions of RAR-β on cell survival and cell proliferation are more complex than currently envisaged. For instance, expression of RAR-β may not be necessarily protective in breast cancer since stromal RAR-β promotes, rather than supresses, mammary gland tumorigenesis [39]. In addition, it has been found that RAR-β contributes axonal regeneration in corticospinal neurons after corticospinal tract injury [8], which is in line with our results in which RAR-β contributes to PTC proliferation and survival. In summary, it seems that the effects of RAR-β on cell survival and cell proliferation are dependent on the cell type and, within a given cell type, they may also be cell-context specific.

In conclusion, RAR-β has a fundamental role in protecting PTC against cisplatin's toxicity and the data presented here strengthen the idea that pharmacological preservation of RAR-β expression through treatment with DIDS might be a promising therapeutic target to alleviate renal complications associated with cisplatin chemotherapy.

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LEGENDS TO FIGURES

**Fig. 1 Treatment with DIDS prevents cisplatin-induced PTC death and inhibition of PTC proliferation but not cisplatin-induced increase in iPGE$_2$** *a) Prevention of cisplatin-induced caspase-3 activation.* Caspase-3 activation was assessed through immunofluorescent analysis of cleaved caspase-3 (red) in cells whose nuclei were stained with DAPI (left panel, original magnification 40x) or through Western blot analysis (right panel; the numbers over the bands represent the mean of the fold change over the control of the densitometric analysis in which protein expression was normalized to β-actin). *b) Prevention of cisplatin-induced loss in cell number.* The number of cells was estimated as a function of the ability to reduce MTT using a calibration curve (inset) in which optical density (O.D.) was plotted against cell number. *c) Prevention of cisplatin-induced cell death.* Bars show the sum, normalized to control values, of the percent of apoptotic and necrotic cells as determined by flow cytometry. The bars include annexin V+/propidium iodide− cells (i.e. early apoptotic cells with preserved plasma membrane

integrity) and annexin V+/propidium iodide+ cells (i.e. late apoptotic/necrotic cells), as determined by flow cytometry. *d) Scanning electron microscopy.* Left: control; Center: extensive blebbing in apoptotic PTC cells upon treatment with cisplatin; Right: Prevention by DIDS. *e) Prevention of cisplatin-induced inhibition of cell proliferation.* Cell proliferation was assessed as the percentage of BrdU-positive nuclei (original magnification 20x) (which was determined through manual count of green-stained cells in five fields in a blind manner) as described in Materials and Methods section. *f) Non-prevention of cisplatin-induced increase in $iPGE_2$.* Left panel: PGT inhibitors DIDS (200 μM) and bromocresol green (BG, 50 μM) inhibit the increase in $iPGE_2$ induced by treatment with $PGE_2$ (1 μM/2 min incubation). $iPGE_2$-dependent immunofluorescence, alone or merged with nuclear staining with DAPI (original magnification, 40X), is shown in the upper panel. Below is shown the quantitative approach to the images using image J software Right panel. Cisplatin-induced increase in $iPGE_2$ is prevented by BG but not by DIDS.

*General information*. 1) Cells were pretreated with DIDS for 1 h and then treated with 25 μM cisplatin for 24 h (unless otherwise indicated). 2) Microphotographs and Western blot autoradiographs are representative examples of at least three independent experiments. 3) Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments. # $P < 0.01$ vs other groups; +++ $P < 0.01$ vs cisplatin and 100 μM DIDS + cisplatin; ++++ $P < 0.01$ vs control and DIDS; + $P < 0.01$ vs control.

**Fig. 2 RAR-β protects against cisplatin-induced PTC death and mediates the preventive effect of DIDS on cisplatin's toxicity** *a) Prevention by DIDS of cisplatin-induced cell death is abolished by RAR-β inhibitor LE-135.* Cells were pre-incubated for 1 h with 2.5 μM LE-135. Then, they were incubated for 1 h with 200 μM DIDS before being exposed to cisplatin. Left panel: Flow cytometry analysis of cell death. Pre-incubation with HIF-1α inhibitor YC-1 (10 μM) did not block the prevention effect of DIDs. Center panel: The number of cells was estimated as a function of the ability to reduce MTT using the calibration curve shown in Fig. 1 b. Right panel: Caspase-3 activation was assessed through Western blot analysis. *b) Prevention by DIDS of cisplatin-induced inhibition of cell proliferation is abolished by RAR-β inhibitor LE-135.* Cell proliferation was assessed as in Fig. 1 d. *c) DIDS increases the expression of RAR-β in both control and cisplatin-treated cells.* PTC were incubated with DIDS (left panel) or they were pre-incubated for 1 h with 200 μM DIDS before being exposed to cisplatin (right panel). Then, expression of RAR-β protein or RAR-β mRNA (*inset*) was determined by Western blot analysis or semiquantitative RT-PCR, respectively. *d*) *Over-expression of RAR-β prevents cisplatin-induced proximal tubular cell death*. Cells were transiently transfected with a mammalian pSG5 expression vector containing or not the cDNA of the wild-type human RAR-β gene. Then cells were exposed to cisplatin for 12 h and caspase-3 activation (left and center panels), and cell death (right panel) were respectively assessed by Western blot analysis/immunofluorescence (original magnification 40x) and flow cytometry of annexin V/PI staining. Inset (left): Expression of RAR-β in PTC cells transfected with plasmid containing or not wild-type RAR-β gene. Inset (right) Cell death induced by cisplatin in cells transfected with RAR-β vector was normalised with respect the value of untreated cells which were also transfected with RAR-β. *e) RAR-β agonist CH55 or RAR pan-agonist all-trans-retinoic acid (ATRA) do not prevent cisplatin's toxicity.* PTC were incubated for 1 h with 2.5 μM CH55 or 10 μM ATRA before being exposed to cisplatin. Then, MTT assay, caspase-3 activation and cell proliferation were determined (original magnification 40x).

*General information:* 1) Cells were treated with 25 μM cisplatin for 24 h, unless otherwise indicated. 2) Flow cytometry analysis of cell death: Bars show the sum, normalized to control values, of the percent of apoptotic and necrotic cells as determined by flow cytometry. The bars include annexin V+/propidium iodide−cells (i.e. early apoptotic cells with preserved plasma membrane integrity) and annexin V+/propidium iodide+ cells (i.e. late apoptotic/necrotic cells). 3) The photographs are representative examples of three independent experiments. Equal protein or mRNA loading was confirmed by assessing the expression of β-actin protein or mRNA. The numbers over the bands represent the mean (fold change over the control) of the densitometric analysis of the three independent experiments in which protein expression or mRNA expression were normalized to β-actin. 4) Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments. # $P < 0.01$ vs other groups; ## $P < 0.01$ vs other groups except control plasmid; #### $P < 0.01$ vs control plasmid + cisplatin ### $P < 0.01$ vs other groups except YC-1+cisplatin, LE-135+cisplatin and LE-135+DIDS+cisplatin; + $P <$

0.01 vs control and DIDS+cisplatin; ++ $P < 0.01$ vs cisplatin-treated groups except YC-1+DIDS+cisplatin; ** $P < 0.01$ vs cisplatin-treated groups except DIDS+cisplatin; *** $P < 0.01$ vs other groups except cisplatin, YC-1+ cisplatin and LE135+cisplatin; **** $P < 0.01$ vs other groups except cisplatin, LE-135+DIDS+cisplatin and LE-135+cisplatin; ***** $P < 0.01$ vs other groups except cisplatin, LE-135+DIDS+cisplatin and YC-1+cisplatin.

**Fig. 3 Human cervical adenocarcinoma HeLa cells are not protected by DIDS against cisplatin** *a) Non-prevention of cisplatin-induced caspase-3 activation.* Caspase-3 activation was assessed through immunofluorescent analysis of cleaved caspase-3 (red) in cells whose nuclei were stained with DAPI (left panel, original magnification 40x) or through Western blot analysis (right panel). *b) Non-prevention cisplatin-induced cell loss.* Left panel: The number of cells was estimated as a function of the ability to reduce MTT using the calibration curve shown in Fig. 1 b (inset) in which optical density (O.D.) was plotted against cell number. Right panel: Cell death, normalized to control values, was determined by flow cytometry. *c) Non-prevention of cisplatin-induced inhibition of cell proliferation.* Cell proliferation was assessed as the percentage of BrdU-positive as described in Fig. 1d. *d) Dimethylsulfoxide partially inhibits cisplatin-induced HeLa cell death, which allows for protection by DIDS.* Cisplatin was solved in either dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) and cell death was assessed as the percentage of cells failing to exclude trypan blue dye. *e) Neither treatment with cisplatin nor with DIDS and cisplatin resulted in changes in iPGE$_2$.* iPGE$_2$-dependent immunofluorescence, alone or merged with nuclear staining with DAPI (original magnification, 40x), is shown in the left panel. The right panel shows the quantitative approach to the images using ImageJ software (note that 200 µM DIDS by its own determined a slight but statistically significant increase in iPGE$_2$). f*) Non-prevention by DIDS of cisplatin-induced inhibition of RAR-β expression.* Left panel: Cells were treated with cisplatin. Right panel: Cells were incubated with DIDS before being exposed to cisplatin. *g) Non-prevention by wild type RAR-β of cisplatin-induced cell death.* Cells were transiently transfected with a mammalian pSG5 expression vector containing or not the cDNA of the wild-type human RAR-β gene. Then cells were exposed to cisplatin and cell death was assessed by flow cytometry of annexin V/PI staining

*General information:* 1) Cells were treated with 25 µM cisplatin for 24 h, unless otherwise indicated. 2) The photographs are representative examples of three independent experiments. Equal protein loading was confirmed by assessing the expression of β-actin. The numbers over the bands represent the mean (fold change over the control) of the densitometric analysis of the three independent experiments in which protein expression was normalized to β-actin. 4) Bars and error bars in graphs: Each bar represents the mean ± SD of 3 different experiments. # $P < 0.01$ vs other groups; * $P < 0.01$ vs control; + $P < 0.01$ vs other groups except control and cisplatin; ++ $P < 0.01$ vs cisplatin-treated groups; +++ $P < 0.01$ vs control, DIDS, cisplatin (DMSO) and DIDS+cisplatin (DMSO).