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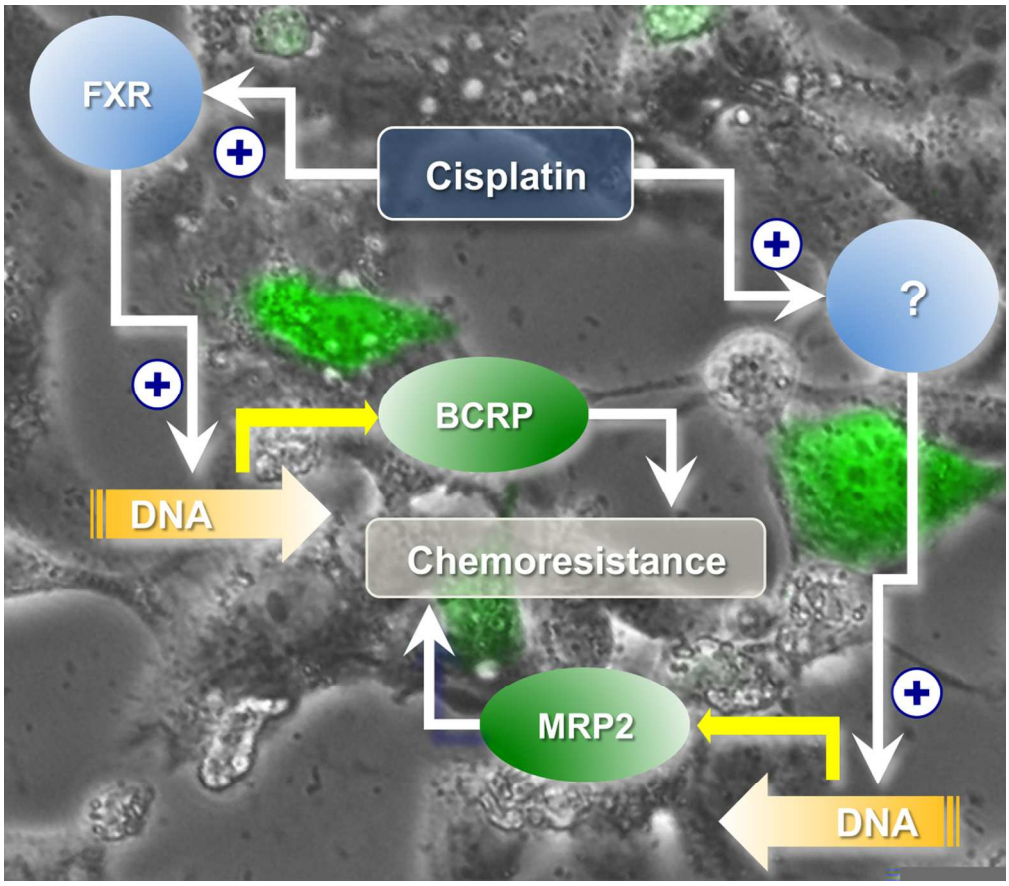

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Cisplatin-induced chemoresistance in colon cancer cells involves FXR-dependent and FXR-independent up-regulation of ABC proteins

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Short title

Role of FXR in colon cancer chemoresistance

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Abbreviations:

ABC, ATP-binding cassette; CDCA, chenodeoxycholic acid; C/EBPβ, CCAAT-enhancer binding protein-β; FXR, farnesoid X receptor; HNF, hepatic nuclear factor; Luc2, firefly luciferase; MOC, mechanism of chemoresistance; MRP, multidrug resistance-associated protein; PI, propidium iodide.

ABSTRACT

Export pumps often limit the usefulness of anticancer drugs. Here we investigated the effect of cisplatin on the expression of ABC proteins in human colon cancer cells. Short-term incubation of Caco-2 and LS174T cells with cisplatin resulted in up-regulation of several ABC pumps, in particular MRP2 and BCRP. In partially cisplatin-resistant cells (LS174T/R) obtained by long-term exposure to cisplatin, MRP2 and BCRP up-regulation was more marked. This was further enhanced when these cells were cultured under maintained stimulation with cisplatin. The MRP2 promoter (MRP2pr) was cloned and partially deleted constructs linked to reporter genes were generated. Transfection of LS174T and LS174T/R cells with these constructs revealed the ability of cisplatin to activate MRP2pr. The intensity of this response was dependent on the conserved MRP2pr region. Basal MRP2pr activity was higher in LS174T/R cells, in which the expression of the transcription factors c/EBPβ, HNF1α, HNF3β and HNF4α, but not PXR, p53, c-Myc, AP1, YB-1, NRF2 and RARα was enhanced. Up-regulation was particularly high for FXR (200-fold) and SHP (50-fold). In LS174T/R cells, GW4064 induced the expression of FGF19, SHP, OSTα/β, but not MRP2 and BCRP, although the sensitivity of these cells to cisplatin was further reduced. In LS174T cells, GW4064-induced chemoresistance was seen only after being transfected with FXR+RXR, when BCRP, but not MRP2, was up-regulated. Protection of LS174T cells against cisplatin was mimicked by transfection with BCRP. In conclusion, in colon cancer cells, cisplatin treatment enhances chemoresistance through FXR-dependent and FXR-independent mechanisms, involving the expression of BCRP and MRP2, respectively.

INTRODUCTION

The mechanisms of chemoresistance (MOC) have recently been classified in five major groups on the basis of whether they involve a reduction in drug uptake (MOC-1a) or enhanced drug export (MOC-1b), a reduction of metabolic pro-drug activation or an increase in drug inactivation (MOC-2), changes in molecular targets (MOC-3), enhanced DNA repair (MOC-4), and a modification in the pro- (MOC-5a) versus anti- (MOC-5b) apoptotic balance [1]. These are present in both healthy tissues, where they are involved in the defense against the chemical stress caused by potentially toxic compounds, and in cancer cells, where they account for the poor response to antitumor drugs.

In the present study, cisplatin was selected as a model antitumor drug due to its well-known ability to induce chemoresistance and because this compound and its derivatives are among the most effective anticancer agents used to treat several types of tumors, including colon cancer [2]. Indeed, chemotherapy regimens based on platinum-derived compounds in combination with other drugs constitute the pharmacological therapy of choice for the treatment of colon cancer [3, 4]. Platinum-based neoadjuvant chemotherapy together with radiotherapy is recommended before surgery in colon cancer at stages II and III [5], and it is maintained after tumor resection in more advanced stages to prevent the high risk of recurrence [6]. However, the pre-existence of drug refractoriness and the frequent development of chemoresistance during treatment are important limitations in the management of these patients [7].

Refractoriness to the platinum-based drugs is the consequence of the complex participation of several MOC. These include MOC-1a. Thus, the expression of the CTR1 copper transporter, which is a major route for cisplatin uptake, in cells derived from human colon adenocarcinoma [8], is decreased in subclonal cells that exhibits acquired resistance to cisplatin [9]. The development of this characteristic may also be due in part to an increase in drug efflux (MOC-1b) by exporting pumps, such as the copper-transporting P-type ATPases ATP7A [10] and ATP7B [11], and the multidrug-resistance related protein 2 (MRP2, gene symbol *ABCC2*) [12]. Enhancement of the amount of glutathione (GSH) and the conjugating activity of GSH S-transferases (MOC-2), the ability of cancer cells to repair damaged DNA more efficiently (MOC-4) and changes in the control of the apoptosis/survival balance (MOC-5) have also been associated with an increased refractoriness to cisplatin [1].

The over-expression of MRP2 is considered to be one of the most important mechanisms accounting for the failure of cisplatin treatment in colon cancer [13]. MRP2 is located at the apical

membrane of several different polarized cells, such as hepatocytes [14], where this pumps plays a key role in the canalicular secretion of glucuronated bilirubin [15]. Because its substrate specificity, MRP2 plays a key role in the elimination from hepatocytes into bile, from kidney proximal tubules into urine, and from intestinal epithelial cells into the intestinal lumen of potentially toxic compounds after being conjugated with GSH, glucuronic acid, or sulfate.

The expression of ABC proteins is regulated in response to many endogenous and xenobiotic compounds, including cisplatin [16]. Most of these inducers are also able to affect the expression of drug-metabolizing phase II enzymes (MOC-2), indicating a coordinated regulation of the genes involved in the phase II and phase III steps of detoxification [17]. Transcriptional regulation of these genes requires the cross-talk among a large variety of transcription factors, including NRF2 [18] and hepatocyte nuclear factors (HNF) [19]. Forming heterodimers with the retinoid X receptor α (RXRα) several nuclear receptors, such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and the constitutive androstane receptor (CAR), are able to bind to hormone response elements in the promoter region of several *ABC* genes [20]. These nuclear receptors are activated by xenobiotics such as rifampicin and dexamethasone (PXR) or phenobarbital (CAR), and also by endogenous compounds such as bile acids (FXR). Thus, up-regulation of ABC proteins in response to the activation of NRF2, HNFs, PXR, CAR or FXR may play a role in preventing the accumulation of potentially toxic compounds in cells expressing these genes.

The initial aim of this study was to investigate the effects of cisplatin on MRP2 expression, and to characterize the mechanism(s) responsible. However, since the results revealed an important up-regulation of the bile acid sensor FXR in response to the treatment with cisplatin, the question as to whether FXR might be involved in the up-regulation of MRP2 and in the reduced sensitivity of colon cancer cells to cisplatin was also investigated.

MATERIALS AND METHODS

Chemicals

Cisplatin (*cis*-diamminedichloroplatinum(II)), chenodeoxycholic acid sodium salt (CDCA), probenecid, propidium iodide (PI) and rifampicin were obtained from Sigma-Aldrich Quimica (Madrid, Spain). Calcein acetoxymethyl ester (calcein AM) was from Invitrogen (Barcelona, Spain). GW4064 was kindly provided by Dr. Luis Alvarez (La Paz University Hospital, Madrid, Spain). According to the suppliers, the purity of these compounds was ≥97%. All other chemicals were of analytical grade.

Cloning procedures

A 2899-bp zone of the 5'-flanking region of the *ABCC2* gene (Z1-MRP2pr) (Supplementary Figure 1; this material is available free of charge via the Internet at http://pubs.acs.org) previously identified as the proximal MRP2 promoter (MRP2pr) (GenBank accession number AF144630) [21] was cloned using DNA obtained from HepG2 cells. Several binding sites for transcription factors in this region have been described [19, 21] or predicted with the Transcript Element Search Software (TESS University of Pennsylvania, PA, USA). In addition, partially deleted MRP2pr constructs of 1423 bp and 873 bp, named here Z2-MRP2pr and Z3-MRP2pr, respectively, were also generated (Figure 1A). All constructs contained the 248-bp sequence located downstream of the transcription start site of the *ABCC2* gene up to the nucleotide prior to the coding region, because deletion of this region is known to abolish MRP2pr activity [21]. MRP2pr constructs were amplified by PCR using the high fidelity AccuPrime Pfx DNA polymerase (Invitrogen), and specific oligonucleotide primers (Supplementary Table 1; this material is available free of charge via the Internet at http://pubs.acs.org), to which attB sites were added to obtain cDNA adapted for Gateway® cloning. PCR products were recombined with the pDONR207 vector (Invitrogen) to generate Entry plasmids, which were further recombined with the pcDNA-DEST47 destination vector (Invitrogen), containing the C-terminal GFP reporter gene. MRP2pr-firefly luciferase (Luc2) fusion plasmids were obtained by Multisite Gateway® cloning (Invitrogen). The pGL4.10[Luc2] plasmid (Promega, Madrid, Spain) was used as template to amplify by PCR the coding sequence of Luc2, by using appropriate primers (Supplementary Table 1). A promoter-less destination vector (pDEST/pL) was obtained from the pcDNA6.2/V5-DEST (Invitrogen) by excluding a fragment of this vector containing the CMV and T7 promoters. The coding sequences of FXR, more precisely the FXRα1- isoform containing exons 1 to 3 and a 12-bp deletion in the exon 5, and RXRα were amplified by PCR from RNA from human liver (HepG2) and placenta (Jeg-3), respectively, by Gateway® technology using gene-specific primers (Supplementary Table 1). The coding sequence of

BCRP had been previously cloned as reported elsewhere [22]. The exact nucleotide sequence of all constructs was confirmed by gel-electrophoresis-based sequencing.

Cell cultures

Caco-2 (HTB-37) and LS174T (CL-188) cell lines, both derived from human colorectal adenocarcinoma, HepG2 (HB-8065), from human hepatoblastoma, JAr cells from human choriocarcinoma (HTB-144), and CHO-K1 (CCL-61), from Chinese hamster ovary, were purchased from the ATCC by way of LGC Standards (Barcelona, Spain) and cultured as recommended by the supplier. A cisplatin-resistant subline (LS174T/R) had previously been obtained in our laboratory [23]. Before using LS174T/R cells they were cultured in the absence of cisplatin for 6 weeks. Alternatively, 10 µM cisplatin was maintained in the culture medium (LS174T/Rms cells). Where indicated the formazan test was used to determine cell viability (CellTiter 96 A_{QUEOUS} Non-Radioactive Cell Proliferation Assay, Promega, Madrid, Spain).

Measurement of MRP-mediated calcein efflux

Calcein efflux assays were carried out to evaluate the functional activity of MRP pumps [24]. Briefly, cells were incubated in 100 µl of uptake medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose and 50 mM HEPES, pH 7.40) containing 1 µM calcein AM and 50 µg/ml PI at 37°C for the indicated time periods. In some experiments, probenecid – a typical MRP inhibitor – was added together with the substrate. Then, 900-µl of ice-cold uptake medium was added to stop the transport process, and intracellular fluorescence was determined immediately by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, Madrid, Spain). Dead cells, whose identification was based on PI staining, were excluded from the data analysis.

Cell transfections and reporter gene assays

Transient transfection of cells, that had been plated (25000 cells/cm^2) 24 h previously was carried out with the lipofectamine LTX and PLUS reagents (Invitrogen). After 14 h in the presence of medium containing DNA-cationic lipid complexes this was replaced by fresh culture medium. Cells were cultured for 72 h before the measurement of promoter activation, which was carried out by determining changes in the fluorescence of cells due to GFP expression by flow cytometry or in the luciferase activity, measured with the Dual-Glo Luciferase Assay System (Promega) with a LAS-4000 image reader (FujiFilm; TDI, Madrid, Spain). The luminescence signal was expressed as arbitrary light units/min corrected by the amount of living cells in each well. Luciferase activity was also normalized by differences in transfection efficiency among the different cultures, as estimated by the *Renilla reniformis* luciferase activity of cells that had been transfected 72 h before with 100 ng pGL4.74[hRluc/TK]

vector (Promega). Photographs depicting GFP-expressing cells were obtained using a Nikon Eclipse TE 2000-S microscope (Nikon Instruments Europe; Amstelveen, The Netherlands).

Measurement of steady-state mRNA levels

Total RNA was isolated using RNA spin columns treated with RNase-free DNase I (GE Healthcare, Madrid, Spain), determined fluorimetrically with the RiboGreen RNA-Quantitation kit (Invitrogen), and used for cDNA synthesis using random primers and SuperScript III reverse transcriptase (SuperScript VILO cDNA Synthesis kit, Invitrogen). Real-time quantitative PCR (QPCR) was performed using gene-specific primers spanning exon-exon junctions in the target mRNA (Supplementary Tables 2 and 3; this material is available free of charge via the Internet at http://pubs.acs.org) and AmpliTaq Gold DNA polymerase (Applied Biosystems, Madrid, Spain) in a 7300 Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were as follows: single cycles at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. Detection of the amplification products was carried out using SYBR Green I. The mRNA abundance of target genes in each sample was normalized on the basis of its GAPDH (Supplementary Table 2).

Western blot analyses

Cells were washed with ice-cold PBS and lysed by incubation for 30 min in ice-cold radioimmunoprecipitation assay buffer (RIPA; 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS dissolved in PBS) supplemented with protease inhibitors (Protease inhibitor cocktail, Sigma-Aldrich). Protein concentrations were determined using a modification of the Lowry method [25]. Cell lysates (20-100 µg protein) were boiled for 7 min, except for the detection of MRP2, for which the samples were not boiled. Immunoblotting analyses were carried out in SDS-PAGE gels, which were then transferred onto nitrocellulose membranes. Blots were probed with primary monoclonal antibodies against human MRP2 (clone M_2III-6), purchased from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY, US), against FXR (clone ab56902) and BCRP (BXP-21), from Abcam (Cambridge, United Kingdom), or against C/EBPβ (clone C-19), HNF1α (clone H-140), HNF3β (clone M-20), HNF4α (clone H-171), SHP (clone H-160), and GAPDH (clone 6C5), all provided by Santa Cruz Biotechnology (Santa Cruz, CA, US). The appropriate horseradish peroxidase-linked secondary antibodies were from Invitrogen. An enhanced chemiluminescence detection system (Hybond ECL; GE Healthcare) was used to visualize the bands.

Statistical methods

Results are expressed as means ± S.D. from at least three different cultures carried out in triplicate. To calculate the statistical significance of the differences, the paired Student *t*-test or the Bonferroni method of multiple-range testing were used, as appropriate.

RESULTS

Effect of cisplatin on the expression of export pumps in colon cancer cells

The effect of cisplatin on the expression of a panel of export pumps was investigated using the colon cancer cell lines LS174T (Table 1) and Caco-2 (Table 2). Under basal conditions both cell lines contained high mRNA levels of ATP7B, ABCA2 and LRP. ATP7A, MDR1, MRP1 and MRP3 were also highly expressed in LS174T cells, but not in Caco-2 cells, whereas the opposite was the case for MRP4, ABCA3, BCRP and MRP2. The expression of the other proteins studied here were medium or low in both cell lines (Table 1 and 2).

The effect of cisplatin was first investigated after short-term exposure (72 h) to concentrations close to the IC_{50} previously determined for this drug in these cell lines [26]. Thus exposure of LS174T cells to cisplatin induced an up-regulation (4 to 12-fold) of many of the proteins, such as BCRP, LRP and several MRP pumps (namely, MRP1, MRP2, MRP3, MRP6 and MRP7) (Table 1). In contrast, several proteins with minor or uncertain role in chemoresistance, such as ABCA2, 3, 6 and 8 were down-regulated (Table 1). This was also found for MRP8 and BSEP, although they were poorly expressed in LS174T cells. When Caco-2 cells were treated with cisplatin, an up-regulation of BCRP, MDR1 and MRPs, namely MRP1, MRP2, MRP3, MRP5 and MRP6, was observed (Table 2) whereas ABCA8 was also down-regulated in these cells (Table 2).

Changes in the expression profile due to long-term exposure to cisplatin (LS174T/R cells) were similar, although in most genes of higher magnitude than those observed after short-term treatment (Table 1). The up-regulation of MRP2 (353-fold) was remarkable. The enhanced expression of other export pumps, although in some cases (ATP7B, MRP1, MRP3, MRP4 and MRP6) strong, was much lower than that of MRP2. When cisplatin was maintained in the culture medium of LS174T/R cells (LS174T/Rms), the changes in the expression pattern were more marked (Table 1). Although the basal expression of BCRP was low this pump was markedly up-regulated in response to short-term incubation cisplatin (12-fold) as well as in LS174T/R (196-fold) and LS174T/Rms (279-fold) cells (Table 1).

Western blot analysis indicated that cisplatin-resistant LS174T/R cells displayed higher levels of MRP2 (Figure 2A) and BCRP (Figure 2B) proteins than LS174T cells. This difference was further marked when resistant cells were cultured under maintained stimulation due to the presence of cisplatin in the culture medium (LS174T/Rms cells) (Figure 2A). This was also seen for BCRP (Figure 2B).

Based on results described above, the role of enhanced expression of MRP2 and BCRP in resistance to cisplatin was investigated. The studies were focused on the three models obtained from LS174T cells. First, we evaluated the functional expression of MRPs in the cells by determining the intracellular accumulation of calcein, a substrate of several members of the MRP family. Flow cytometry analyses revealed that the cellular contents of calcein were lower in LS174T/R than in LS174T cells (Figure 3A and 3B), which was consistent with the higher functional expression of MRPs in the resistant cells. The ability to prevent calcein accumulation was higher in LS174T/Rms cells (Figure 3A and 3B). Probenecid, a well-known MRP inhibitor, significantly increased the accumulation of calcein in these three cell lines, although the probenecid-sensitive fraction of calcein accumulation was higher in LS174T/R and LS174T/Rms than in LS174T cells (Figure 3C).

Determination of MRP2 promoter activity

To investigate the mechanisms accounting for the cisplatin-induced up-regulation of MRP2 the transcriptional activity of the MRP2 promoter was determined in LS174T and LS174T/Rms cells transfected with GFP-reporter gene vectors. Since Chinese hamster ovary cells (CHO-K1) do not express MRP2 these were used as negative controls. Flow cytometry analysis revealed a marked shift in the fluorescence histogram when cells were transfected with the plasmid containing GFP under the control of the CMV promoter (Supplementary Figure 1A, 1B and 1C; this material is available free of charge via the Internet at http://pubs.acs.org). When cells were transfected with Z1-MRP2pr-GFP no increase in the fluorescence of CHO-K1 cells was found (Supplementary Figure 1A). This was consistent with the previously described poor ability of these cells to activate MRP2pr [19]. In contrast, the transfection of LS174T and LS174T/Rms cells with Z1-MRP2pr-GFP resulted in the appearance in the culture of green fluorescent cells and a moderate shift in the GFP-fluorescence histogram, which in the case of LS174T/Rms cells was of higher magnitude (Supplementary Figure 1B and 1C).

To measure the activation of MRP2pr accurately a construct containing luciferase as the reporter gene was used. Luciferase activity was barely detectable in cells transfected with the promoter-less vector (Figure 1B). In contrast, transfection with the Z1-, Z2- or Z3-MRP2pr-Luc2 plasmids into LS174T cells stimulated luciferase activity with similar efficacy for all constructs (Figure 1B). As compared with LS174T cells, a dramatic stimulation of MRP2pr activity was found in LS174T/R cells, which was even higher in LS174T/Rms cells. This occurred despite a similar transfection efficiency in LS174T, LS174T/R and LS174T/Rms cells, as estimated by *Renilla* luciferase expression driven by the herpes virus thymidine kinase (HSV-TK) promoter, was obtained (Figure 1B). The order of MRP2pr transcriptional activity was Z3-MRP2pr>Z1-MRP2pr>Z2-MRP2pr.

Stimulation of the MRP2 promoter by cisplatin

The response to rifampicin (Figure 4A) and cisplatin (Figure 4B) was investigated using LS174T, LS174T/R and LS174T/Rms cells transfected with different fragments of MRP2pr preceding Luc2. Rifampicin was used as a positive control because it is a well-known inducer of MRP2 expression [27] through the activation of PXR [20]. In the present study, rifampicin significantly increased luciferase activity driven by Z1-, Z2- or Z3-MRP2pr in LS174T, LS174T/R and LS174T/Rms cells (Figure 4A). Incubation with cisplatin also resulted in the stimulation of luciferase activity in cells transfected with Z1-MRP2pr or Z3-MRP2pr as compared with each cell type in the absence of cisplatin (Figure 4B). However, cisplatin-induced stimulation was very poor when cells were transfected with Z2-MRP2pr. The concentration of cisplatin used in these experiments was 5 μM because preliminary studies revealed that higher concentrations were toxic for LS174T cells (data not shown).

Expression of transcription factors involved in activation of the MRP2 promoter

Using RT-QPCR the expression of a panel of transcription factors that have been associated with the xenobiotic-mediated induction of cisplatin resistance or the enhanced expression of MRPs was measured in cisplatin-sensitive and -resistant cells (Figure 5). On comparing the results obtained in LS174T, LS174T/R and LS174T/Rms, no significant differences in the mRNA levels of AP-1 (c-Fos/c-Jun heterodimer), NRF2, c-Myc, RARα, PXR, CAR, p53 and YB-1 were found (Figure 5). However, in cisplatin-resistant cells the expression of C/EBPβ, HNF4α, HNF1α and HNF3β was significantly enhanced (2- to 5-fold). The up-regulation was dramatic in the case of FXR (≈200-fold) and SHP (≈50-fold) (Figure 5). The results concerning protein abundance as determined by Western blot analysis were consistent with the enhanced expression of these genes (Figure 1B).

Effect of FXR activation

To elucidate whether FXR up-regulation resulted in enhanced function, the ability of the FXR-mediated pathway to respond to CDCA and GW4060 was investigated. Owing to the presence of cisplatin in the culture medium of LS174T/Rms cells, these were not used in this set of experiments in order to rule out any potential artefact. Under basal conditions, the expression of MRP2 and the well-known FXR target genes FGF19, SHP, OSTα and OSTβ was already higher in LS174T/R than in LS174T cells, except for OSTα (Figure 6). In LS174T cells, with very low levels of FXR (Figures 1 and 5), both GW4064 and CDCA had little effect on the expression of these genes. In contrast, in LS174T/R cells, GW4064 and CDCA stimulated the

expression of FGF19, SHP, OSTα, OSTβ, but not that of MRP2 and BCRP, which were already highly up-regulated in absence of FXR agonists (Figure 6).

FXR activation protects against cisplatin-induced toxicity

The functional repercussions regarding the chemoresistance of the enhanced expression of FXR were investigated. GW4064 did not modify the sensitivity of LS174T cells to cisplatin (Figure 7A). In contrast, GW4064 increased cisplatin resistance in LS174T/R cells (Figure 7A). Whether this effect could be induced in LS174T cells by enhancing the expression of FXR was investigated. In contrast, to the absence of effect in LS174T cells transfected with mock vectors, GW4064 was able to stimulate chemoresistance against cisplatin in LS174T cells transfected with FXR and RXRα (Figure 7B). Thus, no effect of GW4064 on the IC_{50} was observed in LS174T cells both non-transfected and transfected with mock vectors (Figure 7C). In contrast, GW4064 induced a significant increase in the IC_{50} in non-transfected LS174T/R cells and LS174T cells transfected with FXR and RXRα (Figure 7C). This protective effect was detected in spite of the low (5%) efficiency of transfection, as determined by immunofluorescence in parallel plates in which the vector used for transfection contained GFP instead FXR (data not shown).

Role of BCRP in FXR-induced resistance to cisplatin

Finally, we investigated whether FXR-mediated protection against cisplatin toxicity might be due to an enhancement in the expression of ABC proteins other than MRP2. Incubation with GW4064 of LS174T cells transfected with FXR and RXRα resulted in minor changes as regards the most abundantly expressed ABC proteins. A marked up-regulation was only observed for BCRP (27-fold), which under basal conditions, was poorly expressed in these cells (Table 1). Activation of FXR with CDCA was also able to induce BCRP expression (Figure 7D). Using TESS software, the in silico analysis revealed the presence of several putative FXR response elements in DNA region located 5' upstream of *ABCG2* gene (Table 3). When BCRP was transfected in LS174T cells this conferred resistance to mitoxantrone, a well-known BCRP substrate. IC50 value was increased 25-fold (Figure 8A). Similarly, BCRP transfected cells were more resistant to cisplatin whose IC50 value was increased 2.4-fold (Figure 8B).

DISCUSSION

The over-expression of ABC export pumps is one of the major mechanisms accounting for the poor response of gastrointestinal tumors to anticancer agents [1], including cisplatin [16]. In the present study cisplatin was found to induce a typical multidrug-resistance (MDR) phenotype in colon cancer cells, which was characterized by an increased expression of a broad range of ABC proteins. This included pumps with no ability to transport this drug, such as MDR1, MRP1 or MRP3, together with others, such as MRP2, which is known to play a major role in the poor response of colon cancer and other tumors to cisplatin [13], and BCRP, whose role in cisplatin resistance was unknown. Regarding MRP2, a correlation between the expression levels of this protein and the sensitivity to cisplatin of several cell lines has been reported [28, 29]. Moreover, stable transfection of MRP2 cDNA into cells results in the acquisition of resistance to cisplatin [12]. In agreement with these findings, the up-regulation of MRP2 in LS174T/R cells has been previously suggested to be involved in the ability of these cells to reduce the net uptake of cisplatin [9, 23]. This characteristic is abolished by probenecid, which restores the sensitivity of LS174T/R cells to cisplatin-induced toxicity [9].

The question arises as to whether cisplatin is able to activate MRP2 promoter directly. As previously reported [19], we observed that CHO-K1 cells were unable to activate MRP2pr and hence GFP was not synthesized when these cells were transfected with Z1-MRP2pr-GFP. In contrast, as indicated by the shift in the GFP-fluorescence histogram from flow cytometry analysis and the enhancement in luciferase activity when these cells were transfected with Z1-MRP2pr-GFP or Z1-MRP2pr-luciferase, respectively, MRP2pr was activated in LS174T cells. Moreover, in agreement with changes observed in MRP2 mRNA levels, basal MRP2pr activity was higher in LS174T/R cells than in LS174T cells. Maintained stimulation of resistance in LS174T/Rms cells resulted in an increased response to transfection with both Z1-MRP2pr-GFP and Z1-MRP2pr-luciferase.

It has been suggested that DNA-damaging agents induce the up-regulation of the rat *Abcc2* gene [30], and probably also the human ortholog gene *ABCC2* [31], via response elements located at approximately -250 bp upstream of the transcription start site, although the identity of the transcription factors remains unknown. To investigate the sensitivity of different regions of MRP2pr to the treatment with cisplatin the promoter was partially deleted (Z2-MRP2pr). The response of both cisplatin-sensitive and -resistant cells to transfection with this construct was markedly reduced. This suggests the existence of binding sites for inhibitory transcription factors in the sequence located between -1175 and -625 bp. Among the potential inhibitory transcription factors involved in this effect is p53. This is able to inhibit the expression of ABC genes such as *ABCB1* [32] and *ABCC1* [33]. Indeed in silico analysis predicted the existence in

the MRP2pr -1175 to -625 region of several binding sites for p53. Response elements for several transcription factors that may behave as enhancers, such as AP1, HNF1α or NRF2 [19], are present in the deleted region of Z1-MRP2pr (-2650 to -1176). This may account for the recovery of the activity in Z1-MRP2pr as compared to Z2-MRP2pr. Z3-MRP2pr was the construct with the strongest activity, which was probably due to the lack of inhibitory response elements present in Z2-MRP2pr whereas Z3-MRP2pr still contained the majority of binding sites for MRP2pr activators.

The enhanced activity of MRP2pr in LS174T/R cells paralleled changes in the expression of several transcription factors, such as HNF1α and HNF4α. Both have been reported to activate MRP2pr as part of detoxification processes associated with oxidative stress [34]. This is consistent with a link between HNF expression and cisplatin-induced chemical stress, as was observed here. Although HNF3β and C/EBPβ were also increased in LS174T/R cells, they probably do not contribute to resistance to cisplatin in these cells because a negative effect of these transcription factors in the regulation of MRP2, by overriding the stimulatory effect of HNF1α and 4α, is expected to occur [19]. PXR is activated by many xenobiotics, including rifampicin, which through this mechanism enhances MRP expression [20], and induces chemoresistance in tumor cells [35]. Nevertheless, a major direct role of PXR in the MDR phenotype of LS174T/R and LS174T/Rms cells was ruled out because: i) The expression of PXR in LS174T cells was very poor; ii) This was not enhanced in cisplatin-treated cells, whereas, in contrast, other transcription factors were up-regulated; and iii) Removal from the medium of the potential PXR ligand, i.e., cisplatin, did not markedly reduce MDR phenotype of chemoresistant cells.

In contrast, a remarkable finding of the present study was the dramatic up-regulation of FXR in cisplatin-resistant cells which may be also due to activation of NHF-dependent signaling pathway as has been suggested [36]. In LS174T/R cells under basal conditions, and after induction with the FXR agonists CDCA and GW4060, the expression of typical FXR target genes, such as FGF19, SHP, OSTα and OSTβ, was enhanced. This suggests a stimulated activity of the FXR-mediated pathway. However, FXR agonists failed to stimulate MRP2 expression, which suggests that, at least in these human cells, MRP2 is not a target gene of FXR. The reported absence of any correlation between the expression levels of FXR and MRP2 in human liver samples [37] supports this concept. The regulation of Mrp2 expression may be different in rodents, because rat Fxr has been found to bind to an unusual ER-8 element, which is present in the rat Mrp2 promoter [20], but not in the human ortholog MRPpr. Surprisingly, activation of FXR resulted in enhanced expression of BCRP, which up to now was not included as a target gene for this nuclear receptor in spite of the fact that the promoter region contains several putative FXR response elements.

The activation of FXR in LS174T/R cells by GW4064 partly protected the cells against the toxic effect of cisplatin. This could also be reproduced in wild-type cells by transfecting both FXR and RXRα, followed by activation with GW4064. Although FXR is considered to be the natural and specific target for bile acids [38], our results indicate that chemoresistance elicited by cisplatin may involve FXR in a bile acid-independent manner. This suggests that FXR may be part of a complex and poorly understood system of defense to chemical stress. Thus, in this sense a role of FXR in the protection of gastrointestinal epithelia against potentially toxic compounds [39], or even in enhanced resistance to apoptosis of tumor gastric cells [40], has been suggested. The mechanism is poorly understood but this may involve DNA damage-induced activation of MAPKs signalling pathway, which resulted in enhanced activity of specific PKC able to phosphorilate FXR. The transcriptional activity of this nuclear receptor seems to be higher when the protein is phosphorilated [41].

Finally, we wondered about the MOC actually involved in FXR-induced protection against cisplatin toxicity. Besides the up-regulation of MRP2, which probably plays a major role in cisplatin-induced chemoresistance, but through an FXR-independent mechanism, the involvement of other genes must be considered. In this respect, an interesting finding was the ability of cisplatin treatment to induce the expression of BCRP. This was particularly high in cisplatin-resistant cells. Similar results in cells derived from esophageal carcinoma with acquired resistance to cisplatin have been reported [42]. Moreover, FXR activation was also able to up-regulate BCRP. Functional experiments carried out here revealed that indeed BCRP transfection to colon cancer cells conferred protection against a well-known substrate of BCRP, such as mitoxantrone, as well as against cisplatin. However, in LS174T/R cells FXR activation was able to further reduce sensitivity to cisplatin, in spite of the fact that BCRP expression was not further increased. This suggests that other mechanisms are also involved in FXR-mediated enhanced chemoresistance.

The up-regulation in cisplatin-resistant cells of other pumps able to export this drug, such as MRP6 and ATP7B found here, and the previously reported down-regulation of uptake transporters, such as CTR1 or SLC31A1 [9], may contribute to the reduction in the intracellular levels of the drug in these cells (MOC-1). Conjugation with glutathione, which plays an important role in cisplatin inactivation by tumor cells (MOC-2) and which is enhanced in LS174T/R cells [23], and an alteration in the p53 status (MOC-5) may also contribute to FXR-dependent cisplatin-induced MDR phenotype of LS174T/R [23].

In conclusion, in colon cancer cells, treatment with cisplatin induces changes in the expression of a broad range of ABC proteins, in particular MRP2 and BCRP. Both the enhanced

expression of MRP2, which occurs in an FXR-independent manner, and the activation of FXR-mediated mechanisms, which include BCRP up-regulation are involved in the development of cisplatin-induced MDR phenotype.

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FIGURE LEGENDS

Figure 1. (A) Schematic representation of the 5'-flanking region of the human *ABCC2* gene from nucleotides -2650 to +248 relative to the main transcription start site. The open reading frame begins at nucleotide +249. The regions of the promoter cloned into the plasmids upstream of a reporter gene (Z1-, Z2- and Z3-MRP2pr) and the putative binding sites for transcription factors potentially involved in the regulation of MRP2 expression are depicted. (B) Transcriptional activity of MRP2 promoter (MRP2pr)-firefly luciferase (Luc2) fusion plasmids 72 h after being transiently transfected in wild-type LS174T, and cisplatin-resistant cells without (LS174T/R) or with maintained stimulation due to the presence of cisplatin (LS174T/Rms). The constructs contain Z1-, Z2- and Z3-MRP2pr preceding the ORF of the reporter gene. Numbers indicate positions relative to the main transcription start site. A Luc2-expression vector without any promoter was used as a negative control. A vector containing the herpes virus thymidine kinase promoter (HSV-TKpr) and the *Renilla* luciferase reporter gene was used to determine transfection efficiency. The luminescence signal is expressed as arbitrary units of light after normalizing by cell viability and transfection efficiency. Values are means±S.D. from at least 3 independent transfection experiments performed in triplicate. *, $p<0.05$, as compared with luminescence of LS174T cells. †, $p<0.05$, as compared with Z1-MRP2pr.

Figure 2. Representative image of Western blot analysis of MRP2 (A), BCRP (B) and different transcription factors (C) carried out with lysates of the following cells: HepG2 and JAr and Caco-2 (positive controls), and LS174T, and the cisplatin-resistant cells without (LS174T/R) or with (LS174T/Rms) maintained exposure to cisplatin. Detection was performed using appropriate primary monoclonal antibodies. Western blotting of GAPDH was also performed as a control of the protein loaded in each lane.

Figure 3. (A) Time-course of calcein content in wild-type LS174T, and cisplatin-resistant cells without (LS174T/R) or with maintained stimulation due to the presence of cisplatin (LS174T/Rms). The cells were incubated with 1 µM calcein acetoxymethyl ester (calcein AM) and 50 µg/ml of propidium iodide (PI) at 37°C for the indicated time. Values are the mean fluorescence intensities (AUF, arbitrary units of fluorescence) measured by flow cytometry. Dead cells based on PI staining were excluded from the analysis. (B) Representative frequency histogram of calcein fluorescence of non-PI-stained cells after incubation at 37°C for 30 min. (C) Effect of probenecid on calcein content of non-PI-stained cells. Values are the mean fluorescence intensities after incubating the cells with 1 µM calcein AM and 50 µg/ml of PI, with or without 500 µM probenecid, at 37°C for 30 min. Results are expressed as

means±S.D. from at least 3 determinations per data point using cells from 3 different cultures. *, p<0.05, as compared with LS174T cells. †, p<0.05, as compared with cells incubated in the absence of inhibitor.

Figure 4. Induction of activity of the MRP2 promoter (MRP2pr) by rifampicin and cisplatin in in wild-type LS174T (WT), and cisplatin-resistant cells without (LS174T/R) (R) or with maintained stimulation due to the presence of cisplatin (LS174T/Rms) (Rms) cells. Cells were transiently transfected with Z1-, Z2- or Z3-MRP2pr-firefly luciferase fusion plasmids 72 h before the luciferase assay was carried out. Either 10 μM rifampicin (A) or 5 μM cisplatin (B) were added 48 h after transfection. The luminescence signal is expressed as arbitrary units of light after normalizing by cell viability and transfection efficiency. Values are expressed as means±S.D. from at least 3 transfection experiments performed in triplicate. *, p<0.05, as compared with Controls.

Figure 5. Relative expression of different transcription factors measured by RT-QPCR in wild-type LS174T and cisplatin-resistant cells without (LS174T/R) or with maintained stimulation due to the presence of cisplatin (LS174T/Rms). Transcription factors are classified according to the structure of their DNA-binding domains. Levels of mRNA are expressed in comparison with those found in a pool of healthy human liver used as the calibrator, or in LS174T cells (inset). Values are means±S.D. from at least 3 cultures performed in triplicate. *, p<0.05, as compared with LS174T cells.

Figure 6. Effect in LS174T and LS174T/R cells of treatment with FXR agonists on the expression of well-known FXR target genes (FGF19, SHP, OSTα/β) together with MRP2 and BCRP. The levels of mRNA in cells treated with 100 μM chenodeoxycholic acid (CDCA) or 1 μM GW4064 for 24 h were measured by RT-QPCR and expressed in comparison with those found in LS174T cells treated only with DMSO as vehicle. Values are means±S.D. from 3 independent experiments performed in triplicate. *, p<0.05, as compared with LS174T cells. †, p<0.05, as compared with Controls.

Figure 7. Protective effect of FXR activation against cisplatin-induced cell death. A. Wild type (WT) LS174T cells and cisplatin resistant (R) LS174T/R cells were incubated without (Control) or with 1 μM GW4064 for 72 h. B. WT cells were transfected with an empty vector (Mock) or FXR plus RXRα 48 h before being incubated without (Control) or with 1 μM GW4064 for 72 h. C. Comparison of IC_{50} values for cisplatin in WT and R cells with and without transfection and with and without treatment with GW4064 from concentration-response studies similar to those of A and B. The proportion of living cells in comparison with nontreated plates (control = 100%

viability) was determined by the formazan test. IC_{50} was calculated as the cisplatin concentration able to reduce cell viability by 50%. D. BCRP up-regulation in LS174T cells transfected with FXR plus RXRα 48 h before being incubated without (Control) or with 1 µM GW4064 or 50 µM chenodeoxycholic acid (CDCA) for 24 h. Values are means±S.D. from at least 3 experiments performed in triplicate. *, $p<0.05$, on comparing the highest versus the lowest viability. †, $p<0.05$, as compared with or without GW4064.

Figure 8. Chemoresistance induced by BCRP expression against toxic effect induced by mitoxantrone and cisplatin. Using the formazan test cell viability was determined in wild type LS174T cells transfected with an empty vector (Control, open circles) or with a vector containing the complete ORF of human BCRP (closed circles) and incubated with mitoxantrone (A) or cisplatin (B) at the indicated concentrations for 72 h in order to calculate the IC50 value (insets). Values are means±S.D. from 3 experiments performed in triplicate. *, $p<0.05$, as compared with Control.

Supplementary Figure 1. Representative frequency histograms of the GFP fluorescence of CHO-K1 (A), LS174T (B) and LS174T/Rms (C) cells transfected with GFP reporter gene-plasmids. Flow cytometry analyses were carried out 72 h after transfecting the cells with an empty vector (Control) or with plasmids containing the GFP reporter gene, whose expression was driven by the MRP2 promoter (Z1-MRP2pr) or the cytomegalovirus promoter (CMVpr), as a positive control of GFP expression. Insets show representative pictures of GFP expressing cells, obtained by merging the phase-contrast and fluorescence microscopy photographs.

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Table 1. Effect of cisplatin on the expression of drug export pumps.

Expression level	Transporter		ΔCt	Fold-change *vs.* wild-type LS174T			
	Protein	Gene	Wild-type LS174T	LS174T/FXR + GW4064	LS174T + cisplatin	LS174T/R	LS174T/Rms
High	Wilson	*ATP7B*	4.2 ± 0.8	≈1	↑ (2)	↑↑ (7)	↑↑ (7)
	MRP1	*ABCC1*	4.4 ± 0.9	≈1	↑↑ (4)	↑↑ (5)	↑↑ (6)
	ABCA2	*ABCA2*	4.5 ± 0.6	≈1	≈1	≈1	↓ (1/2)
	MDR1	*ABCB1*	4.8 ± 0.9	≈1	↑ (2)	↑ (2)	↑ (3)
	Menkes	*ATP7A*	5.2 ± 1.0	≈1	↑ (2)	≈1	≈1
	LRP	*MVP*	5.3 ± 0.7	≈1	↑↑ (7)	↑ (2)	↑ (2)
	MRP3	*ABCC3*	5.5 ± 0.9	≈1	↑↑ (5)	↑↑ (9)	↑↑↑ (13)
Medium	MRP6	*ABCC6*	6.0 ± 0.9	↓ (1/2)	↑↑ (4)	↑↑ (5)	↑↑ (8)
	MRP2	*ABCC2*	6.1 ± 1.2	≈1	↑↑ (4)	↑↑↑ (353)	↑↑↑ (494)
	ABCA8	*ABCA8*	6.2 ± 0.8	↑↑ (4)	↓↓↓ (1/22)	↓↓↓ (1/20)	↓↓↓ (1/170)
	MRP5	*ABCC5*	6.2 ± 1.0	≈1	↑ (2)	↑ (2)	↑ (3)
	MDR3	*ABCB4*	6.7 ± 0.7	↑ (3)	≈1	↑ (3)	↑↑ (4)
	MRP7	*ABCC10*	6.8 ± 2.7	≈1	↑↑ (6)	↑ (2)	↑ (2)
	ABCA3	*ABCA3*	7.2 ± 1.2	≈1	≈1	↓ (1/2)	↓↓ (1/9)
Low	MRP4	*ABCC4*	9.2 ± 0.4	≈1	≈1	↑↑ (9)	↑↑↑ (13)
	MRP8	*ABCC11*	9.4 ± 1.6	↑ (2)	↓ (2)	↓↓ (1/8)	↓↓↓ (1/27)
	BCRP	*ABCG2*	11.5 ± 2.3	↑↑↑ (17)	↑↑↑ (12)	↑↑↑ (196)	↑↑↑ (279)
	BSEP	*ABCB11*	11.5 ± 1.6	↑ (2)	↓ (1/3)	↓↓ (1/6)	↓↓ (1/8)
	ABCA6	*ABCA6*	12.0 ± 1.0	≈1	≈1	↓ (1/2)	↓ (1/2)

Cells were wild type (LS174T) and cisplatin-resistant cells without (LS174T/R) or with maintained stimulation due to the presence of cisplatin in the culture medium (LS174T/Rms). Some cultures of LS174T cells were exposed to 20 μM cisplatin for 72 h or transfected with FXR and RXRα and then treated with 1 μM GW4064 for 24 h. Levels of mRNA were measured in triplicate by RT-QPCR in samples from 3 different cultures. Ct, threshold cycle. ΔCt is the difference between Ct of the target gene and Ct of GAPDH (21.5±0.7) and it is expressed as means±S.D. The expression level is considered to be high when ΔCt<6.0; medium, ΔCt 6.0 to 9.0; low, ΔCt>9.0. The amount of mRNA was calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between ΔCt of the target gene and ΔCt of a calibrator, which was human liver. Symbols indicating changes of expression are: ↑ or ↓, low; ↑↑ or ↓↓, high; ↑↑↑ or ↓↓↓, very high. ≈1, no significant change (change lower than 1.5-fold).

Table 2. Effect of cisplatin on the expression of drug export pumps.

Expression level	Transporter		ΔCt	Fold-changes *vs.* Caco-2
	Protein	Gene	Caco-2	Caco-2 + cisplatin
High	MRP2	*ABCC2*	3.2 ± 0.3	↑ (2)
	Wilson	*ATP7B*	3.6 ± 0.3	≈1
	MRP4	*ABCC4*	4.6 ± 0.6	↓ (1/2)
	ABCA3	*ABCA3*	5.3 ± 1.4	↓ (1/3)
	LRP	*MVP*	5.4 ± 0.1	≈1
	BCRP	*ABCG2*	5.8 ± 1.2	↑↑ (9)
	ABCA2	*ABCA2*	5.9 ± 1.1	≈1
Medium	MRP3	*ABCC3*	6.9 ± 0.2	↑↑ (4)
	MRP1	*ABCC1*	7.0 ± 0.1	↑ (3)
	MRP5	*ABCC5*	7.0 ± 0.2	↑ (3)
	MRP6	*ABCC6*	8.2 ± 1.0	↑ (2)
	MDR1	*ABCB1*	8.6 ± 0.7	↑ (2)
	Menkes	*ATP7A*	8.7 ± 0.1	≈1
Low	ABCA6	*ABCA6*	9.1 ± 0.4	↓ (1/2)
	MRP7	*ABCC10*	10.4 ± 0.1	≈1
	ABCA8	*ABCA8*	12.1 ± 0.8	↓↓↓ (1/21)
	MRP8	*ABCC11*	12.4 ± 0.1	≈1
	MDR3	*ABCB4*	13.7 ± 1.7	↓↓↓ (ND)
	BSEP	*ABCB11*	16.6 ± 3.1	↓↓↓ (ND)

Steady-state levels of mRNA in Caco-2 cells, exposed or not to 25 μM cisplatin for 72 h, were measured in triplicate by RT-QPCR in samples from 3 different cultures. Ct, threshold cycle. ΔCt is the difference between Ct of the target gene and Ct of GAPDH (20.4±1.1) and it is expressed as means±S.D. The expression level is considered to be high when ΔCt<6.0; medium, ΔCt 6.0 to 9.0; low, ΔCt>9.0. The amount of mRNA of a target gene was calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between ΔCt of the target gene and ΔCt of a calibrator, which was human liver. Symbols indicating changes of expression are: ↑ or ↓, low; ↑↑ or ↓↓, high; ↑↑↑ or ↓↓↓, very high. ≈1, no significant change (change lower than 1.5-fold). ND, not detected.

Table 3. In silico evaluation of potential sites for FXR/RXR interaction with the DNA region located 5' upstream of *ABCG2* gene.

Position	Sequence	RE	Sns	TESS scores			
				La	La/	Lq	Ld
-8973 to -8968	TGAACT	half-site	R	SIU-NCS			
-8755 to -8743	tCACCTnAGGTCA	ER-1	N	SIU-NCS			
-8748 to -8735	AGGTCAnnAGtTCg	DR-2	N	22	1.57	0.917	2
-8290 to -8285	TGAACT	half-site	R	SIU-NCS			
-8222 to -8210	aGACTTnTGACCT	DR-1	R	14.06	1.28	0.894	1.67
-8034 to -8022	cacTCAnTGACCT	IR-1	R	22	1.57	0.917	2
-8022 to -8016	TGAtCCT	half-site	R	16	2	1	0
-7988 to -7983	TGACCc	half-site	R	14	2	1	0
-6813 to -6800	TGAaCTnnTGACCT	DR-2	R	24	1.71	1	0
-6563 to -6551	AGGcCAngagCCa	IR-1	N	SIU-NCS			
-6458 to -6446	aGACtTnTGACCc	DR-1	R	SIU-NCS			
-6151 to -6138	AGGcCAnnAGtTCA	DR-2	N	22	1.57	0.917	2
-5760 to -5755	TGAACT	half-site	R	SIU-NCS			
-5439 to -5426	AGGTCAnnAGtTCg	DR-2	N	22	1.57	0.917	2
-5391 to -5379	TcACCTnAGGTCA	ER-1	N	22	1.57	0.917	2
-4387 to -4382	TGAACT	half-site	N	SIU-NCS			
-4198 to -4181	ctGTCtn(6)TGACCT	IR-6	N	22	1.29	0.917	2
-4155 to -4150	TGAACT	half-site	R	SIU-NCS			
-3994 to -3982	AGGgCAnAGGgCA	IR-1	N	22	1.69	0.917	2
-3879 to -3860	TGACCTn(8)AGGTCc	ER-8	N	SIU-NCS			
-3752 to -3747	TGAACT	half-site	N	SIU-NCS			
-2911 to -2906	TGAACT	half-site	R	SIU-NCS			
-2643 to -2631	GAGTTcnTGtCCT	IR-1	N	SIU-NCS			
-2115 to -2110	TGAACT	half-site	N	SIU-NCS			
-357 to -344	AGGTCAnncGAggT	IR-2	N	12.53	1.25	0.905	1.32

Position is counted 5' upstream of transcription start site.
RE, Response Element; ER, everted repeat; DR, Direct repeat; IR, Inverted repeat.
Sns, Sense of the site; N, normal; R, reverse.
SIU-NCS, Site identified by user, non-calculated scores.
La, Log-likelihood score, higher is better, maximum is 24.
La/, La/Len, higher is better, maximum is 2.0.
Lq, higher is better, maximum is 1.0.
Ld, 0 is best, higher is worse.

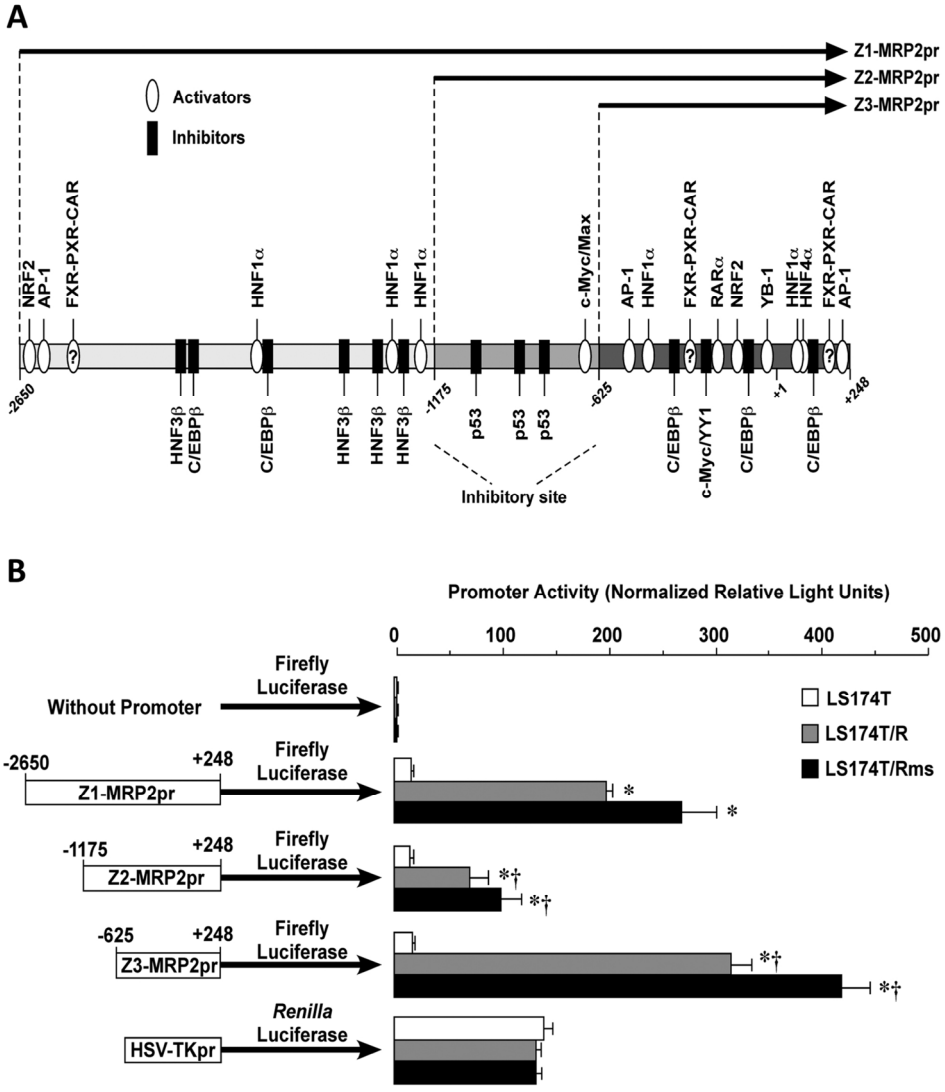

Figure 1

180x215mm (300 x 300 DPI)

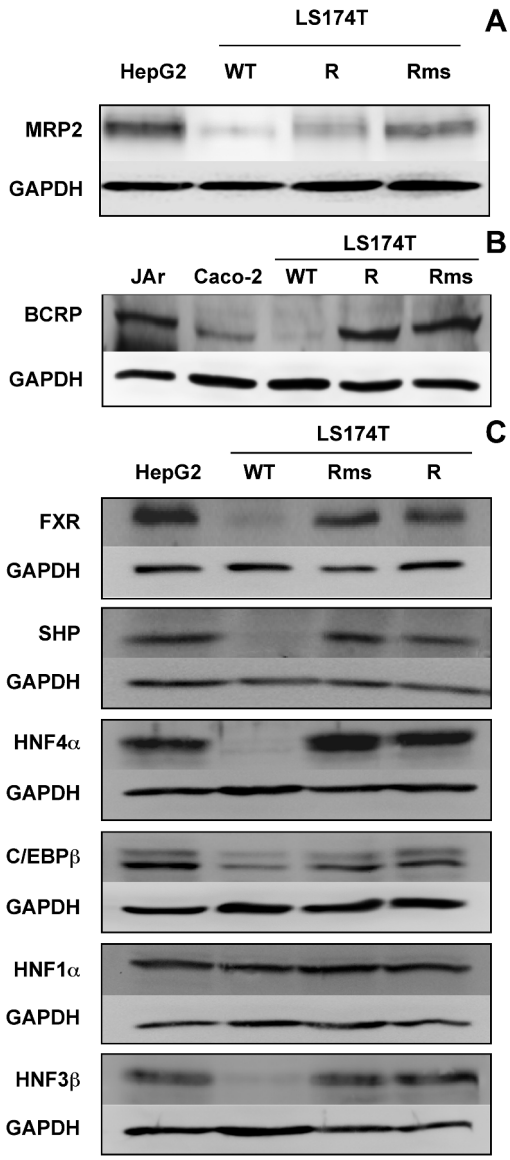

Figure 2

246x586mm (300 x 300 DPI)

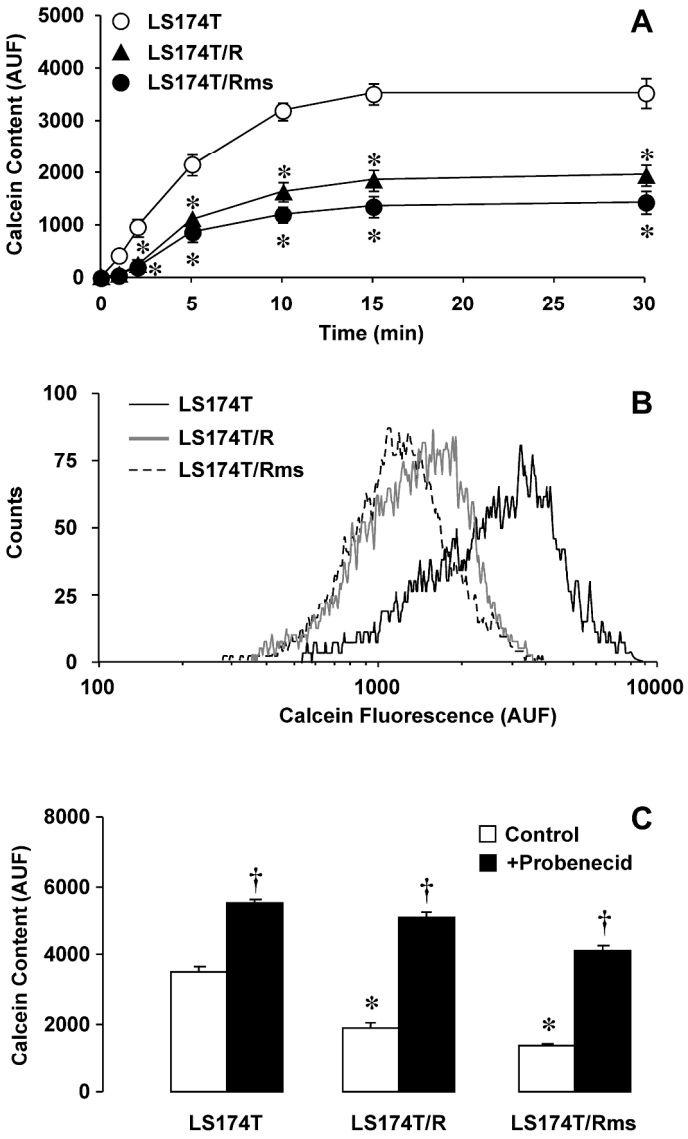

Figure 3

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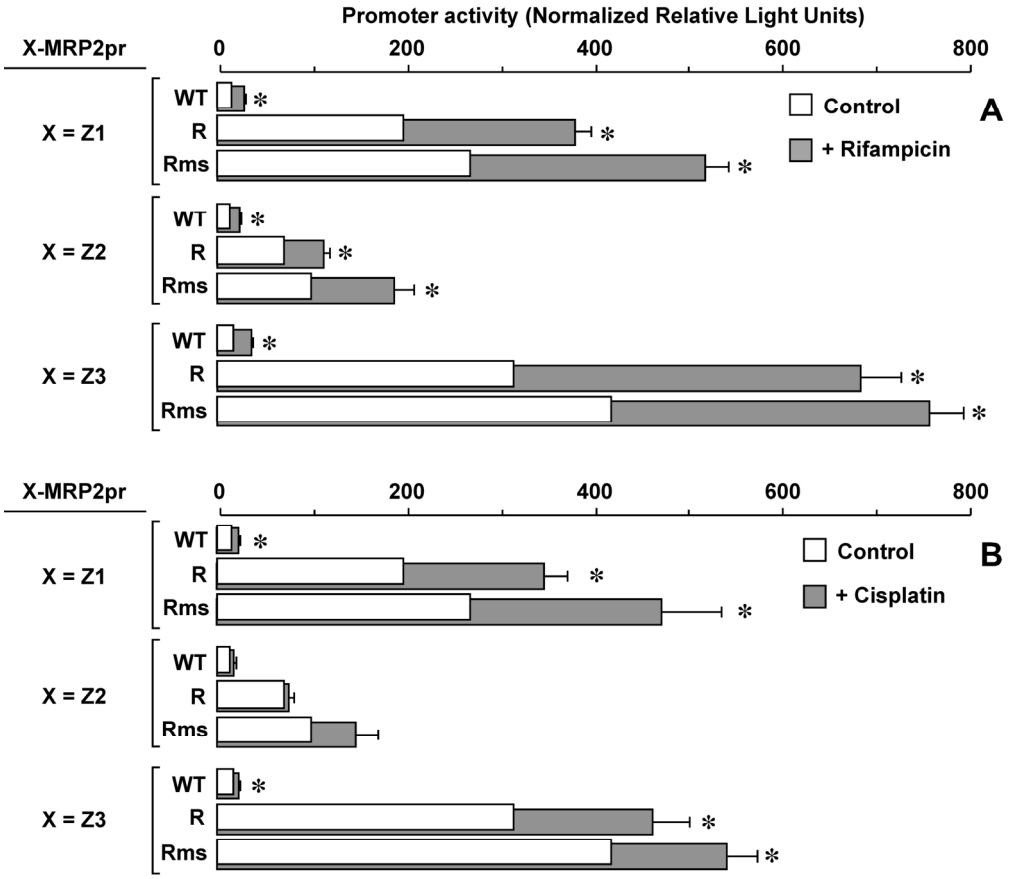


Figure 4

Figure 4
183x172mm (300 x 300 DPI)

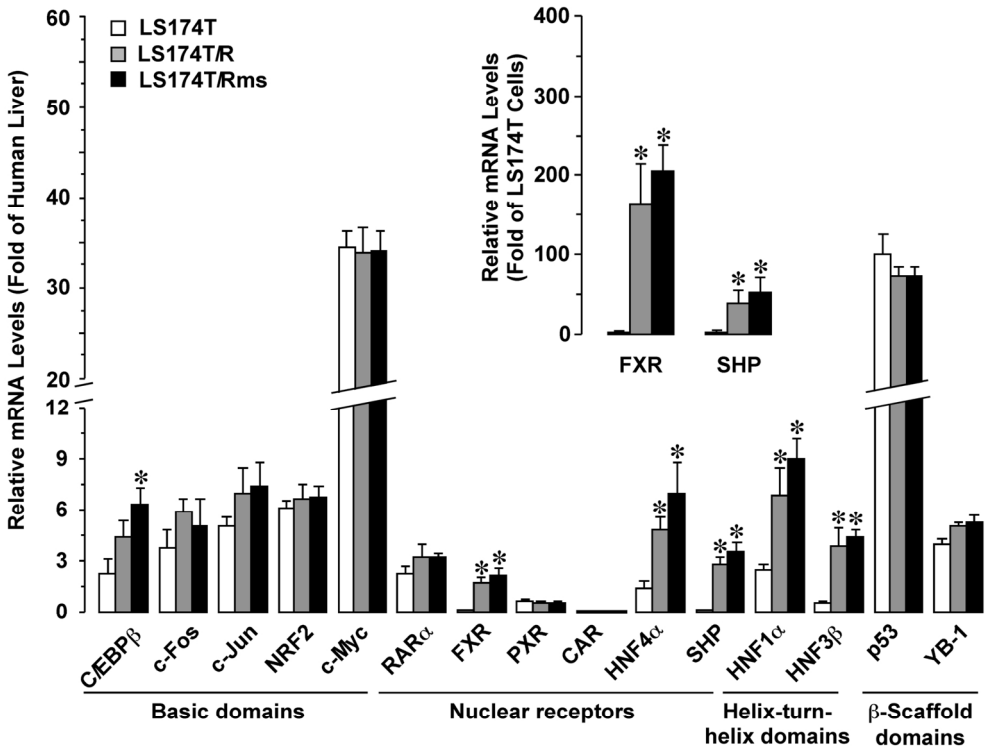


Figure 5

Figure 5
157x128mm (300 x 300 DPI)

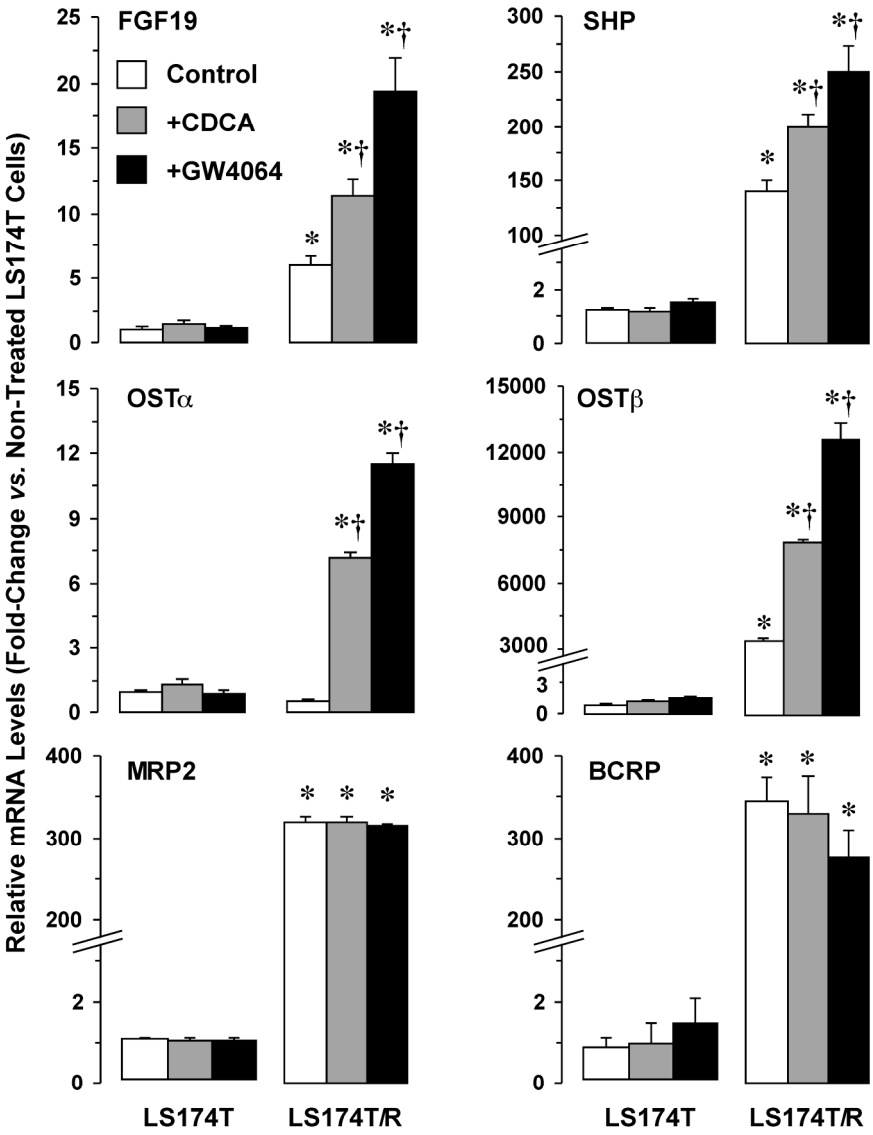


Figure 6

Figure 6
230x318mm (300 x 300 DPI)

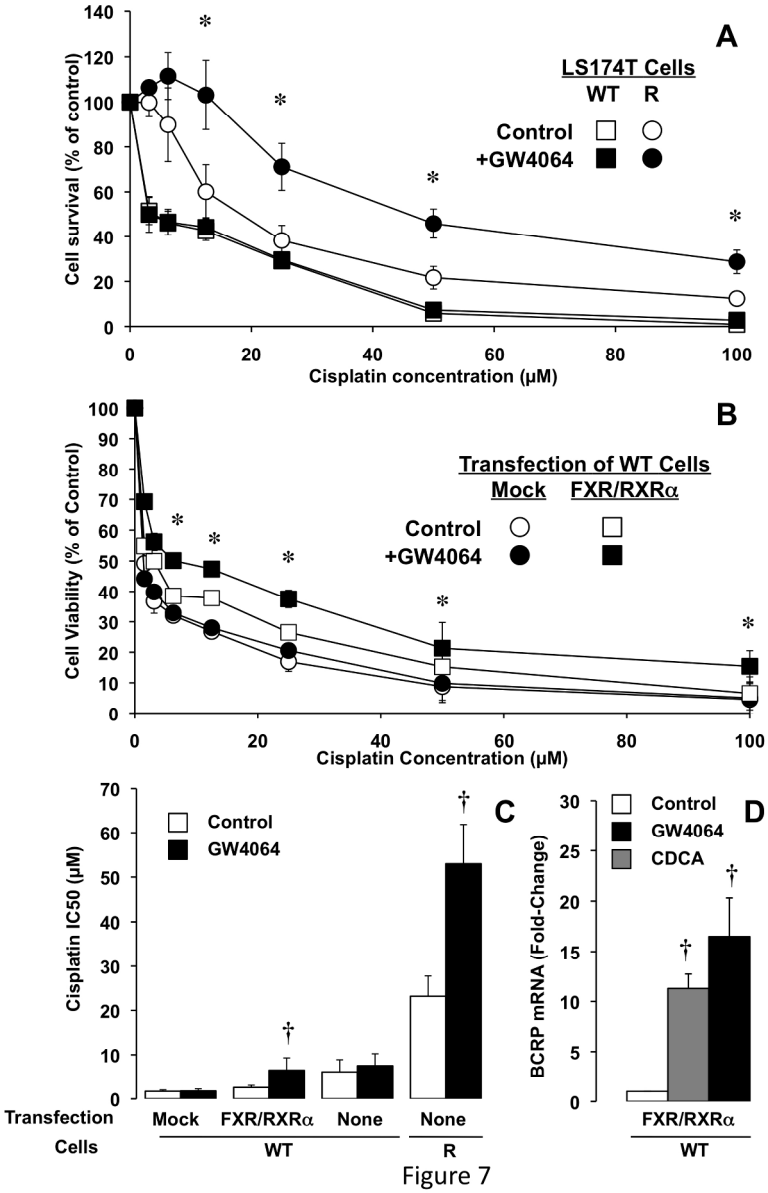


Figure 7

Figure 7
793x1146mm (72 x 72 DPI)

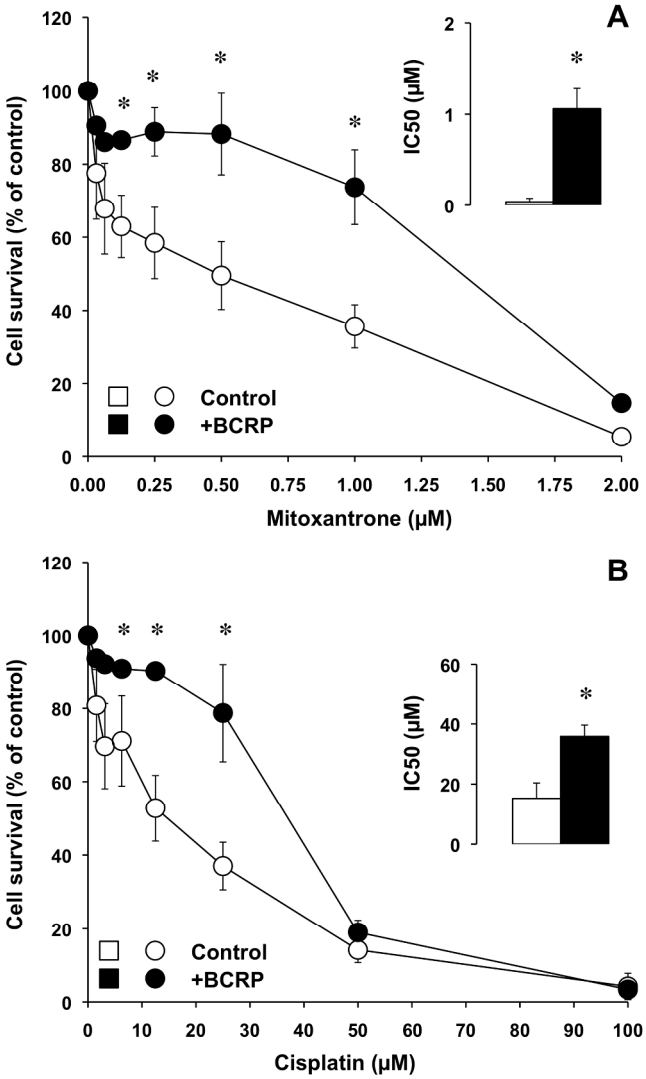
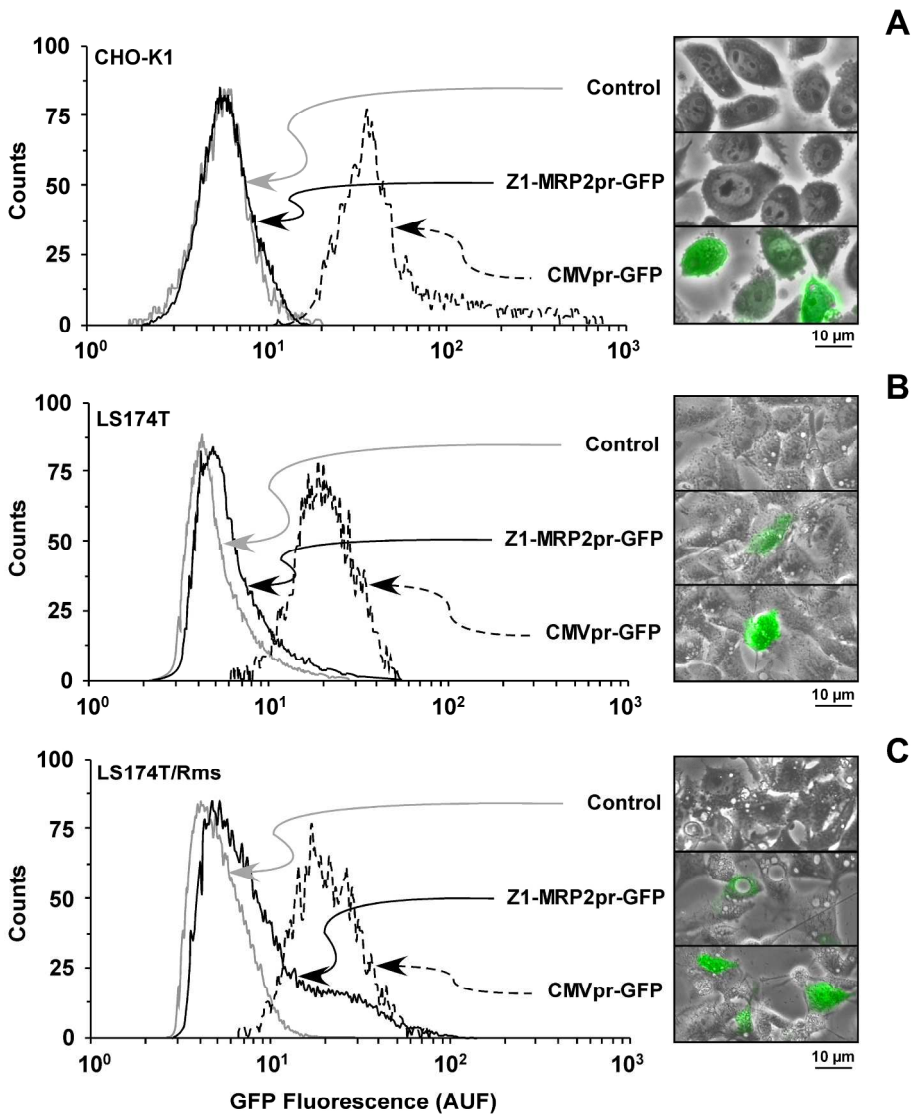


Figure 8

Figure 8
793x1146mm (72 x 72 DPI)



Supplementary Figure 1

223x290mm (300 x 300 DPI)