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UDP-glucuronosyltransferase 1A6 overexpression in breast cancer cells resistant to methotrexate

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ABSTRACT

Methotrexate is a chemotherapeutic agent used in breast cancer treatment, but the occurrence of resistance limits its therapeutic use. A microarrays analysis between sensitive and methotrexate resistant MCF7 and MDA-MB-468 breast cancer cells pointed out the UDP-glucuronosyltransferase 1A (UGT1A) family as a common deregulated node in both cell lines. This family of genes is involved in Phase II metabolism. UGT1A6 was the main isoform responsible for UGT1A family overexpression in these cells. Its overexpression was not due to gene amplification. Transfection of a vector encoding for UGT1A6 in sensitive cells counteracted the cytotoxicity caused by methotrexate. Methotrexate increased the transcriptional activity from a luciferase reporter driven by the UGT1A6 promoter and induced UGT1A6 mRNA and enzymatic activity. Promoter analysis suggested that UGT1A6 induction by methotrexate could be driven by the transcription factors ARNT (HIF-1) and AhR/ARNT. Cells incubated with anticancer drugs susceptible to glucuronidation, such as tamoxifen or irinotecan, together with methotrexate, showed a lesser degree of cytotoxicity, due to UGT1A6 induction. The pharmacological effect of this induction should be taken into account when combining methotrexate with other drugs that are glucuronidated.

1. Introduction

Breast cancer is the most common cancer in women in developed countries, and after lung cancer, the tumour that causes more deaths among females [1]. One of the possible treatments for this cancer is methotrexate (MTX), usually given in combination with cyclophosphamide and 5-flurouracil. MTX is also used in other types of cancer such as acute lymphocytic leukaemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, and head and neck cancer; as well as autoimmune diseases as psoriasis and rheumatoid arthritis [2,3]. However, a major problem of methotrexate, in common with other chemotherapeutic drugs, is the occurrence of drug resistance.

Methotrexate is a folate analogue that reversibly binds to the dihydrofolate reductase (DHFR) enzyme. Dihydrofolate reductase catalyzes the reduction of dihydrofolate to tetrahydrofolate, needed for the synthesis of thymidylate, hypoxantine and glycine [4]. MTX inhibition of DHFR results in the blockage of DNA synthesis and cell growth. Different mechanisms of MTX resistance had been described including *dhfr* locus amplification, mutation of the target, alteration in the degree of polyglutamation, MDR phenotype, and decreased entrance of the drug [3].

With the aim to get further insight into the mechanisms of MTX resistance, functional genomic analysis using microarrays were performed in breast cancer cell lines sensitive and resistant to MTX. We identified UGT1A as the common gene among the differentially expressed genes in the two breast cancer cell lines resistant to MTX.

UDP-glucuronosyltransferases (UGTs) are a family of enzymes involved in phase II metabolism. The addition of a glycosyl group from uridine diphosphoglucuronic acid (UDPGA) renders hydrophobic compounds more soluble for their elimination via bile and urine. UGTs catalyse the glucuronidation of many lipophilic endogenous substrates such as bilirubin and estrogens, and xenobiotics. Anticancer agents such as irinotecan, topotecan, doxorubicin and tamoxifen as well as carcinogens, are glucuroni-

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dated, contributing significantly to the overall availability and pharmacological effect of these xenobiotics [5].

The UGT superfamily is divided into families and subfamilies based on protein sequence identity, where UGT1 and UGT2 families share less than 50% of identity [6]. The UGT1 gene family expresses nine functional UGT1A proteins by alternative splicing of 13 different tandem exons 1 with the common exons 2–5 [7–9]. Thus, all the UGT1A isozymes have a highly conserved "common" region, which is believed to contain the cofactor-binding site, and a variable region containing unique substrate-binding sites. Significant overlap exists in substrate specificity among the UGT family members [10–12]. The UGT1A genes are divided in two clusters, the UGT1A7-10 cluster of genes showing >70% similar in their first exon sequence and <60% similar to the other UGT1A1-6 genes [8].

UGT1A6 metabolizes planar phenols and arylamines. Two types of UGT1A6 formed by alternative splicing have been identified, which differ in the beginning of the first exon [13,14]. Type 1 has a longer and complete exon 1 and 5′UTR, typical of the UGT1A genes; whereas Type 2 suffers alternative splicing in the 5′UTR, removing part of the beginning of exon 1 and resulting in a shorter isoform. UGT1A6 expression is highly modulated by hormones, drugs, and other xenobiotics that serve as ligands for multiple sensors, including the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the aryl hydrocarbon (AhR) receptor, and the transcription factor Nrf2, which responds to oxidative/electrophile stress [14,15].

In this study, the relevance of UGT1A6 overexpression in MTX resistance was analysed in breast cancer cells sensitive and resistant to this drug. Moreover, the induction of UGT1A6 by MTX and the transcription factors involved were studied.

2. Material and methods

2.1. Chemicals

SN-38 and tamoxifen were purchased from Sigma-Aldrich (Madrid, Spain) and dissolved in DMSO. Methotrexate i/v was purchased from Almirall (Barcelona, Spain).

2.2. Cell culture

MCF7 and MDA-MB-468 (abbreviated in this study as MDA-MB) breast cancer cells were grown in Ham's F-12 medium containing 7% foetal bovine serum (GIBCO Invitrogen, Barcelona, Spain), and incubated at 37 °C in a humidified 5% CO_2 atmosphere. MCF7-R and MDA-MB-R were breast cancer cells resistant to 10^{-6} M Methotrexate (MTX), generated in our laboratory upon incubation with stepwise concentrations of MTX, following the methodology previously described in Ref. [16]. These cells were 100-fold resistant compared to sensitive cells. These cell lines were grown in Ham's F-12 medium lacking the final products of DHFR activity: glycine, hypoxanthine and thymidine, (−GHT medium, [17]) containing 7% of dialyzed foetal bovine serum. Cells were detached with 0.05% trypsin (Sigma-Aldrich, Madrid, Spain).

2.3. Microarrays

Gene expression was analyzed by hybridization to the GeneChip® Human Genome U133 PLUS 2.0 from Affymetrix, containing over 54,000 transcripts and variants. Total RNA for cDNA arrays was prepared from triplicate samples of every sensitive and resistant cell line using RNAeasy Mini kit (Qiagen, Madrid, Spain) following the recommendations of the manufacturer. Labelling, hybridization and detection were carried out following the manufacturer's specifications. The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus [18] and are accessible through GEO Series accession number GSE16070 for MCF7 cells and GSE16080 for MDA-MB-468 cells.

2.4. Microarray data analyses

Gene expression analyses were performed using three samples of both sensitive and resistant cell lines. These analyses were carried out with the GeneSpring GX software v 7.3.1 (Agilent Technologies, Madrid, Spain), using the latest gene annotations available (March 2009). Normalization was applied in two steps: 'per chip normalization', by which each measurement was divided by the 50th percentile of all measurements in its array; and 'per gene normalization', by which all the samples were normalized against the median of the control samples (sensitive cells). The expression of each gene was reported as the ratio of each condition relative to control (sensitive) condition after normalization of the data. Then, data were filtered using the control strength, a control value calculated using the Cross-Gene Error model on replicates [9] and based on average base/proportional value. 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. A first filter was performed selecting the genes that displayed a p value, corrected by false discovery rate (Benjamini and Hochberg false discovery rate), of less than 0.05. The output of this analysis was then filtered by fold expression. Thus, lists of genes differentially expressed by at least two fold were generated for each cell line.

2.5. mRNA analysis

Total mRNA was extracted from cells using the Ultraspec™ RNA Kit (Bioteck, Houston, Texas), according to the manufacturer's specifications. The amount of RNA was determined by measuring its absorbance (260 nm) at 25 °C in a Nanodrop ND-1000 spectrophotometer. For the induction experiments, mRNA was extracted 24 h after incubation of 500,000 cells with methotrexate. mRNA was subjected to DNAse treatment to avoid DNA contamination in the PCR step of the RT-PCR analysis.

cDNA was synthesized in a 20 μl reaction mixture containing 0.2–0.5 μg of total RNA, 125 ng random hexamers (Roche, Mannheim, Germany), 10 mM dithiothreitol, 20 units of RNAsin (Promega Biotech Ibérica, Madrid, Spain), 0.5 mM dNTPs (Appli-Chem, Ecogen, Barcelona, Spain), 4 μl of 5× RT buffer and 200 units of M-MLV reverse transcriptase (Invitrogen, Barcelona, Spain). The reaction mixture was incubated at 37 °C for 1 h. Three μl of cDNA mixture were used for PCR amplification by Real Time.

Real Time-PCR: the reaction was performed using the ABI-Prism 7000 Sequence Detection System (Applied Biosystems, Barcelona, Spain). For *ugt1a10*, the reaction was carried out in a final volume of 20 μl, containing 1× Taqman Universal PCR Mastermix (Applied Biosystems, Barcelona, Spain), 1× of TaqMan probe (Applied Biosystems, Barcelona, Spain), 3 μl of cDNA and H_2O. For *ugt1a*, *ugt1a1, ugt1a3-9*, and *ugt1a6* Type 1 and 2, a Sybr Green PCR reaction was performed in a final volume of 20 μl, containing 1× Sybr Universal PCR Mastermix (Applied Biosystems, Barcelona, Spain), 0.25 μM of reverse and forward primers (Ecogen, Barcelona, Spain), 3 μl of cDNA and H_2O. Either for the Taqman or the Sybr Green reactions, the adenosylphosphoribosyl transferase (*APRT*) for mRNA, was used to normalize the results. The primer sequences are listed on additional Table 1. PCR cycling conditions were 2 min at 50 °C, 10 min denaturation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The mRNA amount of the target gene, normalized to APRT, was given by the $\Delta\Delta C_T$ method, where C_T is the threshold cycle indicating the fractional cycle number at which the amount of amplified mRNA reached threshold.

Table 1
Sequences of the primers used in the study.

Primers	For	Rev
UGT1A Ex3-4	TAAGTGGCTACCCCAAAACG	CTCCAGCTCCCTTAGTCTCC
UGT1A1	CATCAGAGACGGAGCATTTTACAC	GTCCGTCAGCATGACATCAAAG
UGT1A3	TAACAGACCCCGTTAACCTCTGC	AAAAGCATGGCAAATGTAGGACAG
UGT1A4	CTTCTGCTGAGATGGCCAGAG	CTGGGTCCATGGAACAGCATAG
UGT1A5	GGGAAGGTGCTGGTGGTG	TGTTTCAAAGAACGATTGAGTGTG
UGT1A6	GGCCTACCATCTGTGTACCTCTTC	ATCCACATCTCTCTTGAGGACAGC
UGT1A7	CTTTGCCAAGGCAGGGAAG	AGAAAATGCACTTCGCAATGG
UGT1A8	CCTATGTGTTTCTCTGCTGCTGAC	TCCAGATCCTCCAGAGTGTATGAG
UGT1A9	AGCCCCCTTCCTCTATGTGT	TGGCATGACTACAACCACCT
UGT1A10	TAQMAN PROBE Hs02516990_s1	
UGT1A6-1 UTR	GCCGTATGACCAAGAAGAGC	GGCTGGGTCTGTGAAAAGAG
UGT1A6-2 UTR	GAAGCTCAGGAGAGGAGTC	GAAGTATAAGTCTAGCCAGTACG
APRT	TAQMAN PROBE Hs00356991_m1	
APRT-H-DNA	CGGGAACCCTCGTCTTTCGCCCCC	GCCTCGGGGGCTCAATCTCACAAC
APRT-H	GCAGCTGGTTGAGCAGCGGAT	AGAGTGGGGCCTGGCAGCTTC

2.6. UGT copy number determination

Genomic DNA from either sensitive or resistant cells was obtained with the Wizard™ Genomic DNA Purification Kit (Promega Biotech Ibérica, Madrid, Spain) following the manufacturer's recommendations. One hundred nanogram of DNA were used for Sybr Green Real Time-PCR amplification. Primers used for UGT1A6 and UGT1A4 were the mRNA For and the UGT1A Ex4 Rev primers (listed on additional Table 1). APRT for DNA was used to normalize the results.

2.7. UGT1A6 activity assay

2.7.1. Preparation of S9 fractions from cell cultures

Cells plated in 100-mm plates were trypsinized, washed with PBS and centrifuged at 800 × *g* for 5 min. The resulting pellet was resuspended in 300 μl of cold potassium-phosphate buffer (100 mM; pH 7.4) and cells were homogenized by sonication. S9 fractions were obtained by centrifugation at 9000 × *g* for 20 min at 4 °C. Protein content of S9 fractions was determined with the Bradford method [19] (Bradford purchased from Sigma-Aldrich, Madrid, Spain) using bovine serum albumin as protein standard.

2.7.2. UDP-glucuronosyltransferase (UGT) activity

UGT activity was measured in S9 fractions prepared from MCF7 and MDA-MB sensitive and resistant cells, using [1-^{14}C]-α-naphthol (American Radiolabeled Chemical Inc., St. Louis, Missouri) as substrate. S9 fractions (125 μg) were incubated in 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$ with 6 μM [1-^{14}C]-α-naphthol and 1 mM UDPGA (Sigma-Aldrich, Madrid, Spain) (final volume 250 μl) at 37 °C for 1 h. Reactions were stopped by addition of 250 μl of acetonitrile and samples were centrifuged for 5 min at 10,000 × *g*. Supernatant samples (100 μl) were analyzed by HPLC coupled to on-line radioactivity detection according to Thibaut et al. [20]. α-Naphthol and α-naphthol-glucuronide were quantified by integrating the area under the radioactive peak.

2.8. Transfection

Cells were plated in 35-mm plates the day before transfection. aODNs were mixed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Roche, Mannheim, Germany) 10 μM for 15 min at RT before lipofecting the cells. siRNAs were mixed with 2 μl of Metafectene (Biontex, Martinsried/Planegg, Germany), and plasmids with 3 μl of Fugene (Roche, Mannheim, Germany) in medium without serum nor antibiotics for 15 min at RT. aODNs sequences were (Sigma-Aldrich, Madrid, Spain): UGT1A1 5′-GCcCTgggaCtCCaCagCCAT-3′; UGT1A3-5 5′-GGgaaCCTggagTcCTgTggCCAT-3′; and aCDK4 5′-ctcAtAtcGAGAGGtAGccaT-3′ (the capital letters contained phosphorothioate linkages). siRNAs sequences were (Thermo, Barcelona, Spain): UGT1A6-A anti strand 5′-CAAGGAAGUUGGCCACUCGTT-3′; UGT1A6-B anti strand 5′-AAAUGAUUGGUAACGGUUCTT-3′; Luciferase sense strand 5′-UAAGGCUAUGAAGAGAUACTT-3; DHFR2-MIS anti strand 5′-AAUGAGGAGGUUGUGGAGGTT-3′. Plasmid pCMV-UGT1A6-1 (Type 1) was purchased from Origene (Rockville, Maryland).

2.9. MTT assay

Cells (30,000) were plated in 35-mm plates in 1 ml of −GHT medium. Methotrexate was added 48 h after transfection. However, it was supplied at the time of plating when performing experiments using SN-38 and Tamoxifen that were added 24 h latter. Seven days after plated, 500 μg of MTT (Sigma-Aldrich, Madrid, Spain) and 5.6 mg of succinate (Sigma-Aldrich, Madrid, Spain) were added and allowed to react for 3 h at 37 °C before the addition of the solubilisation reagent (0.57% acetic acid and 10% SDS in DMSO). Cell viability was measured at 570 nm in a WPA S2100 Diode Array Spectrophotometer.

2.10. Plasmid construction

Two plasmids of different insert length containing the UGT1A6 human promoter were constructed: pUGT1A6-Prom1000 and pUGT1A6-Prom2000. The two promoter inserts of 1071 and 2001 nt in length respectively were generated by PCR amplification, using as template Human Genomic DNA (Promega Biotech Ibérica, Madrid, Spain), and the high-fidelity DNA polymerase Phusion (Finnzymes, Espoo, Finland). UGT1A6 Prom-1000-Fw 5′-cacttgctagcTGGACACAGCTCCTGAAACC-3′; UGT1A6 Prom-1000-Rv 5′-gcacaaagcttTCCAAATCACAGGGCTCCTA-3′; UGT1A6 Prom-2000-Fw 5′-cacttgctagcTCAGCTCCTTCCTCCAACTC-3′; UGT1A6 Prom-2000-Rv 5′-gcacaaagcttAACCACTGCTTTTCACAGGTC-3′; NheI site in Fw primers and HindIII site in Rv are underlined. The fragments were cloned in a pGl4-basic (Promega Biotech Ibérica, Madrid, Spain) vector at the HindIII and NheI sites. The plasmid constructions were sequenced by Macrogen (Korea) using the primers pGL4-Prom-Fw 5′-AAATAGGCTGTCCCCAGTGC-3′; pGL4-Prom-Rv 5′-CGTCTTCGAGTGGGTAGAATG-3′ and UGT1A6 Pr-2000 seq 5′-AGGCTTCTTCTCCCTTCCTG-3′. No difference was observed when comparing the sequences with that reported in Genbank (Accession number NC_000002.11). The Match™ software, using TRANSFAC 6.0 database was used to determine the putative transcription factor binding sites present in UGT1A6 promoter. There are putative ARNT and AhR/ARNT binding sites at −1230 and −1504 nt, respectively, from the UGT1A6 type 2 translational start codon (ATG).

2.11. Luciferase assays

Cells were seeded into 35-mm plates the day before transfection at a density of 2×10^5 cells/well in −GHT medium. Each well was co-transfected with 300 ng of the appropriate plasmid and 10 ng of Renilla plasmid (Promega Biotech Ibérica, Madrid, Spain) using FUGENE™6 (Roche, Mannheim, Germany). Luciferase activity was assayed 30 h after transfection. Cell extracts were prepared by lysing the cells with 200 μl of freshly diluted 1× Reporter Lysis Buffer (Promega Biotech Ibérica, Madrid, Spain). The lysate was centrifuged at 13,000 × *g* for 2 min to pellet the cell debris and the supernatants were transferred to a fresh tube. A 15-μl aliquot of the extract was added to 15 μl of the luciferase assay substrate (Promega Biotech Ibérica, Madrid, Spain) and the luminescence of the samples was read immediately on a Gloomax 20/20 luminometer (Promega Biotech Ibérica, Madrid, Spain), in which the light production (relative light units, RLU) was measured for 5 s. Then, 15 μl of the 1× Renilla substrate in Stop & Glo buffer was added, and light production was measured to normalize the results. A ratio between the luciferase and Renilla RLU was given.

2.12. Binding analysis

2.12.1. Preparation of AhR/ARNT and ARNT duplexes

The probes for the gel-shifts were generated by mixing 25 μg of each single-stranded oligodeoxynucleotides in 150 mM NaCl: AhR-Fw 5′-aggaactcGCGTCccagcca-3′; AhR-Rv 5′-tggctggCACGCgagttcct-3′; ARNT-Fw 5′-ttctcacCACGTactggcta-3′; and ARNT-Rv 5′-tagccag-tACGTGgtgagaa-3′ (the capital letters stand for the transcription factor binding site). After incubation at 90 °C for 5 min, solutions were allowed to cool down slowly until reaching room temperature. Duplexes were purified in a non-denaturing 20% polyacrylamide gel and quantified by their absorbance at 260 nm at 25 °C.

2.12.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from exponentially growing sensitive or resistant cells as described [21]. A double-stranded DNA probe corresponding to the AhR/ARNT or ARNT UGT1A6 promoter sites, was end-labelled with T4 polynucleotide Kinase (New England Biolabs, Ipswich, Massachusetts) and [γ-^{32}P]ATP (3000 Ci/mmol, Perkin Elmer, Madrid, Spain), and used in the gel-shift assays. DNA binding assays were performed as described [22] and were analyzed on a Storm 840 Phosphorimager (Molecular Dynamics). Salmon Testes DNA (Sigma-Aldrich, Madrid, Spain) was used as a non-specific competitor in a 1:8 non-specific/specific ratio. In the supershift experiments, 1 μg of rabbit polyclonal antibody AhR or ARNT (both from Santa Cruz, Heidelberg, Germany) was added to the reaction mixture. Both antibodies were added at the same time as the probe and AhR was incubated OVN at 4 °C and ARNT was incubated for 30 min at room temperature.

2.13. Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using Student's t-test using InStat software for Mac OS X. Results were considered significant if $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

3. Results

3.1. Differential gene expression between cells sensitive and resistant to MTX

Microarray analyses between sensitive and resistant cells to 10^{-6} M methotrexate were performed using MCF7 and MDA-MB-468 breast cancer cell lines. A list of the 2-fold differentially expressed genes that were statistically significant and passed the Benjamini and Hochberg-FDR was obtained for each cell line. Both cell lines overexpressed UGT1A, a 24-fold increase in MCF7-R and a 28-fold increase in MDA-MB-R. Validation of UGT1A family overexpression was carried out by RT-Real Time PCR (Fig. 1). Among the members of the UGT1A family a different expression pattern was observed. MCF7-R (Fig. 1A) and MDA-MB-R (Fig. 1B) shared UGT1A1, UGT1A3, UGT1A4, UGT1A5 and UGT1A6 overexpression. UGT1A6 was the family member that showed the greatest increase between resistant and sensitive cells in both cell lines, 15-fold increase in MCF7-R cells and 46-fold in MDA-MB-R. UGT1A6 is composed of two isoforms that differ on the 5′UTR and the transcription start site, Type 1 and Type 2. MCF7-R cells showed a noticeable increase in Type 1 UGT1A6, a 12-fold increase, whereas MDA-MB-R had an increase in both Type 1 and Type 2 UGT1A6, 55- and 40-fold increase, respectively (Fig. 2A MCF7 and Fig. 2B MDA-MB).

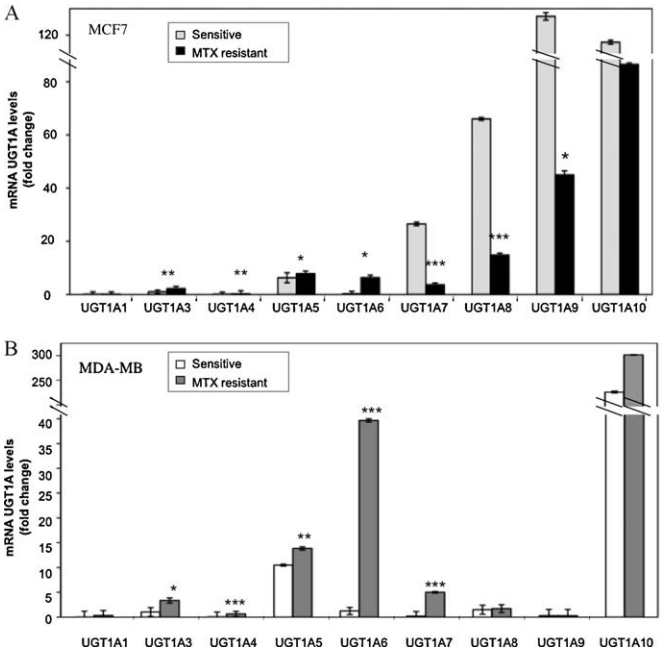

Fig. 1. UGT1A mRNA levels. Basal mRNA levels of the different members of the UGT1A family in sensitive or 10^{-6} M MTX resistant cells. Prior to RT-Real Time PCR, mRNA was submitted to DNAse treatment. Expression data of UGT1A family members are referred to UGT1A3 mRNA levels of sensitive cells, whose value was taken as 1. (A) MCF7-S and MCF7-R. (B) MDA-MB-S and MDA-MB-R. Data represent the mean ± SEM of at least four experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

3.2. Copy number

We explored whether or not UGT1A6 overexpression was due to gene amplification by Real Time-PCR. No changes in copy number were found between sensitive and resistant cells in both cell lines for either UGT1A6 or UGT1A4, another UGT family member with a noticeable overexpression (Fig. 2C MCF7 and Fig. 2D MDA-MB), suggesting an increase in the transcription rate.

3.3. UGT1A activity

We studied whether the noticeable increase in UGT1A6 mRNA levels in MTX resistant cells was followed by a rise in UGT1A6 activity. The measurement of UGT1A activity, determined by [1-^{14}C]-α-naphthol-glucuronide formation, showed a clear increase in MCF7-R and MDA-MB-R cells, of 2-fold and 9-fold, respectively (Fig. 2E).

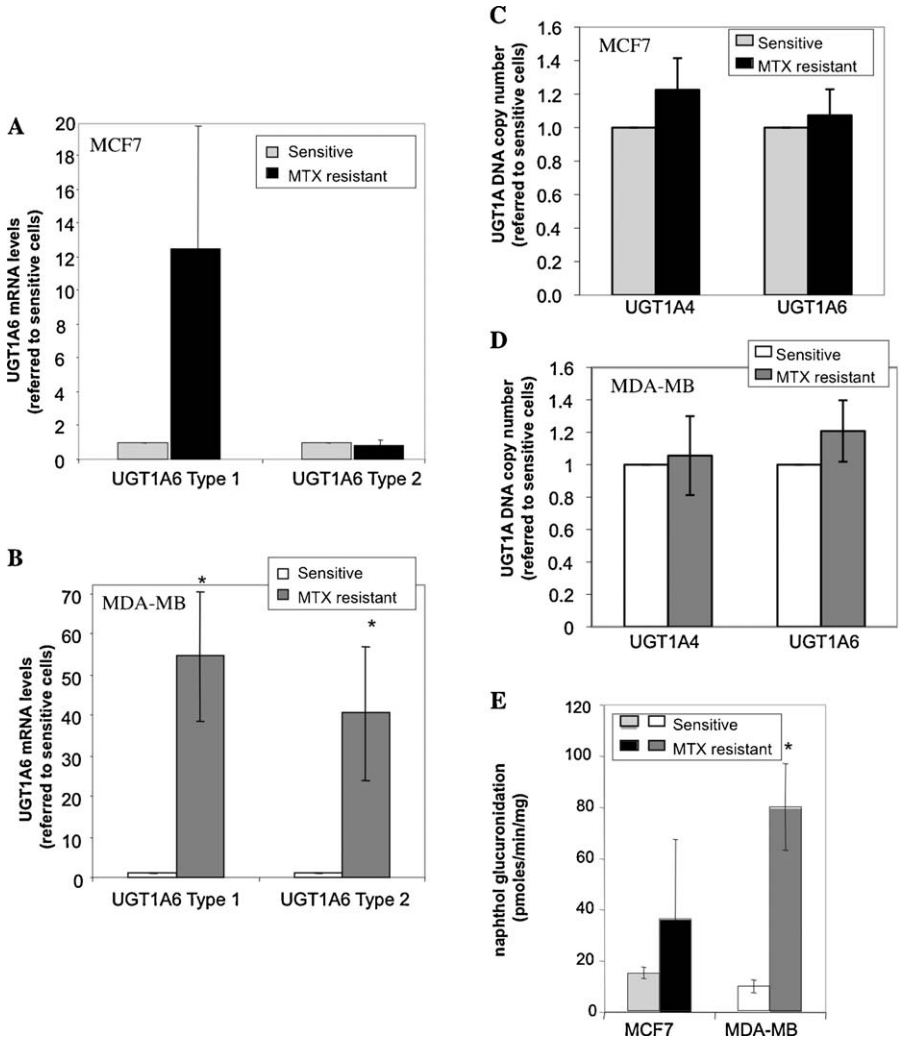

Fig. 2. Type 1 and type 2 UGT1A6 mRNA levels, copy number and UGT activity. Basal UGT1A6 mRNA levels of sensitive and 10^{-6} M MTX resistant cells; (A) MCF7 and (B) MDA-MB. Prior to RT-Real Time PCR, mRNA was submitted to DNAse treatment. Data are referred to sensitive cells and represent the mean ± SEM of three experiments. (C and D) UGT1A6 and UGT1A4 copy number of sensitive and MTX resistant cells. (C) MCF7 and (D) MDA-MB. Data are referred to sensitive cells and represent the mean ± SEM of five experiments. (E) UGT1A activity. The S9 fraction of MDA-MB and MCF7 cells, either sensitive or resistant, was incubated with [1-^{14}C]-α-naphthol and UGT1A activity was measured by HPLC. Data represent the mean ± SEM of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

3.4. Inhibition of UGT1A6 expression and MTX sensitization

To study the role of UGT1A6-1 (Type 1) in MTX resistance in breast cancer cells, inhibition of its expression was carried out by siRNAs. Two different siRNAs against UGT1A6-1 were designed to ensure the specificity of the results. No increases in sensitivity to MTX were observed in MCF7-S or R and MDA-MB-S or R upon UGT1A6-1 siRNA transfection (data not shown). Then, we studied the influence of the other overexpressed UGTs in MTX resistance. As with UGT1A6, no differences in sensitivity to MTX were observed when inhibiting UGT1A1 or UGT1A3-5 in MCF7-S or R and MDA-MB-S or R (data not shown). The functionality of the inhibiting molecules against UGT1A6-1 and UGT1A1 or UGT1A3-5 was confirmed by the decrease in the mRNA levels of the specific UGT1A targets (data not shown). The lack of an increased sensitivity to MTX after down regulation of the overexpressed UGTs found in resistant cells suggests a redundant action among the different UGT isoforms, as a siRNA targeting the whole UGT1A family sensitized cells to MTX [23].

3.5. UGT1A6-1 overexpression and MTX resistance

The influence of UGT1A6-1 overexpression in MTX resistance was studied in sensitive breast cancer cells by the transfection of a plasmid containing the UGT1A6-1 gene. Sensitive breast cancer cells incubated with 3×10^{-8} M MTX plus 1 μg of pCMV-UGT1A6-1 displayed a significant increase in cell survival referred to cells treated with MTX alone, of 20% in MCF7 (Fig. 3A) and 48% in MDA-MB cells (Fig. 3B). This rise in cell survival was specific of UGT1A6-1 as the empty vector did not cause any effect. The increase in UGT1A6-1 mRNA levels upon plasmid transfection was confirmed by RT-Real Time PCR (data not shown).

3.6. Effect of MTX on UGT1A mRNA levels

We studied whether MTX was able to induce UGT1A gene expression. MCF7-S and MDA-MB-S cells were incubated for 24 h with 3×10^{-8} M MTX, and then UGT1A mRNA levels were analysed by RT-Real Time-PCR. MCF7-S (Fig. 4A) and MDA-MB-S (Fig. 4B) showed a similar pattern of induction of the UGT1A family. In both cases, when measuring the mRNA levels of the UGT1A family as a pool (1A*), an increase was observed, of 2-fold in MCF7-S MTX treated cells and 3.8-fold in MDA-MB-S. However, when determining the mRNA levels of each specific member of the UGT1A family upon MTX incubation, we observed two patterns of expression, one for the UGT1A1-6 cluster for which induction was the predominant response, and another for the UGT1A7-10 cluster

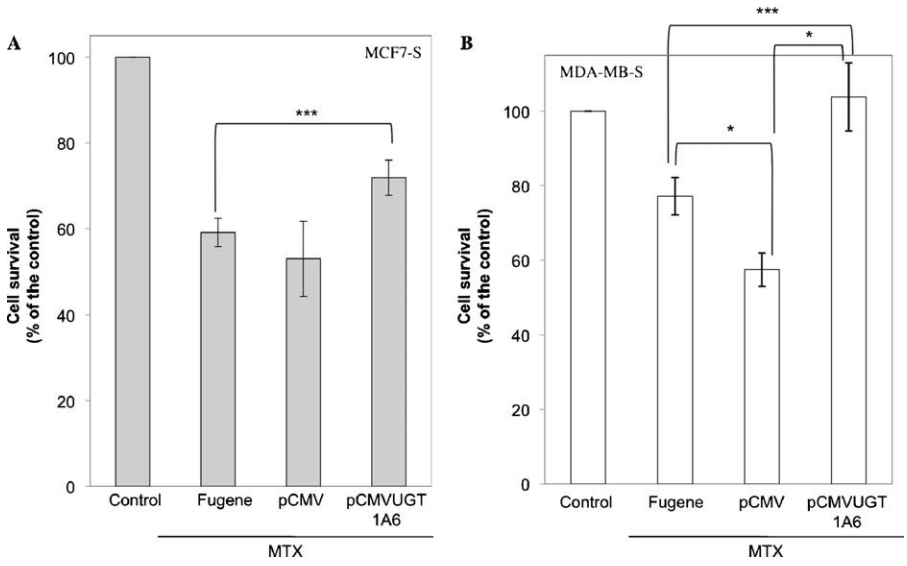

Fig. 3. Methotrexate cytotoxicity upon UGT1A6-1 plasmid transfection. 30,000 sensitive cells were incubated in −GHT medium. The following day, cells were transfected with the appropriated plasmid using Fugene. 48 h after transfection, cells were treated with 3×10^{-8} M MTX. The empty vector pCMV was used as a negative control. Data represent the fold change referred to cells not treated with MTX. MTT assays were performed a week after transfection. (A) MCF7-S cells. (B) MDA-MB-S cells. Data represent the mean ± SEM of at least five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

for which inhibition was prevalent. Both cell lines had in common the increase in the expression of UGT1A1, UGT1A4 and UGT1A6, and the decrease in the expression of UGT1A8, UGT1A9 and UGT1A10. In MCF7-S and MDA-MB-S cells treated with MTX, UGT1A6 was the family member that presented the highest rise in expression, 2.4-fold for MCF7-S and 5.7-fold for MDA-MB-S.

3.7. UGT1A activity upon MTX incubation

We analysed whether the induction that MTX exerted on UGT1A6 mRNA levels was translated into a higher glucuronidation activity. Since MDA-MB-S cells showed a greater response to MTX induction, they were selected for the enzymatic determination.

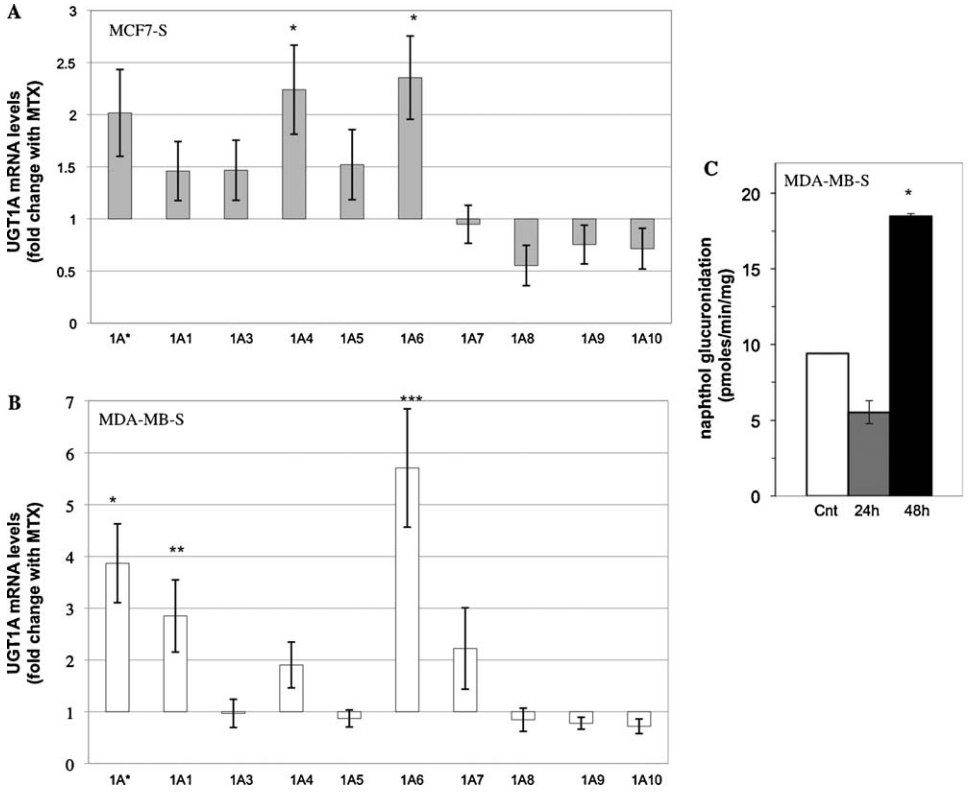

Fig. 4. UGT1A mRNA and activity after MTX incubation. (A and B) 500,000 sensitive cells were incubated for 24 h with 3×10^{-8} M MTX. Total mRNA was obtained and submitted to DNAse treatment prior to RT-Real Time PCR. Data represent the fold change referred to cells non-treated with MTX. (A) MCF7-S cells. (B) MDA-MB-S cells. Data represent the mean ± SEM of at least three experiments. (C) UGT1A activity. MDA-MB sensitive cells were incubated for 24 and 48 h with 3×10^{-8} M MTX. The microsomal fraction was obtained and UGT1A activity was analysed through [1-^{14}C]-α-naphthol glucuronidation. Data represent the mean ± SEM of two experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

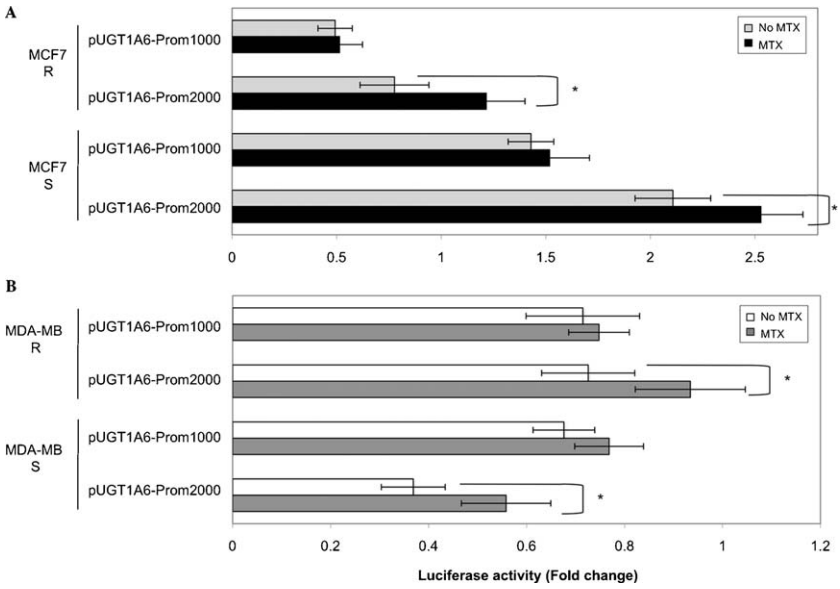

Fig. 5. Analysis of UGT1A6 promoter by luciferase activity. 200,000 cells were incubated in −GHT medium. The following day, cells were transfected with the appropriated plasmid using Fugene and incubated either in the presence or in the absence of MTX. Thirty hours latter dual luciferase assays were performed. The control was the empty vector, pGl4. Renilla was used to normalise the data. (A) MCF7 cells and (B) MDA-MB cells. Data represent the mean ± SEM of at least three experiments. $^{*}p < 0.05$.

Therefore, these cells were incubated in −GHT medium for different periods of time with 3×10^{-8} M MTX. As shown in Fig. 4C, a noticeable increase in the glucuronidation levels of [1-^{14}C]-α-naphthol- was observed at 48 h, despite an initial decrease at 24 h.

3.8. Effect of MTX on UGT1A6 transcriptional activity

MTX induced UGT1A6 expression, so we proceeded to study if that induction was the result of a transcriptional activation. Two different luciferase reporter plasmids containing 1071 bp or 2001 bp of the UGT1A6 promoter were constructed. Cells were transfected with these plasmids and the Renilla plasmid to normalize the results. In both cell lines, MTX caused an increase in promoter activity but only from the 2001 bp fragment (Fig. 5A MCF7 and Fig. 5B MDA-MB). This increase in transcription activity was observed either in sensitive or resistant cells. The luciferase activity from the empty vector (pGL4) remained unchanged when incubated with MTX (data not shown).

3.9. Transcription factors binding to the UGT1A6 promoter

The luciferase assays indicated that the region responsible for MTX induction was located between the −1071 to −2001 bp of the UGT1A6 promoter region. Computational analysis using the Transfac database revealed the presence of binding sites for two transcription factors that could be involved in MTX induction, AhR/ARNT and ARNT, which are related to xenobiotics metabolism and oxidative stress. Nuclear extracts from MCF7 and MDA-MB sensitive and resistant to MTX were incubated with two radiolabelled probes derived from the UGT1A6 promoter containing the binding sites for either AhR/ARNT or ARNT. A similar behaviour was observed in both breast cancer cell lines with both probes (Figs. 6A and 7A). Sensitive cells showed three retardation bands using the AhR/ARNT as well as ARNT probes. When these cells were treated with 3×10^{-8} M MTX for 24 h, only the band with the lowest mobility could be observed in the gel shift with a intensity higher than in untreated cells. Resistant cells showed this same retardation pattern of a unique shifted band that did not change when these cells were depleted of MTX for 24 h. Supershift assays were performed to assess the specificity and identity of the retardation bands. When MCF7-S nuclear extracts were incubated with an AhR antibody, the three retardation bands disappeared, indicating that the antibody blocked the formation of the AhR/ARNT-probe complex (Fig. 6B). MCF7-S nuclear extracts incubated with the ARNT antibody produced a super-shifted band due to the reduced mobility of the Ab-ARNT-probe complex, as well as the disappearance of the upper band of the three bands present in the gel-shift (Fig. 7B).

3.10. Influence of MTX in UGT1A metabolism of other drugs

Tamoxifen and the active metabolite of irinotecan, SN-38, are two drugs metabolized by UGT1A. The IC50 for SN-38 was 0.75 nM in MDA-MB cells and 0.75 μM in MCF7 cells, the IC50 for TMX was 0.35 μM in MCF7 cells, and the IC50 for MTX was 6×10^{-8} M in both cell lines. When MDA-MB-S or MCF7-S cells were incubated with increasing concentrations of SN-38, a noticeable cell death was observed (Figs. 8A and 9A). However, cells incubated with SN-38 together with 2×10^{-8} M MTX, showed a cell survival recovery of 22% with 0.01 μM SN-38 in MDA-MB-S (Fig. 8A), reaching a 43% at 0.025 μM SN-38 in MCF7-S (Fig. 9A), probably due to SN-38 increased glucuronidation upon MTX incubation. MDA-MB-R and MCF7-R cells did not present significant changes in cell survival, when incubated with SN-38 and MTX 1×10^{-6} M (Fig. 8B and Fig. 9B). This effect could be explained by the fact that methotrexate resistant cells show already high levels of UGT1A6 regardless of the presence of MTX. Tamoxifen was assayed in MCF7 cells only as the mechanism of action of this drug needs ER+ cells (Fig. 10). Similar to SN-38 behaviour, sensitive cells incubated with tamoxifen together with MTX had a higher cell survival than those incubated with tamoxifen alone, reaching a 36% of cell survival recovery when incubated with 1 μM tamoxifen (Fig. 10A). Resistant cells did not show significant changes in cell survival due to the presence or absence of MTX (Fig. 10B). The vehicle of both drugs, DMSO, did not cause significant changes in cell survival.

4. Discussion

Drug resistance constitutes a drawback of cancer treatment. Microarray analysis of two different breast cancer cell lines resistant to methotrexate, MCF7-R and MDA-MB-R, pointed out UGT1A, as the

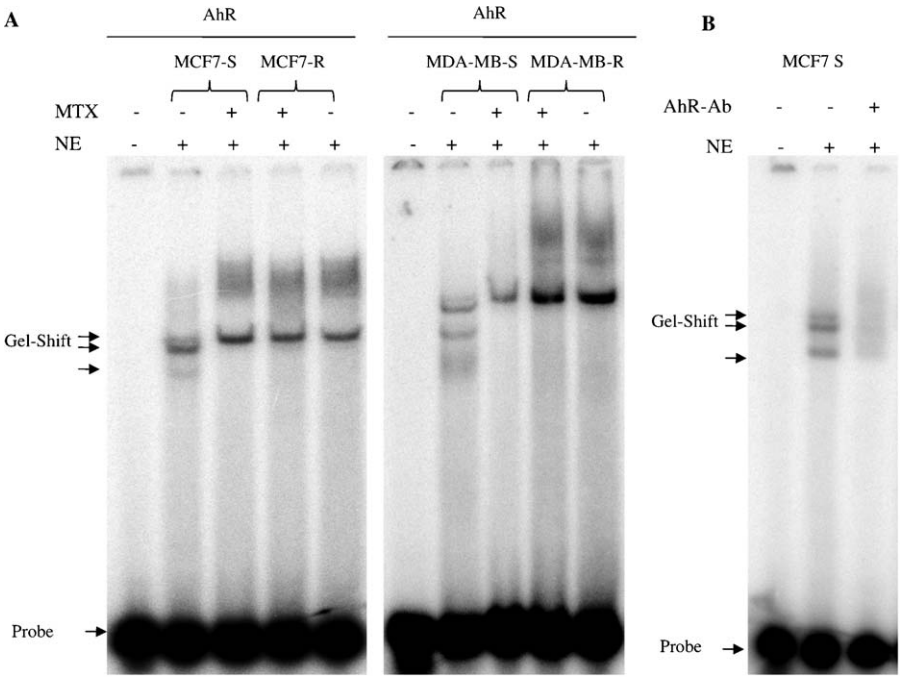

Fig. 6. Binding of AhR to UGT1A6 promoter. Cells were incubated with or without MTX for 24 h prior to nuclear extracts harvest. Nuclear extracts were incubated with a radiolabelled probe corresponding to UGT1A6 AhR/ARNT promoter sequence. (A) MCF7 sensitive and resistant and MDA-MB sensitive and resistant. (B) AhR supershift. Incubation of MCF7-S nuclear extracts with AhR antibody. Shifted and supershifted bands are indicated by arrows.

only gene family in common between them. Additionally, it is worth noting that ABCG2 MTX transporter was about 10-fold overexpressed in MCF7-R cells, although no significant changes in ABCG2 expression were observed in MDA-MB-R.

Among the different members of UGT1A family, the cluster formed by UGT1A1 through 6, and specifically UGT1A6, showed to be the main responsible for the rise of UGT1A expression in breast cancer resistant cells. UGT1A6 is a major UGT in humans that mediates glucuronidation [24] and is responsible for the metabolism of drugs, such as acetaminophen [25] and valproate [26]; carcinogens, such as benzo(a)pyrene; and endogenous substrates, including serotonin [27] and 5-hydroxytryptophol [28]. Glucuronidation is one of the main phase II clearance mechanisms for drugs, dietary natural and chemical products as well as environmental substances in humans. Glucuronidation increases water solubility of compounds, enabling their elimination of the body via urine or bile acids.

UGT1A family could somehow contribute to MTX metabolism to a certain degree, as it shares a phenolic structure common to other UGT1A substrates. This effect could be hypothesized from the increased survival observed in sensitive cells treated with MTX when UGT1A6 was overexpressed. However, no increase in sensitivity to MTX was observed when a siRNA against UGT1A6 was used. Different aODNs towards the remaining members of the

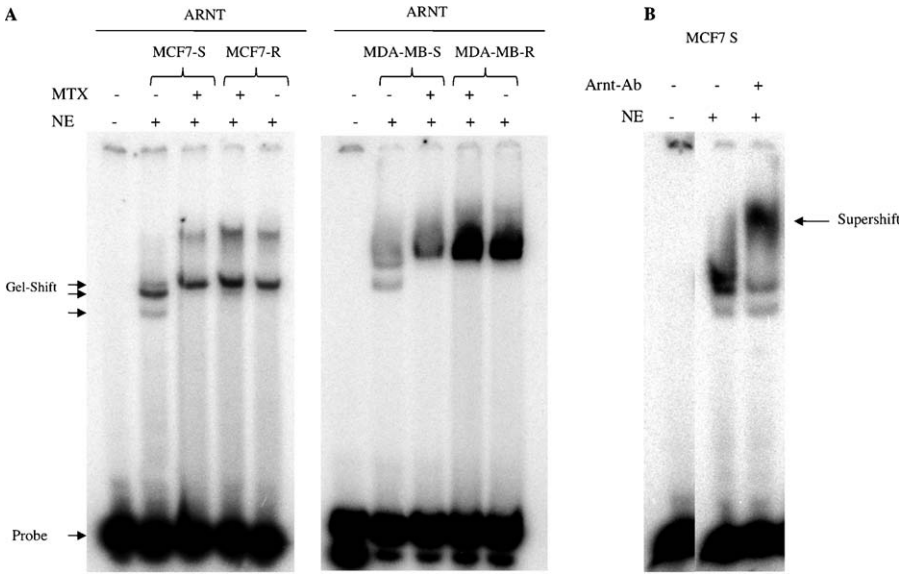

Fig. 7. Binding of ARNT to UGT1A6 promoter. Cells were incubated with or without MTX for 24 h prior to nuclear extracts preparation. Nuclear extracts were incubated with a radiolabelled probe corresponding to UGT1A6 ARNT promoter sequence. (A) MCF7 sensitive and resistant and MDA-MB sensitive and resistant. (B) ARNT supershift. Incubation of MCF7-S nuclear extracts with ARNT antibody. Shifted and supershifted bands are indicated by arrows.

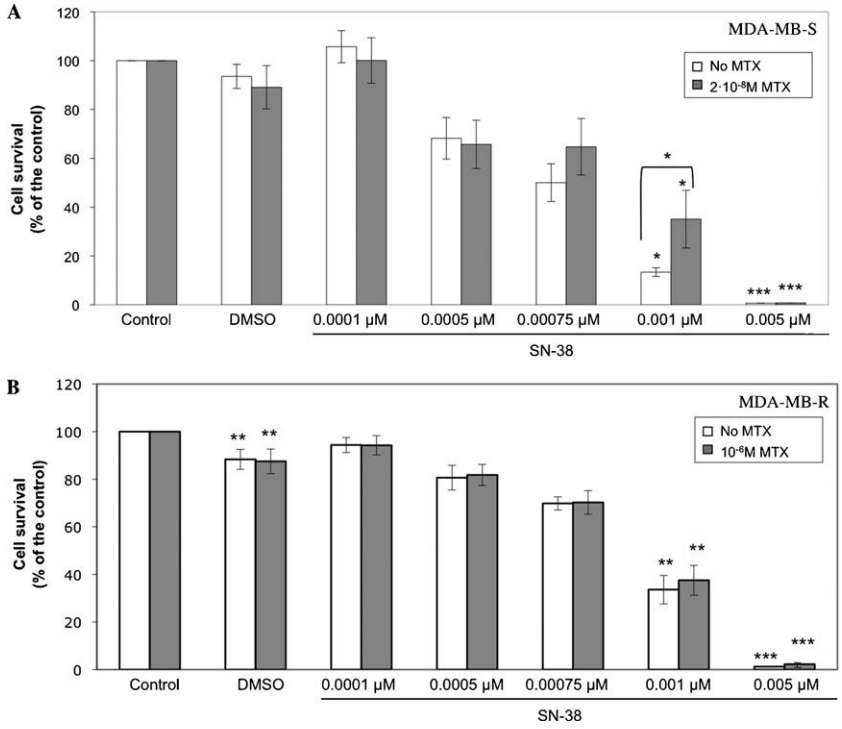

Fig. 8. SN-38 cytotoxicity in the presence of MTX in MDA-MB cells. 30,000 cells were incubated in −GHT medium either in the absence or in the presence of MTX. The following day, cells were incubated with the appropriated SN-38 concentration. The vehicle of SN-38, DMSO was tested alone at the concentrations used. MTT assays were performed a week after adding MTX. (A) MDA-MB-S cells and (B) MBA-MB-R cells. Data represent the mean ± SEM of four experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

UGT1A1-6 cluster were also tested, to evaluate whether another family member was responsible for MTX metabolism, but increase in sensitivity was neither observed. The lack of sensitization towards MTX when UGT1A6 was downregulated could be explained by the redundant action of the different UGTs isoforms. In previous studies, when MTX-treated cells were transfected with a siRNA targeting the whole UGT1A family, an increase in cell sensitivity was observed, supporting the hypothesis of UGT1A redundancy [23]. On the other hand, it is difficult to discriminate the contribution of a single UGT isoform in the metabolism of an UGT substrate. No isoform-specific substrates, UGT isoform-specific chemical inhibitors or inhibitory antibodies are available [29].

UGT1A overexpression in both breast cancer cell lines resistant to MTX was not due to gene amplification, but to an increase in UGT1A transcription that led to an increased UGT1A activity. UGT1A family is characterised by its induction by a wide range of compounds.

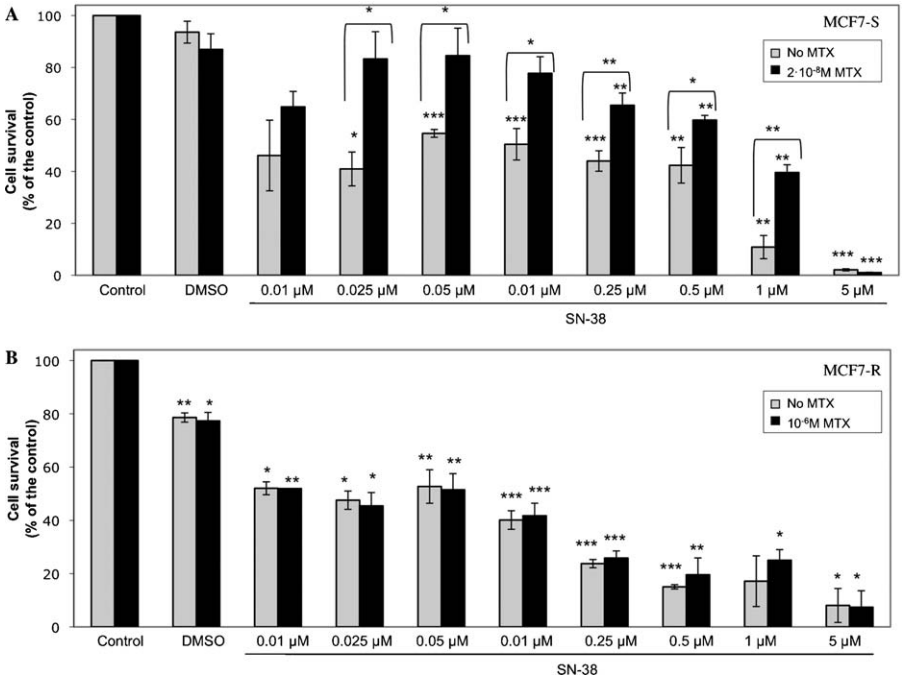

Fig. 9. SN-38 cytotoxicity in the presence of MTX in MCF7 cells. (A) MCF7-S cells and (B) MCF7-R cells. Data represent the mean ± SEM of four experiments. Other conditions as in Fig. 8.

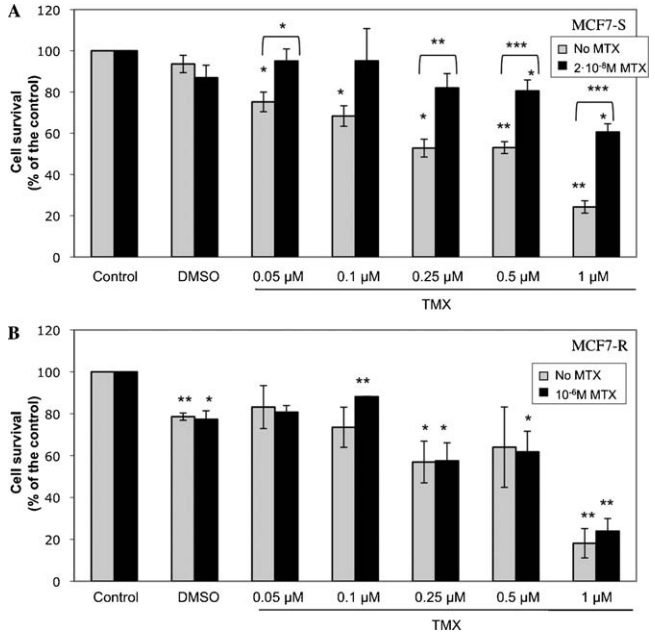

Fig. 10. Tamoxifen cytotoxicity in the presence of MTX in MCF7 cells. 30,000 cells were incubated in −GHT medium in the absence or in the presence of MTX. The following day, cells were incubated with the appropriated tamoxifen concentration. The vehicle of tamoxifen, DMSO was tested alone at the concentrations used. (A) MCF7-S cells and (B) MCF7-R cells. Data represent the mean ± SEM of four experiments. Other conditions as in Fig. 8.

Among the chemicals that induce UGT1A6 expression we can find: flavonoids, sulforaphane [30], LPS [31], 3-methylcholanthrene (3-MC) [32,33], benzo[a]pyrene (B[a]P), dioxin [34], b-naphthoflavone (BNF) [35], oltipraz [36–38], and rifampicin [39]. MTX could be an UGT1A6 inducer according to our results of increased mRNA levels, luciferase activity and UGT1A enzymatic activity.

The different transcription factors involved in UGT1A induction include activator protein 1 (AP-1) [32], constitutive active receptor (CAR) [40], pregnane X receptor (PXR) [41], peroxisome proliferator-activated receptor (PPAR) [42], antioxidant response element (ARE) through Nrf2 [36,43] and aryl hydrocarbon receptor (AhR) [44]. AhR was described to provoke moderate UGT1A6 induction in humans and rodents [14,45–48]. This transcription factor is located in the cytoplasm and upon activation by ligand binding, it enters the nucleus and interacts with the AhR nuclear translocator protein (ARNT), forming the AhR/ARNT heterodimer that binds to a specific DNA sequence called xenobiotic response element (XRE, 5′-GCGTG-3′) [49]. AhR/ARNT binding to XRE activates the expression of a battery of genes involved in drug and hormone metabolism, e.g., CYP1A1, CYP1B1, CYP1A2, ALDH3, GSTA2, NAD(P)H:quinone reductase and UGT1A6 [50,51]. TCDD (dioxin) is one of the compounds that induce UGT1A6 via AhR binding to the XRE as it has been observed in primary hepatocyte cultures and in colon carcinoma Caco-2 cells [14]. Some AhR agonists cause coordinate induction of both phase-I CYPs and UGTs to attenuate the generation of mutagenic benzo[a]pyrene metabolites, facilitating detoxification of the carcinogen [52]. In addition, UGTs may be responsible for homeostatic control of AhR ligands, such as bilirubin [52]. AhR can also induce UGT1A6 expression through Nrf2 binding to the ARE element present in the UGT1A6 promoter [43], however no induction in response to MTX was observed in the luciferase assays for the first 1000 bp region, where the ARE site is located. Our luciferase and gel-shift results support the idea that MTX could be inducing UGT1A6 expression through AhR/ARNT heterodimers and ARNT itself. ARNT can also bind to other ligands like hypoxia-inducible factor 1 (HIF-1), which binds to a promoter sequence (5′-CACGT-3′) different from XRE. It has been shown that down-regulation of HIF-1α by siRNA sensitizes MCF7 cells to MTX [53]. Furthermore, up-regulation of the hypoxia pathway by HIF not only confers an aggressive phenotype but also contributes to resistance to radiotherapy and chemotherapy [54]. The potential mechanisms for HIF-mediated drug resistance include the interference with the apoptotic pathway [55], the upregulation of the multidrug resistance transporter P-glycoprotein [56], and a poor drug delivery in functionally deficient vessels [54]. Thus, MTX could induce UGT1A6 through HIF, thus causing a phenotype more resistant to this drug. Another possibility would be that MTX, instead of directly activating AhR or ARNT, could bind to an ARNT inhibitor such as the short heterodimer partner (SHP) and SMRT, or activate ARNT through coactivators as CBP and ERAP140. SHP has been shown to inhibit a reporter activity induced by TCDD, an UGT1A6 substrate, in RL95-2 cells [51].

MTX induction of UGT1A6 may have important toxicological, pharmacological and physiological consequences, as it would decrease the bioavailability of many dietary constituents and drugs susceptible to glucuronidation, such as irinotecan and tamoxifen, which would become less active when administered simultaneously with MTX, as indicated by our results. UGTs have been reported to be responsible in part for the resistance to chemotherapeutic drugs such as daunorubicin [57] and mycophenolic acid [58], and as shown here they constitute an important element in MTX resistance in breast cancer cells. This type of cross-resistance between MTX and other chemotherapeutics has significant pharmacological repercussions and could represent a handicap to tumour treatment, since methotrexate is given in combination with other drugs such as tamoxifen to treat breast cancer. Additionally, any other drug susceptible to glucuronidation, such as paracetamol, given to a patient treated with MTX, would decrease its bioavailability and therapeutic effect.

In summary, we show that UGT1A6 is overexpressed in breast cancer cells resistant to methotrexate, and that this drug induces UGT1A6 mRNA and enzymatic activity, through a mechanism mediated by ARNT and AhR/ARNT. The pharmacological effect of this induction should be taken into account when combining methotrexate with other drugs susceptible to glucuronidation.

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