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Transcriptional regulation of aldo-keto reductase 1C1 in HT29 human colon cancer cells resistant to methotrexate: Role in the cell cycle and apoptosis

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ABSTRACT

While studying differentially expressed genes between sensitive and 10^{-5} M Methotrexate (MTX) resistant HT29 human colon cancer cells, we identified some members of the aldo-keto reductase (AKR) superfamily. The study was followed with the member AKR1C1 (EC 1.1.1.213), validating its increase in mRNA and protein levels in MTX resistant cells. The genomic content for AKR1C1 remained unchanged between sensitive and resistant cells, thereby excluding a mechanism of AKR1C1 gene amplification. Thus, we cloned the AKR1C1 human promoter and performed luciferase experiments that revealed a transcriptional regulation of the gene in the resistant cells. Computational studies showed a putative binding site for the transcription factor Sp1. The co-transfection of Sp1 or Sp3 with different constructs of AKR1C1 promoter deletions, including and excluding the proximal GC-box, demonstrated a key role for these factors in regulating AKR1C1 transcriptional activity. Gel-shift assays revealed an increase in Sp1 and Sp3 binding in resistant compared to sensitive cells, without differences in Sp1 protein levels. Dephosphorylation of the extracts coincided with a decrease in Sp1 binding, which is consistent with a process of regulation of Sp1 by phosphorylation. We also investigated the possible relationship between AKR1C1 expression and MTX action. Overexpression of AKR1C1 counteracted the S-phase accumulation of cells and apoptosis caused by MTX treatment. This suggests a role of AKR1C1 in cell proliferation. Finally, overexpression of AKR1C1 in MTX sensitive HT29 cells conferred resistance to the chemotherapeutic agent and silencing of AKR1C1 by means of iRNA technology sensitized the cells to MTX.

1. Introduction

Dihydrofolate reductase (DHFR), a key enzyme of the folate cycle and the one carbon unit metabolism [1–3], catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tertrahydrofolate (THF) [4]. DHFR enzymatic activity is necessary for the biosynthesis of purines and thymidylate that are needed for cell proliferation. Methotrexate (MTX) is a 4-amino 10-methyl analog of folic acid that inhibits DHFR activity by competing with DHF for the active site. MTX was one of the first antimetabolite drugs developed and nowadays continues to play an important role in the chemotherapy of

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Abbreviations: AKR1C1, aldo-keto reductase 1 member C1; MTX, methotrexate; DHFR, dihydrofolate reductase; Sp1, specificity protein 1; APRT, adenine phosphoribosyltransferase.

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human malignancies such as acute lymphoblastic leukemia, lymphoma, osteosarcoma, breast cancer, and head and neck cancer [5,6]. Unfortunately, the efficacy of this chemotherapeutic agent is often compromised by the development of resistance in cancer cells.

The identification of suitable genes to target in combination with MTX could be a strategy to minimize the development of resistance. To this end, we studied the gene expression profile in MTX resistance using the HG U133A 2.0 cDNA microarrays from Affymetix containing 22.300 transcripts. The human colon adenocarcinoma cell line HT29 was chosen for this study because it can be adapted to grow in high concentrations of MTX [7] and concomitantly develop amplification of the *dhfr* gene [8]. Among the genes whose expression is changed in cells with acquired resistance to 10^{-5} M MTX, we noted some members of the aldo-keto reductase (AKR) superfamily.

Members of the AKR superfamily are monomeric cytoplasmic proteins of about 320 amino acid residues, which have related structures and common evolutionary origins [9]. These enzymes are present from prokaryotes to eukaryotes and they share similar sequences and properties [10–12]. The AKRs have been proposed to be involved in detoxification processes [11,13–15], as they can catalyze the NAD(P)H-dependent oxidoreduction of a wide range of substrates [16]. Substrate specificity is dictated by the loops at the back of the structure. Fourteen families of AKRs exist, and the AKR1 family contains many of the human isoforms.

AKRs have previously been related to cancer. Hsu et al. [17] showed that AKR1C1, also known as Dihydrodiol dehydrogenase (DDH), is highly overexpressed in NSCLC patients and its high expression correlates with a poor prognostic outcome. Overexpression of AKR1C1 has also been found in esophageal cancer, and has been associated with disease progression [18]. This gene is upregulated in HER-2/neu-positive breast tumors, which could suggest an enhanced activation of the cellular detoxification processes within the breast tumor microenvironment [19]. In addition, AKRs contribute to polycyclic aromatic hydrocarbons (PAH)-induced oral carcinogenesis [20], as induced AKR1C isozymes can convert PAH *trans*-dihydrodiols to deleterious *O*-quinones that can cause oxidative DNA damage as well as change-in-function mutations in the p53 tumor suppressor gene [21].

AKR1C1 overexpression has also been related to drug-resistance in a variety of cancers. It has been proposed that the high similarity between the chemical structures of anticancer drugs and some compounds that can be metabolized by AKR1C1 could indicate that these drugs may be subject to this enzyme activity [17]. Several reports are on accordance with this hypothesis. On one hand, Ax et al. detected AKR1C1 overexpression in daunorubicin-resistant human stomach cancer cells and suggested an association of this enzyme to drug-resistance, which they postulated to be mediated through drug detoxification in these cancer cells [22], as it had been also proposed for NSCLC [17]. On the other hand, a study on human ovarian cancer cell lines indicated that overexpression of AKR1C1 was closely associated with resistance to cisplatin and probably to disease progression [23]. The same investigators suggested that an increase in AKR1C1 activity would be sufficient to detoxify ROS, induced by cisplatin, and could lead to apoptosis-related development of drug-resistance [24]. Chen et al. [25] also correlated AKR1C1 expression with cisplatin-based chemotherapy resistance using epithelial ovarian cancer patient samples. In addition, Hung et al. [26] concluded that resistance to cisplatin, adriamycin and radiotherapy in lung adenocarcinoma cells was closely associated with AKR activity. Furthermore, increased expression of AKR1C1 in ethacrynic acid-induced drug-resistant human colon cancer cells has been also identified [27,28], and it has been proposed that this overexpression may give rise to an enhanced capacity to metabolize exogenous and endogenous substrates, thereby contributing to the drug-resistant phenotype.

In this work we detected an overexpression of AKR1C1 in HT29 MTX-resistant cells and proceeded with the study of the mechanism of action for this effect. We found that there is a transcriptional regulation of AKR1C1 in the resistant cells, which is mainly dependent on the transcription factor Sp1, and that AKR1C1 overexpression counteracts the effects of MTX both at the level of S-phase cell arrest and apoptosis.

2. Materials and methods

2.1. Cell culture

Human colon adenocarcinoma cell line HT29 was routinely grown in Ham's F12 medium supplemented with 7% fetal bovine serum (FBS, both from Gibco) at 37 °C in a 5% CO_2 humidified atmosphere. Cells resistant to 10^{-5} M MTX (HT29-R) were previously obtained in the laboratory upon incubation with stepwise concentrations of MTX (Lederle) in selective DHFR medium (–GHT medium) lacking glycine, hypoxanthine and thymidine, the final products of DHFR activity. This medium was supplemented with 7% dialyzed fetal bovine serum (above two from GIBCO). The starting conditions for obtaining the resistant cells were 3×10^6 HT29 parental cells treated with a concentration of 10^{-8} M MTX. The cells that survived were allowed to grow until subconfluence, and 70% of this population was incubated with 3×10^{-8} M MTX. The same process was repeated increasing the concentration of MTX by a factor of three until reaching 10^{-5} M. This subline is freely available upon request.

2.2. Microarrays

Gene expression was analyzed by hybridization to The GeneChip® Human Genome U133A 2.0 microarrays from Affymetrix, containing 22,300 transcripts and variants. Total RNA for cDNA arrays was prepared from triplicate samples using RNAeasy Mini kit (Qiagen) following the recommendations of the manufacturer. Labeling, hybridization and detection were carried out following the manufacturer's specifications.

2.3. Microarray data analysis

Quantification was carried out with GeneSpring GX 7.3 software (Silicon Genetics), which allows multi-filter comparisons using data from different experiments to perform the normalization, generation of restriction lists and the functional classification of

the differentially expressed genes. Normalization was applied in two steps: (i) ''per Chip normalization'' by which each measurement was divided by the 50th percentile of all measurements in its array; and (ii) ''per Gene normalization'' by which all the samples were normalized against the median of the control samples. The expression of each gene is reported as the ratio of the value obtained after each condition relative to control condition after normalization of the data. Then data were filtered using the control strength, a control value calculated using the Cross-Gene Error Model based on replicates [29]. Measurements with higher control strength are relatively more precise than measurements with lower control strength. Genes that did not reach this value were discarded. Additional filtering was performed to determine differentially expressed genes. On one hand, a restriction p-value of less than 0.05 was applied. The output of this analysis was then filtered by fold expression, selecting specifically those genes that had a differential expression of at least 5-fold.

2.4. RT-PCR

Levels of AKR1C1 mRNA were determined by RT-PCR under quantitative conditions. Total RNA was extracted from cells (4×10^6) using Ultraspec™ RNA reagent (Biotecx) following the recommendations of the manufacturer. Complementary DNA was synthesized in a total volume of 20 μl from RNA samples by mixing 1 μg of total RNA, 125 ng of random hexamers (Roche), in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units of RNasin (Promega), 0.5 mM dNTPs (AppliChem), 200 units of M-MLV reverse transcriptase (Invitrogen) and 50 mM Tris–HCl buffer, pH 8.3. The reaction mixture was incubated at 37 °C for 60 min and the cDNA product was used for subsequent PCR amplification with specific primers. A standard 50 μl mixture contained 5 μl of the cDNA mixture, 1.2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 μCi of [α-^{32}P]dATP (3000 Ci/mmol, Amersham Ibérica), 1.5 units of Taq polymerase (Ecogen), 500 ng of each primer and 20 mM Tris–HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq DNA polymerase were separated from primers and dNTPs by using a layer of paraffin (Fluka) (reaction components contact only when paraffin fuses, at 60 °C). PCR was performed in an MJ Research Thermocycler equipped with peltier system and temperature probe. Preliminary experiments were carried out using different number of cycles to determine the linear conditions of PCR amplification. The sequences of the forward and reverse specific primers used for PCR amplification and the length of the PCR product, are given below.

AKR1C1: 5′-GGTCACTTCATGCCTGTCCTG-3′ and 5′-GTCTTCTCTC-TTCACACTGCCATC-3′ (197 bp)
APRT: 5′-GCAGCTGGTTGAGCAGCGGAT-3′ and 5′-AGAGTGGGGCC-TGGCAGCTTC-3′ (253 bp)

[α-^{32}P]dATP was used in the PCR to produce a radioactive product that could be detected with great sensitivity during the exponential phase of the reaction. After an initial denaturation for 2 min at 94 °C, PCR was performed for 20 cycles. Each cycle consisted of denaturation at 92 °C for 1 min, primer annealing at 59 °C for 1 min, and primer extension at 72 °C for 1 min. A final 7-min extension step at 72 °C was performed. Five microliters of each PCR sample was electrophoresed on a 1-mm-thick 5% polyacrylamide gel. The gels were dried and placed on contact with europium screens that were scanned using phosphorimaging. The expression of AKR1C1 mRNA is reported upon normalization using the APRT mRNA as internal control.

2.5. Preparation of cytoplasmic and total extracts for Western-blotting

Cytoplasmic extracts were obtained from HT29 or HT29-R cells. Cells were collected in ice-cold F-12 medium and centrifuged at 800 × g for 5 min. The cell pellet was gently resuspended in 3 ml of hypotonic buffer (HB = 15 mM NaCl, 60 mM KCl, 0.5 mM EDTA, 1 mM PMSF, 1 mM beta-mercaptoethanol, 15 mM Tris–HCl, pH 8). After centrifugation in the same conditions as above, it was resuspended in 100 μl of hypotonic buffer containing 0.1% Triton X-100 and centrifuged at 13,000 × g for 5 min. The resulting supernatant corresponded to the cytoplasmic extract. The entire procedure was carried out at 4 °C. Five microliters of the extract were used to determine protein concentration by the Bradford assay (Bio-Rad). The extracts were frozen in liquid N_2 and stored at −80 °C. One hundred micrograms of both cytoplasmic extracts were resolved on SDS 12%-polyacrylamide gels [30] and transferred to PVDF membranes (Immobilon P, Millipore) using a semidry electroblotter. The membranes were probed with an antibody against AKR1C1 (Santa Cruz Biotechnology). Signals were detected by secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence, as recommended by the manufacturer (Amersham). To normalize the results, blots were reprobed with an antibody against PPARγ.

Total extracts from HT29 cells, either sensitive or MTX-resistant, were used to assay Sp1 protein levels. Cells were collected in ice-cold F-12 medium and centrifuged at 800 × g for 5 min. The cell pellet was gently resuspended in 3 ml of HB and centrifugated in the same conditions than above. Afterwards, cells were resuspended in 100 μl of deoxycholate buffer (100 mM NaCl, 10 mM NaH_2PO_4 pH 7.4, 1 M PMSF, 1% triton, 0.1% SDS, 0.5% deoxicholate) and centrifugated at 13,000 × g for 10 min. The resulting supernatant corresponded to the total extract. The entire procedure was carried out at 4 °C. Protein concentration was determined by the Bradford assay and 40 μg of both sensitive and resistant HT29 cells total extracts were resolved on SDS 7%-polyacrilamide gels. Transference to PVDF membranes was followed with incubation with an antibody against Sp1 (PEP2, Santa Cruz Biotechnology) and detection was accomplished by secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence.

2.6. Determination of AKR1C1 and DHFR copy number

HT29 or HT29-R cells (3×10^6) were washed twice in NaCl/P_i and scraped in 2 ml lysis buffer (400 mM NaCl, 1 mM EDTA, 0.1% SDS, and 10 mM Tris/HCl, pH 7.4). Four hundred micrograms proteinase K was added to the lysate, which was then incubated for 2 h at 37 °C. Two purification steps and nucleic acid precipitation were followed by a 2 h-incubation at 37 °C in 40 μg/ml RNAse DNAse free, and the same three steps were repeated after this incubation to obtain the genomic DNA. Five hundred nanograms of this DNA were used for PCR

amplification in a 50 μl reaction containing 1.2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 μCi of [α-^{32}P]dATP (3000 Ci/mmol, Amersham Ibérica), 1.5 units of Taq polymerase (Ecogen), 500 ng of each primer and 20 mM Tris–HCl, pH 8.5. The specific primers used for amplification were:

AKR1C1: 5′-GGTCACTTCATGCCTGTCCTG-3′ and 5′-CTGTGACAAGATGGCATTGCAG-3′ Annealing in exon 1 and intron 2, respectively (PCR product of 194bp).
APRT:5′-CGGGAACCCTCGTCTTTCGCC-3′ and 5′-GCCTCGGGGGCTCAATCTCAC-3′ Annealing in intron 1 and intron 2, respectively (PCR product of 357 bp).

PCR was performed in an MJ Research Thermocycler equipped with peltier system and temperature probe and following the program described: after an initial denaturation for 3 min at 94 °C, PCR was performed for 22 cycles. Each cycle consisted of denaturation at 92 °C for 30 s, primer annealing at 59 °C for 1 min, and primer extension at 72 °C for 1 min. A final 7-min extension step at 72 °C was performed. Five microliters of each PCR sample was electrophoresed and scanned with Phosphorimaging.

DHFR copy number was assayed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with 100 ng of genomic DNA and the assays-on-demand from Applied Biosystems: HS00758822-s1 for DHFR and HS00356991-m1 for APRT. The reaction was performed following the manufacturers recommendations.

2.7. Luciferase constructs

According to the NCBI mRNA reports, there could be complete AKR1C1 mRNA species initiating at −184 nt (NM_001353) and −17 nt (BC_00216) relative to the translational start. Taking into account this information, we proceeded to clone the AKR1C1 promoter from HT29 resistant cells. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit v 2.0 (Applied Biosystems). No difference was observed when comparing this sequence with that reported in Genbank (Accession number AB032150). The Match™ software with TRANSFAC 6.0 database was used to determine the putative transcription factor binding sites present in AKR1C1 promoter. There is a putative Sp1 binding site at −74 from the translational start codon (ATG). Deletion constructs of the AKR1C1 promoter region were engineered by unidirectional cloning of PCR fragments from the AKR1C1 promoter between the NheI and XhoI sites of the reporter luciferase vector pGL3-basic (Promega). These PCR fragments were generated using a common reverse primer and three different forward primers. The forward primers were preceded by an arbitrary sequence (in lowercase, see below) including a NheI restriction site (underlined below) and the reverse primer followed a similar structure but contained a *Xho*I restriction site (underlined) in the arbitrary sequence.

Akr-pr241: 5′-tcaagtcaggctagcCTTCAGAGGAAATTAATGAGTG-3′
Akr-pr84: 5′-tcaagtcaggctagcGTGTGCTCAGGGGCGTTGCCAG-3′
Akr-prDeletionSp1: 5′-tcaagtcaggctagcGCTCATTGCTCTTATAGCCTGTG-3′
Akr-prRev: 5′-cagtgctgcctcgagGTCACTAGCCTGGCTGGCAAATG-3′

The PCR fragments generated were of 241 bp when using Akr-pr241, of 84 bp when Akr-pr84 was used and of 63 bp for the Akr-prDeletionSp1 primer. The 2 first constructs contained a putative Sp1 binding site while the third construct did not.

2.8. Transfections, co-transfections and luciferase assays

HT29 cells, either sensitive or MTX-resistant, were seeded into 6-well plates the day before transfection at a density of 2×10^5 cells/well in Ham's F12 medium containing 7% fetal bovine serum. Transfection was performed using FUGENE™ 6

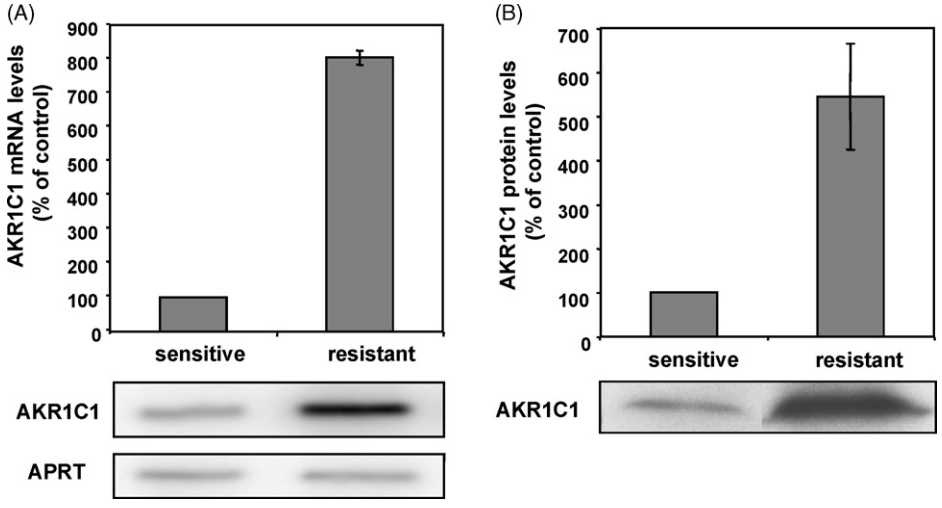

Fig. 1 – Validation of AKR1C1 upregulation. The overexpression of AKR1C1 was confirmed at the level of mRNA and protein in HT29 cells resistant to 10^{-5} M MTX. (A) AKR1C1 mRNA levels: 1 μg of total RNA was used as starting material for the reverse transcriptase reaction. Quantitative RT-PCR was carried out as described in Section 2. Phosphorimaging analysis was used to quantify the intensity of the radioactive bands. Results are shown as mean ± S.E.M. for triplicate samples. (B) AKR1C1 protein levels: Western blot assays of AKR1C1 were performed using 100 μg of cytoplasmatic extracts from either sensitive or MTX-resistant HT29 cells and AKR1C1 antibody (Santa Cruz). Results are shown as mean ± S.E.M. for triplicate samples.

(Roche). For each well, 6 μl of FUGENE™ 6 in 100 μl of serum-free medium was incubated at room temperature for 5 min. The mixture was added to increasing amounts of each promoter construct and incubated at room temperature for 20 min before addition to the cells. In co-transfections, 500 ng of each promoter construct was mixed together with 2 μg of Sp1 or Sp3 before the addition of FUGENE™ 6 in serum-free medium. Luciferase activity was assayed 30 h after transfection.

In all cases, cell extracts were prepared by lysing the cells with 200 μl of freshly diluted 1x Reporter Lysis Buffer (Promega). The lysate was centrifugated at 13,000 × *g* for 2 min to pellet the cell debris and the supernatants were transferred to a fresh tube. A 10-μl aliquot of the extract was added to 25 μl of the luciferase assay substrate (Promega) and the luminiscence of the samples was read immediately on a Gloomax 20/20 luminometer (Promega), in which the light production (relative light units, RLU) was measured for 10 s. Each transfection was performed in triplicate. Protein concentration was determined by the Bradford assay and used to normalize the results.

2.9. Gel-shift assays

Nuclear extracts were prepared from exponentially growing sensitive or resistant HT29 cells as described [31]. The probes were constructed by PCR amplification of different regions of AKR1C1 promoter sequence, bearing or not the putative Sp1 binding site (underlined), using different forward primers and a common reverse primer. A shorter ds probe of 22 bp was generated by annealing complementary single-stranded oligodeoxynucleotides corresponding to the putative Sp1 binding site present in the AKR1C1 promoter, using the AKR-pr84 and its complementary sequence.

AKR-pr84: 5′-GTGTGCTCAGGGGCGTTGCCAG-3′
AKR-prDeletionSp1: 5′-GCTCATTGCTCTTATAGCCTGTG-3′
AKR-prRev: 5′-GTCACTAGCCTGGCTGGCAAATG-3′

All the probes were gel-purified, end-labeled with T4 polynucleotide Kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), and used in the gel shift assays. DNA binding assays were performed as described [32] and were visualized using a phosphorimaging.

In the supershift experiments, 1 μg of rabbit polyclonal antibody PEP-2 or 1 μg of rabbit polyclonal antibody D-20 (against Sp1 and Sp3, respectively) (both from Santa Cruz) were added to the reaction mixture 15 min after the addition of the probe and incubated for an additional period of 15 min.

For phosphatase treatments, nuclear extracts were incubated with different amounts of Alkaline Phosphatase (0.2–1 U) (Sigma) for 30 min on ice. The reaction was stopped by the addition of 100 mM NaF, 10 mM sodium pyrophosphate and 2 mM Na_3VO_4 before proceeding to the binding reaction thereby preventing the dephosphorylation of the probe. Control extracts were also incubated with the phosphatase inhibitors before proceeding to the gel retardation assays.

2.10. Functional validations

2.10.1. Transfection of siRNAs against AKR1C1

HT29 cells (30,000) were plated in 1 ml of –GHT medium. Transfection was performed 18 h later with a specific siRNA designed against AKR1C1 RNA (siAKR). For each well, 4 μl of Lipofectamine (Invitrogen) in 100 μl of serum free –GHT medium were mixed in Eppendorf tubes with 100 nM siAKR in 100 μl of serum free –GHT medium, and this mixture was incubated at room temperature for 20 min before addition to the cells. MTX (5×10^{-8} M) was added 48 h after siRNA treatment and cells were incubated for seven days before the MTT assays were performed [33]. When screening for AKR1C1 mRNA levels after siRNA treatment, 10,000 cells were incubated with increasing amounts of either siAKR (3–100 nM) or siAKR-4MIS (3–30 nM) maintaining a 3:1 (μl of Lipofectamine: μg siRNA) ratio and following the procedure previously described. Cells were collected 48 h after siRNA treatment for quantitative RT-PCR. The specificity of siAKR was tested at different levels: (i) computationally since BLAST analysis of this sequence reported AKR1C1 as the only mRNA hit; (ii) siAKR treatment did not cause significant effects on mRNA levels for other cellular genes such as Sp3, TBK1 (tank binding kinase) or cyclophilin (data not shown); and iii) siAKR-4MIS bearing 4 mismatches with respect to siAKR (underlined) did not produce a decrease in AKR1C1 mRNA. The sequences for both siRNAs were:

siAKR: 5′-AUCACCAAGCAGGAGAGAUTT-3′
siAKR-4MIS: 5′-AUGUGGAAGCAGGAGAGAUTT-3′

2.10.2. Transfection of an expression plasmid coding forAKR1C1

HT29 cells were seeded into 6-well plates at a density of 2×10^4 cells/well in 2 ml of HAM F12 selective medium. 18 h later, transfections with an expression plasmid for AKR1C1 (pCMV6-XL5-AKR1C1, kindly provided by Dr. Simpkins, Temple University, Philadelphia) in the presence or absence of MTX were performed. The overexpression of AKR1C1 was monitored by determining its mRNA levels after 24 and 48 h upon transfection of this plasmid. For each well, 3 μl of FUGENE™ 6 (Roche) in 100 μl of serum free –GHT medium was incubated at room temperature for 5 min. Then, this mixture was added to 10 μg of the DNA and, after 20 min at room temperature, added to the cells. When combining pCMV6-XL5-AKR1C1 transfection and MTX treatment, 3×10^{-8} M MTX was added 24 h after transfection. The effect of FUGENE™ 6 in MTX effectiveness was assessed with the corresponding controls. Seven days later, the viability was measured by the MTT assay.

2.11. Cell cycle analysis

HT29 sensitive cells (10^5) were seeded into 6-well plates in 1 ml of –GHT selective medium. Eighteen hours later, transfections with 10 μg of pCMV6-XL5-AKR1C1 were performed as previously described. MTX (3×10^{-8} M) was added 1 h after transfection. Forty-eight hours later, nuclei were stained with 50 μg/ml propidium iodide (Sigma) and cell cycle distribution was monitored on a Beckman Coulter Epics XL flow cytometer.

2.12. Apoptosis

Seeding and treatment of HT29 sensitive cells with pCMV6-XL5-AKR1C1 was performed as in cell cycle assays. AKR1C1 plasmid was allowed to express for 24 h and MTX was added

during the last 6 h of treatment. Caspase 3/7 activation was assayed with Caspase-Glo 3/7 Assay Kit (Promega) in a Goomax 20/20 luminometer (Promega), expressed as percentage of relative light units (RLU) referred to the control and normalized by protein content determined by the Bradford assay.

3. Results

3.1. Members of an AKR subfamily are overexpressed in HT29 MTX-resistant cells

The expression profile of the 22.300 transcripts and variants included in the HG U133A 2.0 from Affymetrix was compared between sensitive HT29 cells and resistant to 10^{-5} M MTX. Among the differentially expressed genes that passed the filters described in Section 2 using the specific software GeneSpring GX v7.3.1, we directed special attention on some members of the AKR1 family among the list of genes overexpressed more than 5-fold (Table 1). We continued the studies with AKR1C1 and, as shown in Fig. 1, we validated its overexpression in MTX resistant cells at the mRNA and protein level by quantitative RT-PCR and Western Blot analysis, respectively.

3.2. AKR1C1 and DHFR copy-number in HT29 sensitive and MTX-resistant cells

To test whether AKR1C1 overexpression in HT29 MTX-resistant cells was due to gene amplification, we used quantitative PCR to determine the copy number for AKR1C1. No variation was observed in the genomic content for this gene between HT29 sensitive and resistant cells, as shown in Fig. 2A. DHFR copy number was assayed as a control of gene amplification in an AbI Prism 7000 Sequence Detection System, and a 11-fold increase in DHFR copy number was observed in HT29 resistant cells (Fig. 2B). DHFR amplification we demonstrate is in accordance with bibliography describing this phenomenon as a mechanism for MTX-resistance [34–37].

3.3. Transcriptional regulation of AKR1C1 promoter

Since gene amplification was not the process responsible for AKR1C1 overexpression in the resistant cells, we proceeded to test whether this effect was due to gene promoter transcriptional regulation. We cloned different deletion constructs of the human AKR1C1 5′-flank into the pGL3 luciferase reporter, and these constructs were transiently

Table 1 – List of genes overexpressed more than 5-fold in HT29 cells resistant to 10^{-5} M MTX

Genbank	Symbol	Gene name	Ratio	Raw expression	*p*-Value	Gene function
NM_002414	CD99	CD99 antigen	37.3	26 to 1048	1.4E−04	Cell adhesion
M33376	AKR1C2	Aldo-keto reductase 1C2	21.4	154 to 3274	1.6E−04	Bile acid transporter
S68290	AKR1C1	Aldo-keto reductase 1C1	19.3	206 to 3972	2.4E−04	Xenobiotic metabolism
NM_000767	CYP2B6	Cytochrome P450, 2B6	16.8	21 to 359	3.4E−04	Monooxygenase
AL533838	MGC8685	Tubulin	14.6	17 to 261	1.1E−03	Structural molecule
NM_020299	AKR1B10	Aldo-keto reductase 1B 10	13.5	62 to 844	1.5E−04	Aldehyde reductase
AB028021	FOXA2	Forkhead box A2	11.2	27 to 322	1.2E−03	Transcription factor
M28882	MCAM	Melanoma cell adhesion	10.5	22 to 246	8.8E−05	Cell adhesion
NM_000791	DHFR	Dihydrofolate reductase	10.1	18 to 199	8.4E−04	Dihydrofolate reductase
NM_000029	AGT	Angiotensinogen 8	8.7	51 to 480	7.5E−04	Endopeptidase inhibitor
NM_012446	SSBP2	ssDNA binding protein 2	8.4	19 to 174	1.7E−03	ssDNA binding
NM_001216	CA9	Carbonic anhydrase IX	8.0	60 to 517	1.5E−04	Carbonate dehydratase
AF054841	TM4SF7	Transmembrane 4, 7	7.9	72 to 615	2.9E−03	–
BF726531	AQP5	Aquaporin 5	7.7	144 to 1166	2.9E−03	Transporter
M25915	CLU	Clusterin	7.5	76 to 619	2.2E−04	–
NM_000712	BLVRA	Biliverdin reductase A	7.4	37 to 297	4.5E−05	Biliverdin reductase
NM_001958	EEF1A2	e. elongation factor 1A2	6.5	281 to 1951	1.2E−03	Translation elongation
BC006471	AF1Q	ALL1-fused from chr1q	6.4	20 to 141	3.0E−04	–
NM_000047	ARSE	Arylsulfatase E	6.4	54 to 374	2.9E−03	Arylsulfatase
U73936	JAG1	Jagged 1	6.3	79 to 527	3.8E−03	–
AI471375	PRKCA	Protein kinase C, alpha	6.1	19 to 129	9.7E−04	Protein kinase C
AF018081	COL18A1	Collagen, type XVIII, A1	6.1	32 to 208	1.1E−03	Cell adhesion
AW192795	MUC5AC	Mucin 5, A and C	5.9	83 to 535	2.2E−03	–
NM_015974	CRYL1	Crystallin, lambda 1	5.6	48 to 292	5.1E−04	Oxidoreductase
NM_000610	CD44	CD44 antigen	5.6	124 to 758	3.2E−04	Hyaluronic acid binding
NM_001975	ENO2	Enolase 2 gamma	5.5	38 to 227	1.8E−03	PEP hydratase
BC005902	BLVRA	Biliverdin reductase A	5.3	53 to 307	3.7E−04	Biliverdin reductase
AB018580	AKR1C3	Aldo-keto reductase 1C3	5.2	411 to 2.150	1.5E−04	Prostaglandin metabolism
NM_005165	ALDOC	Aldolase C	5.0	73 to 396	3.9E−03	Fructose-P2 aldolase

It is shown the GenBank accession number of genes in the AKR superfamily that were overexpressed in HT29 cells resistant to 10^{-5} M MTX. The ratio column corresponds to the fold change in expression of each gene relative to the control. The change in the actual numerical values of the expression between sensitive (left) and resistant (right) cells is given in the row expression column. The last column indicates the functional categories to which the genes belong. Results are the mean of three independent experiments performed for each condition. *t*-test *p*-value was less than 0.001 for all the samples.

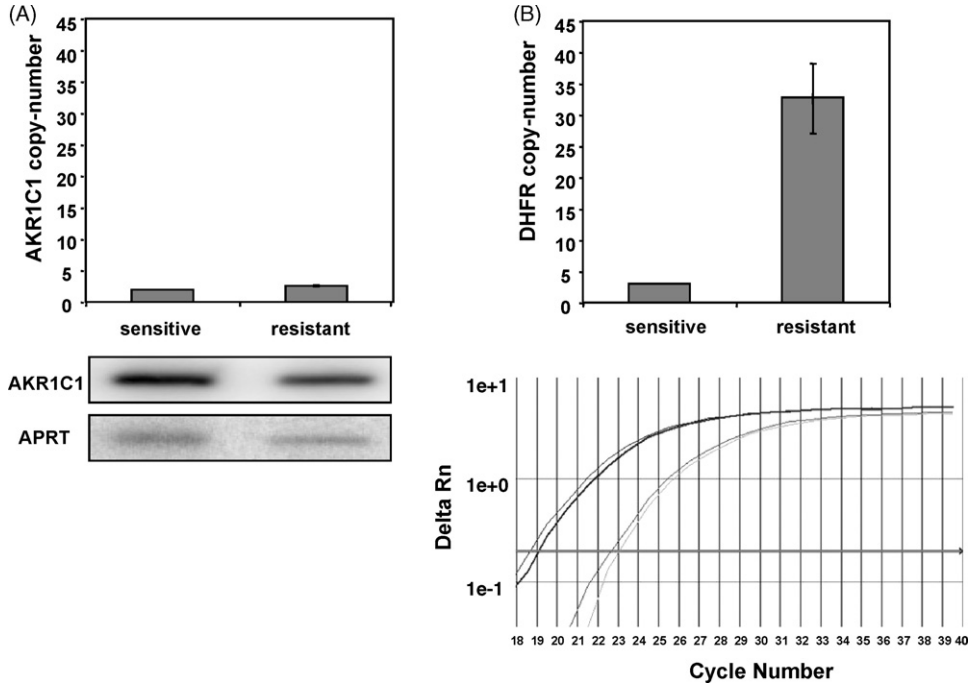

Fig. 2 – AKR1C1 and DHFR copy-number in HT29 cells. Genomic DNA prepared from HT29 cells, either from sensitive or MTX-resistant, was used to determine the AKR1C1 and DHFR copy number in both cell types. (A) AKR1C1 copy number was performed by quantitative PCR as described in Section 2. The intensity of the radioactive bands was quantified by using phosphorimaging and the APRT signal was used to normalize the results (mean ± S.E.M. for triplicate samples). (B) DHFR copy number was determined by Real-time PCR using 100 ng of each DNA for the reaction, which was carried out as described in Section 2. Results are shown as mean ± S.E.M. for triplicate samples. The two amplification curves to the left of the graph correspond to the resistant cells, and the two to the right correspond to sensitive cells.

transfected into HT29 cells, either sensitive or MTX-resistant. When a construct containing 241 bp of the AKR promoter was used (AKR-pr241), a 1400-fold increase with respect to the empty vector was observed in the resistant

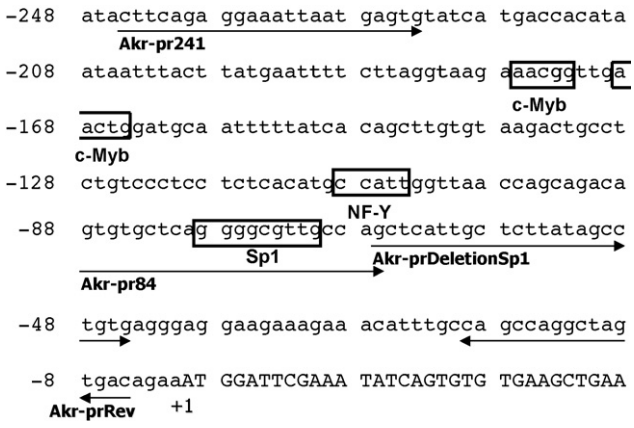

Fig. 3 – Human AKR1C1 promoter sequence. AKR1C1 5′-flank was obtained from Genbank (accession number AB032150). Putative transcription factors binding sites present in the AKR1C1 promoter were searched using the Match™ software and the TRANSFAC 6.0 database. There is a putative Sp1 binding site at −74 from the translational start codon (ATG) (+1). Primers used for the generation of luciferase constructs and gel shift probes, including and excluding the GC-box, are designed as arrows.

cells (Fig. 4A). A 55-fold increase resulted from the transfection of a construct bearing only 84 bp of the promoter (AKR-pr84) in HT29 resistant cells (Fig. 4B). On the other hand, only a 5-fold increase was produced (Fig. 4C) when the transfected construct (AKR-prDeletionSp1) did not include a putative Sp1 binding site, which was present in the two previous constructs. However, when any of the 3-abovementioned constructs was transfected in HT29 sensitive cells, the promoter activity was not significantly different from the luciferase activity obtained when an empty vector was transfected (Fig. 4, A–C). Next, co-transfection studies of AKR-pr84 or AKR-prDeletionSp1 together with an expression vector for Sp1 were performed. As shown in Fig. 5A, co-transfection of AKR-pr84 with Sp1 in HT29 resistant cells led to an increase in promoter activity of 4-fold relative to the AKR-pr84 construct alone. However, co-transfection of AKR-prDeletionSp1 together with Sp1 showed no variation in promoter activity. If these co-transfection experiments were performed in HT29 sensitive cells, no significant increases were observed in any of the conditions. When the co-transfections were performed with an expression vector for Sp3, an increase in AKR1C1 transcription was observed although to about half of the activity detected upon co-transfection with Sp1 (Fig. 5B). These results demonstrated that AKR1C1 promoter was mainly regulated in the resistant cells by Sp1 binding to its GC-box located at −74 from the translational start codon (Fig. 3) and also by Sp3 but to a lesser extend.

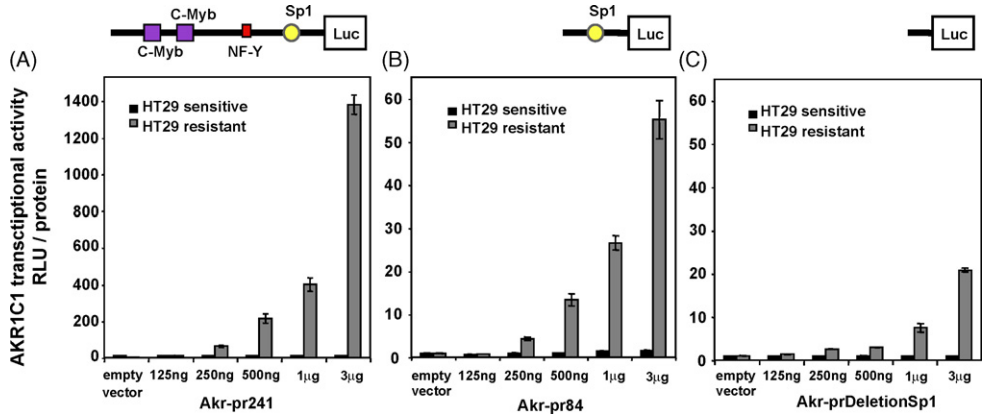

Fig. 4 – Transcriptional regulation of AKR1C1 promoter. (A, B and C) Transfections of deletion constructs of the AKR1C1 promoter into HT29 cells. Sensitive or resistant to MTX cells were transiently transfected using the amounts of plasmid DNA shown in the figure. Thirty hours after transfection, luciferase (Luc) activity was assayed. The protein content was used to normalize the luc activity (relative light units, RLU) for each sample and is expressed relative to that of pGL3 basic vector (mean ± S.E.M. for triplicate wells). A schematic representation of each deletion construct used in the luciferase assays is shown on top of the figure.

3.4. *Binding analysis of AKR1C1 promoter*

By computational analysis, a putative Sp1 binding site was found in the AKR1C1 promoter sequence (Fig. 3). A probe of 84 bp (AKR-pr84) containing nucleotides −88 to −5 relative to ATG that included this GC box was used in gel shift assays to test for Sp1 binding. As shown in Fig. 6A, 2 bands were obtained by using nuclear extracts from either HT29 sensitive or resistant cells, and the binding of the upper band was almost four times stronger in resistant cells compared to sensitive HT29 cells. This binding was not obtained when using a probe that expanded from nucleotide −63 to −5 from the ATG, which excluded the putative Sp1 binding site (AKR-prDeletionSp1) (Fig. 6B). The binding pattern obtained in the gel-shift analysis was characterized by using antibodies against Sp1 (PEP-2) and Sp3 (D-20). When the probe AKR-pr84 was used, the upper band was supershifted with the Sp1 antibody, thus corresponding to binding by Sp1 (Fig. 6C). The lower band corresponded to binding by Sp3, as it disappeared completely when the Sp3 antibody was used. A shorter probe of 24 bp containing the Sp1 binding site in the AKR1C1 promoter was also used. By using this probe, we confirmed the higher Sp1 and Sp3 binding with the resistant cells nuclear extract, as well as the characterization of the two obtained bands (Fig. 6D). The consensus sequence for Sp1 binding site was additionally used in the gel-shift analysis. By using this probe and nuclear extracts from HT29 resistant cells, three bands were observed. The upper band corresponded to binding by Sp1, as it was competed by Sp1 antibody, and the two lower bands corresponded to binding by Sp3, as they both disappeared when an Sp3 antibody was used (Fig. 6E).

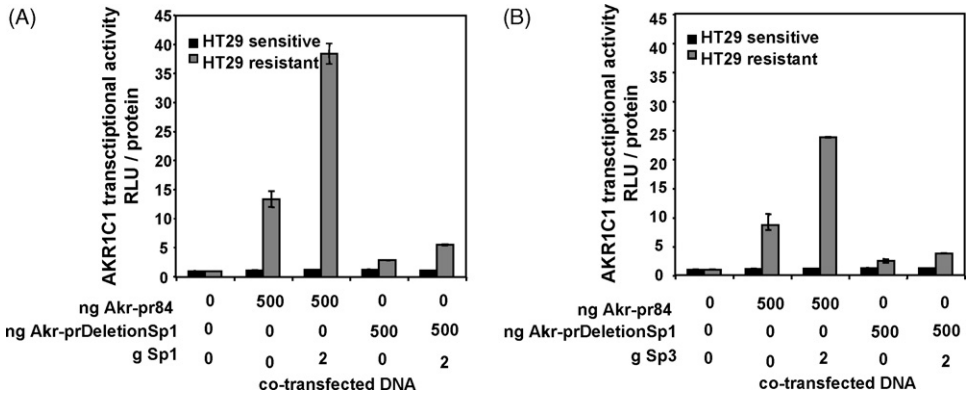

Fig. 5 – AKR1C1 transcriptional activity upon co-transfection with Sp1 or Sp3. (A) Co-transfections of Sp1 plus Akr-pr84 or Akr-prDeletionSp1 in the sensitive and resistant HT29 cells. Five hundred nanograms of each construct was transfected alone or in combination with an expression plasmid for Sp1. Luc activity was also assayed 30 h after transfection and the results were treated as in Fig. 4. (B) Co-transfections of Sp3 plus Akr-pr84 or Akr-prDeletionSp1 in the sensitive and resistant HT29 cells. Five hundred nanograms of each construct were transfected alone or in combination with an expression plasmid for Sp3. Luc activity was assayed 30 h after transfection and the results were treated as above.

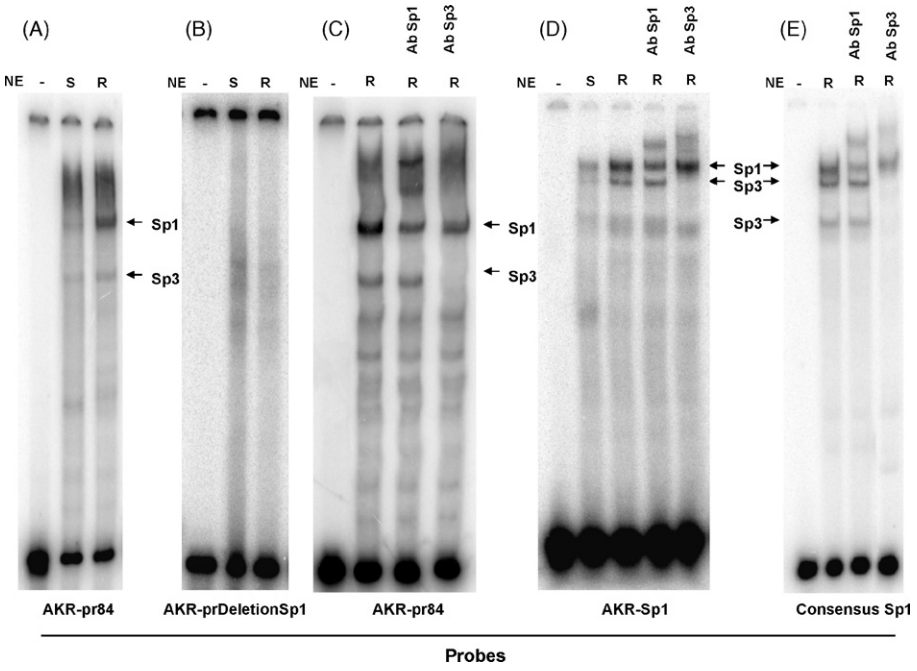

Fig. 6 – Characterization of Sp1 binding to AKR1C1 promoter. Binding reactions were performed with 20,000 cpm of each ds probe, 2 μg nuclear extracts (NE) from exponentially growing HT29 sensitive (S) or MTX-resistant (R) cells and 1 μg poly [d(I-C)] as the nonspecific competitor. Supershift mobility assays were performed in the presence of specific antibodies against either Sp1 or Sp3 (Ab Sp1 or Ab Sp3, respectively). Shifted and supershifted bands are indicated by arrows. The characteristics of each probe used are described in Section 2.

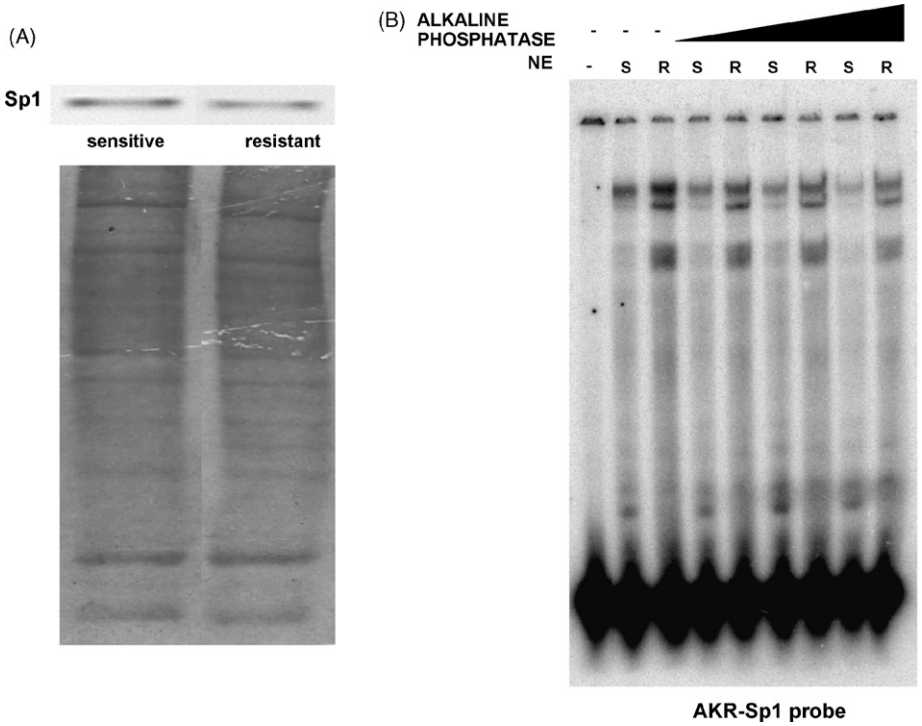

Fig. 7 – Sp1 protein levels and effect of phosphatase treatment. (A) Sp1 protein levels determined by Western analysis. Forty micrograms of total protein extracts from HT29 cells (either sensitive or resistant to MTX) were processed using the PEP2 Ab against Sp1. The stained gel after the transfer is shown as loading control. (B) Effect of phosphatase treatment on Sp1 binding to the AKR1C1 promoter. Two micrograms of nuclear extract (NE) from either sensitive (S) or MTX-resistant (R) HT29 cells were incubated with increasing amounts of alkaline phosphatase for 30 min on ice. The reaction was stopped by the addition of 100 mM NaF, 10 mM sodium pyrophosphate and 2 mM Na_3VO_4. Immediately afterwards, the binding reaction was carried out (lanes 4 to 9) using the AKR-Sp1 probe. Control extracts were also incubated with the phosphatase inhibitors before proceeding to the gel retardation assays (lanes 2 and 3).

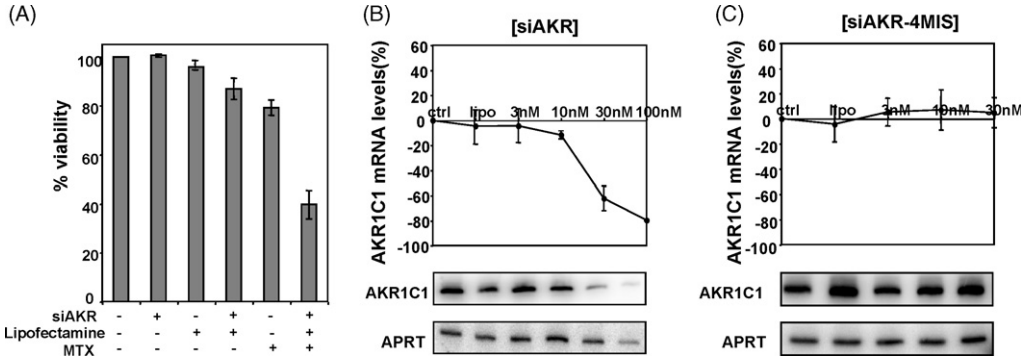

Fig. 8 – Effect of siRNA against AKR1C1 RNA on MTX sensitivity in HT29 cells. (A) Effect of siRNA against AKR1C1 (siAKR) on MTX sensitivity. Incubation with 100 nM siAKR was performed alone or in combination with 5×10^{-8} M MTX. The drug was added 48 h after siRNA treatment, and cell viability was determined 7 days after by the MTT assay. Results are expressed as percentage viability referred to the untreated cells. (B and C) Effects of siAKR or siAKR-4 MIS (a siRNA with 4 mismatches with respect to siAKR) on AKR1C1 mRNA. After 48 h of siRNA treatment, RNA levels were determined by quantitative RT-PCR. The intensity of the radioactive bands was quantified by phosphorimaging and the results (mean ± S.E.M. for triplicate samples) were normalized using the signal corresponding to the APRT mRNA. The values are expressed as percentage of AKR1C1 mRNA levels referred to the control (untreated cells) (lipo is the abbreviation of Lipofectamine).

As the levels of Sp1 protein were unchanged between resistant and sensitive cells (Fig. 7A), we explored whether or not the higher Sp1 binding in HT29 MTX-resistant cells was due to enhanced Sp1 phosphorylation. Incubation of nuclear extracts with alkaline phosphatase was performed for 30 min and stopped before proceeding to the binding reaction to avoid dephosphorylation of the probe. As can be observed in Fig. 7B, dephosphorylation of the extracts led to a decrease in the binding of Sp1 and Sp3. Thus, the stronger binding present in the resistant cells might be due to an increase in the phosphorylation state of these transcription factors.

3.5. *Effect of siRNAs against AKR1C1 on methotrexate sensitivity*

Given that AKR1C1 was overexpressed in cells resistant to MTX, we investigated the role of decreasing its mRNA on the sensitivity to this chemotherapeutic agent. Treatment with 100 nM siAKR increased the sensitivity of HT29 cells towards MTX by about 50% (Fig. 8A). This treatment reduced 80% the levels of AKR1C1 RNA after 48 h in the resistant cells, containing increased levels of AKR1C1 mRNA, while a 4-mismatch siRNA against AKR1C1 did not produce any significant reduction in its mRNA levels (Fig. 8B and C, respectively).

3.6. *Effect of overexpression of AKR1C1 on Methotrexate sensitivity*

Transient transfection with an expression vector for AKR1C1 (pCMV5-XL6-AKR1C1) in sensitive HT29 cells was performed in the presence or in the absence of 3×10^{-8} M MTX. The transfection of the expression vector for AKR1C1 reverted by 50% the effect of methotrexate (Fig. 9), thus providing evidence that AKR1C1 can confer increased resistance of HT29 cells toward MTX.

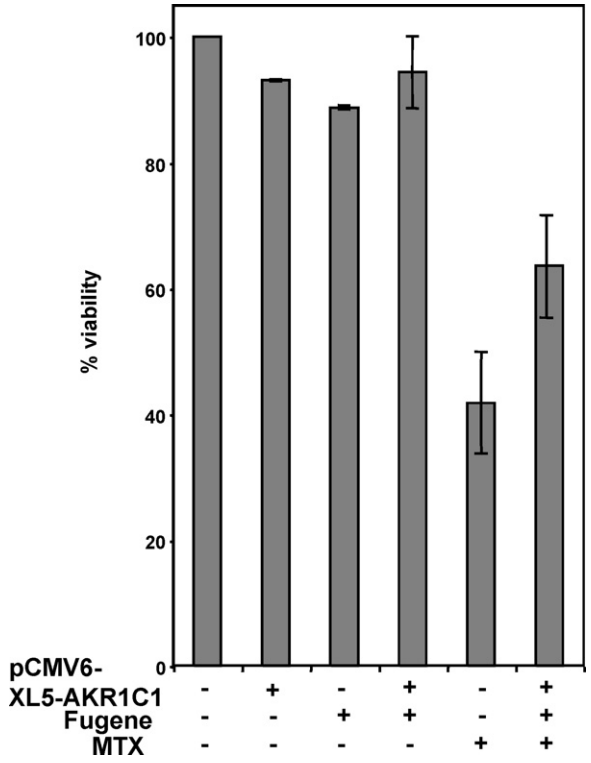

Fig. 9 – Effect of AKR1C1 overexpression on MTX sensitvity in HT29 cells. AKR1C1 was overexpressed by means of an expression plasmid (pCMV6-XL5-AKR1C1). Ten micrograms of this plasmid were transfected using FUGENE™ 6 into HT29 sensitive cells, either alone or in combination with 3×10^{-8} M MTX, added 24 h after plasmid treatment. MTT assay was performed after 7 days of AKR1C1 expression and percentage viability was referred to the untreated cells (mean ± S.E.M. for triplicate samples).

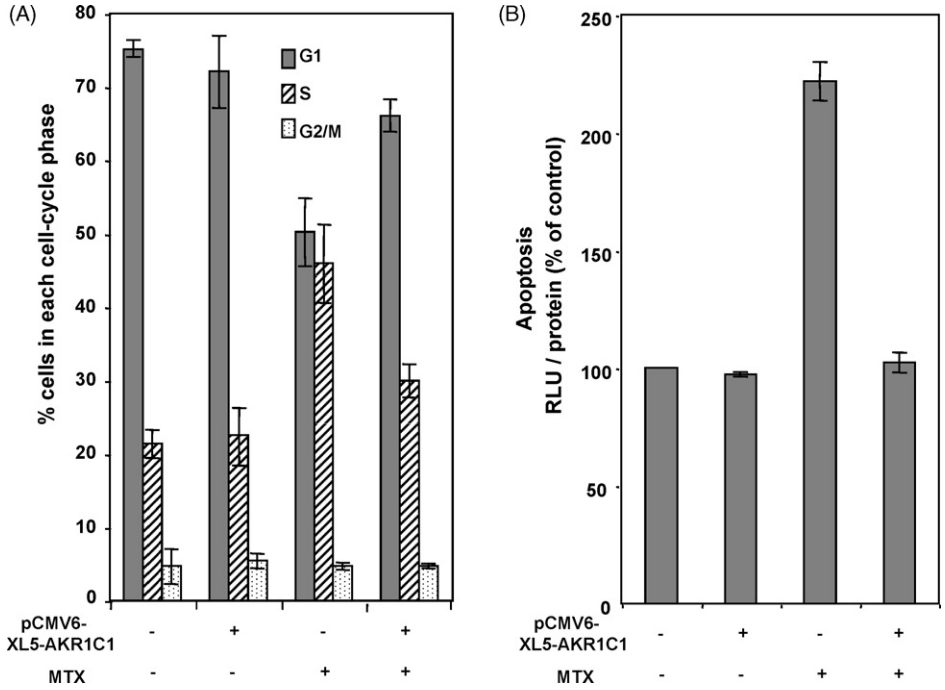

Fig. 10 – Changes in the cell cycle and in the apoptosis caused by overexpression of AKR1C1. (A) Changes in cell cycle distribution. Ten micrograms of pCMV6-XL5-AKR1C1 were transfected into HT29 sensitive cells. MTX (3×10^{-8} M) was added 1 h after plasmid treatment. After 48 h of drug incubation, cells were collected, stained with propidium iodide and analyzed for DNA content using flow cytometry. Results are shown as mean ± S.E.M. from at least three independent experiments. (B) Changes in apoptosis. Overexpression of AKR1C1 was performed by transfecting 10 μg of expression plasmid, either alone or in combination with 3×10^{-8} M MTX, for 24 h in HT29 sensitive cells. MTX was added during the last 6 h of treatment. Caspase 3/7 activation was finally measured and expressed as percentage of relative light units (RLU) referred to the control and normalized by protein content (mean ± S.E.M. from at least three independent experiments).

3.7. *AKR1C1 counteracts MTX-induced S-phase arrest and apoptosis*

Cell cycle distribution after treatment with MTX and/or pCMV6-XL5-AKR1C1 was monitored after a 48-h treatment. Incubation with MTX alone induced the S-phase arrest, as previously described by others [38], while cells in G1 phase were significantly decreased. However, if AKR1C1 expression plasmid was transfected in combination with MTX, the percentage of cells in S-phase was significantly lower and those in G1 increased with respect to MTX alone (Fig. 10A). These results suggest that AKR1C1 may serve to facilitate the progression throughout the cell cycle and thus stimulate cell proliferation, counteracting the effects caused by MTX. This facilitation for cell progression was also explored by testing the percentage of apoptotic cells. In this way, MTX caused an increase in apoptosis, as measured by caspases 3/7 activation, while overexpression of AKR1C1 reversed the apoptosis caused by the chemoterapeutic agent (Fig. 10B).

4. Discussion

The aim of this work was to study the regulation of AKR overexpression that we found in HT29 cells resistant to methotrexate using functional genomics. We focused on the analysis of AKR1C1 since it was one of the two most differentially expressed genes within the AKR family and had higher raw values of expression than AKR1C2. Moreover, the gene function attributed to AKR1C1 (xenobiotics metabolism) was more in accordance with the resistance process than the functional category in which AKR1C2 was included (bile acid transport). It is of note that the increase in RNA and AKR1C1 protein were at the same level, about 7-fold, which suggests that the overexpression is caused mainly by an increase in mRNA abundance rather than by a translational control. The increase in mRNA was not due to an amplification of the *akr1c1 locus*. This is at variance with respect to the *dhfr* gene, which had undergone an amplification process of about 10-fold compared to the sensitive cells.

Given this result it became apparent that a transcriptional regulation has taken place. In this regard, from the luciferase experiments two facts can be concluded: (i) there is a vast increase in transcriptional activity originating from the AKR1C1 promoter in the resistant cells compared with sensitive cells; and (ii) AKR1C1 transcriptional activation in the resistant but not in the sensitive cells depends at least in part on Sp1 and also on Sp3. The gel-shift experiments also corroborate the role of Sp1and Sp3 in regulating AKR1C1 in the resistant cells. As there was no difference in the levels of Sp1 protein between resistant and sensitive cells, we

explored the phosphorylation status of this transcription factor in the two types of cells, considering that it has been reported that phosphorylated Sp1 shows a higher binding than the non-phosphorylated form [32]. Indeed, the increase in Sp1 binding in the resistant condition could be due to a higher phosphorylation state of Sp1 as dephosphorylation of the extracts coursed with a decrease in its binding. In this direction, we searched for the differential expression, in the microarray data, of those kinases and phosphatases known to control the phosphorylation state of Sp1 [39]. Within the kinases, PKC alpha was overexpressed. It is interesting to note that kinase has already been related to methotrexate resistance [40] through a mechanism involving transcription factor Sp1 [32].

Trying to mimic the overexpression of AKR1C1 present in the resistant cells, we transiently transfected an expression vector for this gene into sensitive cells, which resulted into a decrease in sensitivity toward methotrexate. This result supported the idea that the overexpression of AKR1C1 may contribute, at least partially, to the resistant phenotype. On the other hand, decreasing AKR1C1 mRNA levels by means of iRNA technology sensitizes the cells toward MTX.

Given that a relationship between AKR and proliferation had been reported [41,42], we studied and demonstrated that AKR1C1 transfection counteracts the cell cycle S-phase arrest caused by MTX, suggesting that AKRC1C activity may be needed for the cells to progress throughout the cell cycle. This could be a strategy, concomitant with the amplification of the *dhfr locus*, for the resistant cells to bypass the metabolic pressure exerted by MTX on nucleotide synthesis. Since AKR1C1 is functionally involved in the metabolism of xenobiotics, its activity might be acting either on MTX directly or on one of its metabolites to decrease the concentration of DHFR inhibitor.

In addition, AKR activity correlates with apoptosis; Chow et al. [43] showed that inhibition of AKR1C1 expression by iRNA increased the apoptosis and drug (bleomycin)-sensitivity of A431 skin carcinoma cells, indicating an important role for AKR1C1 in tumor cells self-protective mechanisms and tumor progression. Our results are in keeping with this possibility since overexpression of AKR1C1 counteracts the apoptosis induced by MTX. In this respect, the expression levels for BID and BAX found in the microarrays for the resistant cells were decreased to ratios of 0.6 and 0.7, respectively, compared to the expression in the sensitive cells. Overexpression of AKR1C1 also confers resistance to cisplatin and adriamycin in NSCLC cells, probably through activation of protein kinase C and altered control of DNA repair and apoptosis [40]. Furthermore, a related aldo-keto reductase AKR7A5 from mouse protects V79 cells against apoptosis induced by 4-hydroxynonenal [44].

In summary, AKR1C1 upregulation may represent a mechanism, parallel to DHFR amplification, which can contribute to the establishment of MTX resistance. Its overexpression partially reverses both the MTX-induced S-phase arrest of the cell cycle and the apoptosis caused by this chemoterapeutic agent. The increase in AKR1C1 mRNA is transcriptionally regulated, at least in part by Sp1 transcription factor. Silencing of AKR1C1 by iRNA technology improves the sensitivity toward MTX.

Acknowledgements

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