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**UDP-GLUCURONOSYLTRANSFERASE 1A6 OVEREXPRESSION IN BREAST  
CANCER CELLS RESISTANT TO METHOTREXATE**

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**Category: Antibiotics and Chemotherapeutic**

## ABSTRACT

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

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4 Breast cancer is the most common cancer in women in developed countries, and after lung  
5 cancer, the tumour that causes more deaths among females [1]. One of the possible treatments  
6 for this cancer is methotrexate (MTX), usually given in combination with cyclophosphamide  
7 and 5-fluorouracil. MTX is also used in other types of cancer such as acute lymphocytic  
8 leukaemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, and head and neck  
9 cancer; as well as autoimmune diseases as psoriasis and rheumatoid arthritis [2, 3]. However,  
10 a major problem of methotrexate, in common with other chemotherapeutic drugs, is the  
11 occurrence of drug resistance.  
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26 Methotrexate is a folate analogue that reversibly binds to the dihydrofolate reductase (DHFR)  
27 enzyme. Dihydrofolate reductase catalyzes the reduction of dihydrofolate to tetrahydrofolate,  
28 needed for the synthesis of thymidylate, hypoxanthine and glycine [4]. MTX inhibition of  
29 DHFR results in the blockage of DNA synthesis and cell growth. Different mechanisms of  
30 MTX resistance had been described including *dhfr* locus amplification, mutation of the target,  
31 alteration in the degree of polyglutamation, MDR phenotype, and decreased entrance of the  
32 drug [3].  
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46 With the aim to get further insight into the mechanisms of MTX resistance, functional  
47 genomic analysis using microarrays were performed in breast cancer cell lines sensitive and  
48 resistant to MTX. We identified UGT1A as the common gene among the differentially  
49 expressed genes in the two breast cancer cell lines resistant to MTX.  
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58 UDP-glucuronosyltransferases (UGTs) are a family of enzymes involved in phase II  
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1 metabolism. The addition of a glycosyl group from uridine diphosphoglucuronic acid  
2 (UDPGA) renders hydrophobic compounds more soluble for their elimination via bile and  
3 urine. UGTs catalyse the glucuronidation of many lipophilic endogenous substrates such as  
4 bilirubin and estrogens, and xenobiotics. Anticancer agents such as irinotecan, topotecan,  
5 doxorubicin and tamoxifen as well as carcinogens, are glucuronidated, contributing  
6 significantly to the overall availability and pharmacological effect of these xenobiotics [5].  
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17 The UGT superfamily is divided into families and subfamilies based on protein sequence  
18 identity, where UGT1 and UGT2 families share less than 50% of identity [6]. The UGT1 gene  
19 family expresses nine functional UGT1A proteins by alternative splicing of 13 different  
20 tandem exons 1 with the common exons 2–5 [7-9]. Thus, all the UGT1A isozymes have a  
21 highly conserved “common” region, which is believed to contain the cofactor-binding site,  
22 and a variable region containing unique substrate-binding sites. Significant overlap exists in  
23 substrate specificity among the UGT family members [10-12]. The UGT1A genes are divided  
24 in two clusters, the UGT1A7-10 cluster of genes showing >70% similar in their first exon  
25 sequence and < 60% similar to the other UGT1A1-6 genes [8].  
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41 UGT1A6 metabolizes planar phenols and arylamines. Two types of UGT1A6 formed by  
42 alternative splicing have been identified, which differ in the beginning of the first exon [13,  
43 14]. Type 1 has a longer and complete exon 1 and 5'UTR, typical of the UGT1A genes;  
44 whereas Type 2 suffers alternative splicing in the 5'UTR, removing part of the beginning of  
45 exon 1 and resulting in a shorter isoform. UGT1A6 expression is highly modulated by  
46 hormones, drugs, and other xenobiotics that serve as ligands for multiple sensors, including  
47 the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the aryl  
48 hydrocarbon (AhR) receptor, and the transcription factor Nrf2, which responds to oxidative  
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/electrophile stress [14, 15].

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4 In this study, the relevance of UGT1A6 overexpression in MTX resistance was analysed in  
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6 breast cancer cells sensitive and resistant to this drug. Moreover, the induction of UGT1A6 by  
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9 MTX and the transcription factors involved were studied.  
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## 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<sub>2</sub> atmosphere. MCF7-R and MDA-MB-R were breast cancer cells resistant to 10<sup>-6</sup>M Methotrexate (MTX), generated in our laboratory upon incubation with stepwise concentrations of MTX, following the methodology previously described in [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



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NCBI's 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 50<sup>th</sup> 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<sup>TM</sup> RNA Kit (Bioteck, Houston, Texas), according to the manufacturer's specifications. The amount of RNA was determined

1 by measuring its absorbance (260 nm) at 25°C in a Nanodrop ND-1000 spectrophotometer.

2 For the induction experiments, mRNA was extracted 24 hours after incubation of 500,000  
3 cells with methotrexate. mRNA was subjected to DNase treatment to avoid DNA  
4 contamination in the PCR step of the RT-PCR analysis.  
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7 cDNA was synthesized in a 20 µl reaction mixture containing 0.2-0.5 µg of total RNA, 125  
8 ng random hexamers (Roche, Mannheim, Germany), 10 mM dithiothreitol, 20 units of  
9 RNAsin (Promega Biotech Ibérica, Madrid, Spain), 0.5 mM dNTPs (AppliChem, Ecogen,  
10 Barcelona, Spain), 4 µl of 5X RT buffer and 200 units of M-MLV reverse transcriptase  
11 (Invitrogen, Barcelona, Spain). The reaction mixture was incubated at 37°C for 1 hour. Three  
12 µl of cDNA mixture were used for PCR amplification by Real Time.  
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15 Real Time-PCR: the reaction was performed using the ABI-Prism 7000 Sequence Detection  
16 System (Applied Biosystems, Barcelona, Spain). For *ugt1a10*, the reaction was carried out in  
17 a final volume of 20 µl, containing 1X Taqman Universal PCR Mastermix (Applied  
18 Biosystems, Barcelona, Spain), 1X of TaqMan probe (Applied Biosystems, Barcelona,  
19 Spain), 3 µl of cDNA and H<sub>2</sub>O. For *ugt1a*, *ugt1a1*, *ugt1a3-9*, and *ugt1a6* Type 1 and 2, a Sybr  
20 Green PCR reaction was performed in a final volume of 20 µl, containing 1X Sybr Universal  
21 PCR Mastermix (Applied Biosystems, Barcelona, Spain), 0,25 µM of reverse and forward  
22 primers (Ecogen, Barcelona, Spain), 3 µl of cDNA and H<sub>2</sub>O. Either for the Taqman or the  
23 Sybr Green reactions, the adenosylphosphoribosyl transferase (*APRT*) for mRNA, was used to  
24 normalize the results. The primer sequences are listed on Additional Table 1. PCR cycling  
25 conditions were 2 min at 50°C, 10 min denaturation at 95 °C, followed by 40 cycles of 15 sec  
26 at 95 °C and 1 min at 60 °C. The mRNA amount of the target gene, normalized to *APRT*, was  
27 given by the  $\Delta\Delta C_T$  method, where  $C_T$  is the threshold cycle indicating the fractional cycle  
28 number at which the amount of amplified mRNA reached threshold.  
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## 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 ng 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 800g 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 9,000g 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-<sup>14</sup>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<sub>2</sub> with 6 µM [1-<sup>14</sup>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 10,000g. Supernatant

1 samples (100  $\mu$ l) were analyzed by HPLC coupled to on-line radioactivity detection according  
2 to Thibaut et al. [20].  $\alpha$ -Naphthol and  $\alpha$ -naphthol-glucuronide were quantified by integrating  
3 the area under the radioactive peak.  
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## 7 8 9 **2.8. Transfection.**

10 Cells were plated in 35-mm plates the day before transfection. aODNs were mixed with N-  
11 [1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Roche,  
12 Mannheim, Germany) 10  $\mu$ M for 15 min at RT before lipofecting the cells. siRNAs were  
13 mixed with 2  $\mu$ l of Metafectene (Biontex, Martinsried/Planegg, Germany), and plasmids with  
14 3  $\mu$ l of Fugene (Roche, Mannheim, Germany) in medium without serum nor antibiotics for 15  
15 min at RT. aODNs sequences were (Sigma-Aldrich, Madrid, Spain): UGT1A1 5'-  
16 GCcCTgggaCtCCaCagCCAT-3'; UGT1A3-5 5'- GGgaaCCTggagTcCTgTggCCAT -3'; and  
17 aCDK4 5'-ctcAtAtcGAGAGGtAGccaT-3' (the capital letters contained phosphorothioate  
18 linkages). siRNAs sequences were (Thermo, Barcelona, Spain): UGT1A6-A anti strand 5'-  
19 CAAGGAAGUUGGCCACUCGTT -3'; UGT1A6-B anti strand 5'-  
20 AAAUGAUUGGUAACGGUUCTT -3'; Luciferase sense strand 5'-  
21 UAAGGCUAUGAAGAGAUACTT-3; DHFR2-MIS anti strand 5'-  
22 AAUGAGGAGGUUGUGGAGGTT-3'. Plasmid pCMV-UGT1A6-1 (Type 1) was purchased  
23 from Origene (Rockville, Maryland).  
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## 49 **2.9. MTT assay.**

50 Cells (30,000) were plated in 35-mm plates in 1 ml of -GHT medium. Methotrexate was  
51 added 48 hours after transfection. However, it was supplied at the time of plating when  
52 performing experiments using SN-38 and Tamoxifen that were added 24 hours latter. Seven  
53 days after plated, 500  $\mu$ g of MTT (Sigma-Aldrich, Madrid, Spain) and 5.6 mg of succinate  
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(Sigma-Aldrich, Madrid, Spain) were added and allowed to react for 3 hours 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'-cacttgctagcTCAGCTCCTTCCTCCA ACTC-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  $2 \times 10^5$  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 FUGENET<sup>TM</sup>6 (Roche, Mannheim, Germany). Luciferase activity was assayed 30 h after transfection. Cell extracts were prepared by lysing the cells with 200  $\mu$ l of freshly diluted 1x Reporter Lysis Buffer (Promega Biotech Ibérica, Madrid, Spain). The lysate was centrifuged at 13,000g for 2 min to pellet the cell debris and the supernatants were transferred to a fresh tube. A 15- $\mu$ l aliquot of the extract was added to 15  $\mu$ l 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  $\mu$ l of the 1X 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  $\mu$ g 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'-tagccagtACGTGgtgagaa-3' (the capital letters stand for the transcription factor binding site). After incubation at 90°C for 5 minutes, 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 [ $\gamma$ - $^{32}$ 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  $\mu$ g 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 $\pm$ 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 2B MDA-MB).

#### 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 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 [ $1\text{-}^{14}\text{C}$ ]- $\alpha$ -naphthol-glucuronide formation, showed a clear increase in MCF7-R and MDA-MB-R cells, of 2-fold and 9-fold, respectively (Fig.2E).

### 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  $3\cdot 10^{-8}\text{M}$  MTX plus  $1\mu\text{g}$  of pCMV-UGT1A6-1 displayed a

1 significant increase in cell survival referred to cells treated with MTX alone, of 20% in MCF7  
2 (Fig.3A) and 48% in MDA-MB cells (Fig.3B). This rise in cell survival was specific of  
3 UGT1A6-1 as the empty vector did not cause any effect. The increase in UGT1A6-1 mRNA  
4 levels upon plasmid transfection was confirmed by RT-Real Time PCR (data not shown).  
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### 10 11 12 **3.6. Effect of MTX on UGT1A mRNA levels.**

13 We studied whether MTX was able to induce UGT1A gene expression. MCF7-S and MDA-  
14 MB-S cells were incubated for 24 hours with  $3 \cdot 10^{-8}$ M MTX, and then UGT1A mRNA levels  
15 were analysed by RT-Real Time-PCR. MCF7-S (Fig.4A) and MDA-MB-S (Fig.4B) showed a  
16 similar pattern of induction of the UGT1A family. In both cases, when measuring the mRNA  
17 levels of the UGT1A family as a pool (1A\*), an increase was observed, of 2-fold in MCF7-S  
18 MTX treated cells and 3.8-fold in MDA-MB-S. However, when determining the mRNA  
19 levels of each specific member of the UGT1A family upon MTX incubation, we observed  
20 two patterns of expression, one for the UGT1A1-6 cluster for which induction was the  
21 predominant response, and another for the UGT1A7-10 cluster for which inhibition was  
22 prevalent. Both cell lines had in common the increase in the expression of UGT1A1,  
23 UGT1A4 and UGT1A6, and the decrease in the expression of UGT1A8, UGT1A9 and  
24 UGT1A10. In MCF7-S and MDA-MB-S cells treated with MTX, UGT1A6 was the family  
25 member that presented the highest rise in expression, 2.4-fold for MCF7-S and 5.7-fold for  
26 MDA-MB-S.  
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### 51 **3.7. UGT1A activity upon MTX incubation.**

52 We analysed whether the induction that MTX exerted on UGT1A6 mRNA levels was  
53 translated into a higher glucuronidation activity. Since MDA-MB-S cells showed a greater  
54 response to MTX induction, they were selected for the enzymatic determination. Therefore,  
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these cells were incubated in –GHT medium for different periods of time with  $3 \cdot 10^{-8}$  M MTX.

As shown in figure 4C, a noticeable increase in the glucuronidation levels of [1- $^{14}$ C]- $\alpha$ -naphthol- was observed at 48 hours, despite an initial decrease at 24h.

### 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 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 (Fig.6A and 7A). Sensitive cells showed three retardation bands using the AhR/ARNT as well as ARNT probes. When these cells were treated with  $3 \cdot 10^{-8}$  M MTX for 24 hours, only the band with the lowest

1 mobility could be observed in the gel shift with a intensity higher than in untreated cells.  
2 Resistant cells showed this same retardation pattern of a unique shifted band that did not  
3 change when these cells were depleted of MTX for 24 hours. Supershift assays were  
4 performed to assess the specificity and identity of the retardation bands. When MCF7-S  
5 nuclear extracts were incubated with an AhR antibody, the three retardation bands  
6 disappeared, indicating that the antibody blocked the formation of the AhR/ARNT-probe  
7 complex (Fig.6B). MCF7-S nuclear extracts incubated with the ARNT antibody produced a  
8 super-shifted band due to the reduced mobility of the Ab-ARNT-probe complex, as well as  
9 the disappearance of the upper band of the three bands present in the gel-shift (Fig.7B).  
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### 24 **3.10. Influence of MTX in UGT1A metabolism of other drugs.**

25 Tamoxifen and the active metabolite of irinotecan, SN-38, are two drugs metabolized by  
26 UGT1A. The IC<sub>50</sub> for SN-38 was 0.75 nM in MDA-MB cells and 0.75 μM in MCF7 cells,  
27 the IC<sub>50</sub> for TMX was 0.35 μM in MCF7 cells, and the IC<sub>50</sub> for MTX was 6·10<sup>-8</sup>M in both  
28 cell lines. When MDA-MB-S or MCF7-S cells were incubated with increasing concentrations  
29 of SN-38, a noticeable cell death was observed (Fig.8A and 9A). However, cells incubated  
30 with SN-38 together with 2·10<sup>-8</sup>M MTX, showed a cell survival recovery of 22% with 0.01  
31 μM SN-38 in MDA-MB-S (Fig.8A), reaching a 43% at 0.025 μM SN-38 in MCF7-S  
32 (Fig.9A), probably due to SN-38 increased glucuronidation upon MTX incubation. MDA-  
33 MB-R and MCF7-R cells did not present significant changes in cell survival, when incubated  
34 with SN-38 and MTX 1·10<sup>-6</sup>M (Fig.8B and 9B). This effect could be explained by the fact  
35 that methotrexate resistant cells show already high levels of UGT1A6 regardless of the  
36 presence of MTX. Tamoxifen was assayed in MCF7 cells only as the mechanism of action of  
37 this drug needs ER+ cells (Fig.10). Similar to SN-38 behaviour, sensitive cells incubated with  
38 tamoxifen together with MTX had a higher cell survival than those incubated with tamoxifen  
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1 alone, reaching a 36% of cell survival recovery when incubated with 1 $\mu$ M tamoxifen  
2 (Fig.10A). Resistant cells did not show significant changes in cell survival due to the presence  
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4 or absence of MTX (Fig.10B). The vehicle of both drugs, DMSO, did not cause significant  
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6 changes in cell survival.  
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#### 4. DISCUSSION

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4 Drug resistance constitutes a drawback of cancer treatment. Microarray analysis of two  
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6 different breast cancer cell lines resistant to methotrexate, MCF7-R and MDA-MB-R, pointed  
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8 out UGT1A, as the only gene family in common between them. Additionally, it is worth  
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10 noting that ABCG2 MTX transporter was about 10-fold overexpressed in MCF7-R cells,  
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12 although no significant changes in ABCG2 expression were observed in MDA-MB-R.  
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19 Among the different members of UGT1A family, the cluster formed by UGT1A1 through 6,  
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21 and specifically UGT1A6, showed to be the main responsible for the rise of UGT1A  
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23 expression in breast cancer resistant cells. UGT1A6 is a major UGT in humans that mediates  
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25 glucuronidation [24] and is responsible for the metabolism of drugs, such as acetaminophen  
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27 [25] and valproate [26]; carcinogens, such as benzo(a)pyrene; and endogenous substrates,  
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29 including serotonin [27] and 5-hydroxytryptophol [28]. Glucuronidation is one of the main  
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31 phase II clearance mechanisms for drugs, dietary natural and chemical products as well as  
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33 environmental substances in humans. Glucuronidation increases water solubility of  
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35 compounds, enabling their elimination of the body via urine or bile acids.  
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40 UGT1A family could somehow contribute to MTX metabolism to a certain degree, as it  
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42 shares a phenolic structure common to other UGT1A substrates. This effect could be  
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44 hypothesized from the increased survival observed in sensitive cells treated with MTX when  
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46 UGT1A6 was overexpressed. However, no increase in sensitivity to MTX was observed when  
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48 a siRNA against UGT1A6 was used. Different aODNs towards the remaining members of the  
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50 UGT1A1-6 cluster were also tested, to evaluate whether another family member was  
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52 responsible for MTX metabolism, but increase in sensitivity was neither observed. The lack  
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54 of sensitization towards MTX when UGT1A6 was downregulated could be explained by the  
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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.

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,

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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-1 $\alpha$  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].



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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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**COMPETING INTERESTS**

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24 The authors declare that they have no competing interests.  
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**FOOTNOTES**

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## FIGURE LEGENDS

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5 **Figure 1. UGT1A mRNA levels.** Basal mRNA levels of the different members of the  
6 UGT1A family in sensitive or  $10^{-6}$ M MTX resistant cells. Prior to RT-Real Time PCR,  
7 mRNA was submitted to DNase treatment. Expression data of UGT1A family members are  
8 referred to UGT1A3 mRNA levels of sensitive cells, whose value was taken as 1. A) MCF7-S  
9 and MCF7-R. B) MDA-MB-S and MDA-MB-R. Data represent the mean  $\pm$  SEM of at least  
10 four experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$ , or \*\*\*  $p < 0.005$ .  
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23 **Figure 2. Type 1 and type 2 UGT1A6 mRNA levels, copy number and UGT activity.**  
24 Basal UGT1A6 mRNA levels of sensitive and  $10^{-6}$ M MTX resistant cells; A) MCF7 and B)  
25 MDA-MB. Prior to RT-Real Time PCR, mRNA was submitted to DNase treatment. Data are  
26 referred to sensitive cells and represent the mean  $\pm$  SEM of three experiments. C) and D)  
27 UGT1A6 and UGT1A4 copy number of sensitive and MTX resistant cells. C) MCF7 and D)  
28 MDA-MB. Data are referred to sensitive cells and represent the mean  $\pm$  SEM of five  
29 experiments. E) UGT1A activity. The S9 fraction of MDA-MB and MCF7 cells, either  
30 sensitive or resistant, was incubated with  $[1-^{14}\text{C}]\text{-}\alpha\text{-naphthol}$  and UGT1A activity was  
31 measured by HPLC. Data represent the mean  $\pm$  SEM of three experiments. \*  $p < 0.05$ , \*\*  $p <$   
32 0.01, or \*\*\*  $p < 0.005$ .  
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50 **Figure 3. Methotrexate cytotoxicity upon UGT1A6-1 plasmid transfection.** 30,000  
51 sensitive cells were incubated in -GHT medium. The following day, cells were transfected  
52 with the appropriated plasmid using Fugene. 48 hours after transfection, cells were treated  
53 with  $3 \cdot 10^{-8}$ M MTX. The empty vector pCMV was used as a negative control. Data represent  
54 the fold change referred to cells not treated with MTX. MTT assays were performed a week  
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1 after transfection. A) MCF7-S cells. B) MDA-MB-S cells. Data represent the mean  $\pm$  SEM of  
2 at least five experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$ , or \*\*\*  $p < 0.005$ .  
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7 **Figure 4. UGT1A mRNA and activity after MTX incubation.** A) and B) 500,000 sensitive  
8 cells were incubated for 24 hours with  $3 \cdot 10^{-8}$ M MTX. Total mRNA was obtained and  
9 submitted to DNase treatment prior to RT-Real Time PCR. Data represent the fold change  
10 referred to cells non treated with MTX. A) MCF7-S cells. B) MDA-MB-S cells. Data  
11 represent the mean  $\pm$  SEM of at least three experiments. C) UGT1A activity. MDA-MB  
12 sensitive cells were incubated for 24 and 48 hours with  $3 \cdot 10^{-8}$ M MTX. The microsomal  
13 fraction was obtained and UGT1A activity was analysed through [ $1-^{14}$ C]- $\alpha$ -naphthol  
14 glucuronidation. Data represent the mean  $\pm$  SEM of two experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  
15 or \*\*\*  $p < 0.005$ .  
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32 **Figure 5. Analysis of UGT1A6 promoter by luciferase activity.** 200,000 cells were  
33 incubated in -GHT medium. The following day, cells were transfected with the appropriated  
34 plasmid using Fugene and incubated either in the presence or in the absence of MTX. Thirty  
35 hours latter dual luciferase assays were performed. The control was the empty vector, pGI4.  
36 Renilla was used to normalise the data. A) MCF7 cells and B) MDA-MB cells. Data represent  
37 the mean  $\pm$  SEM of at least three experiments. \*  $p < 0.05$ .  
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49 **Figure 6. Binding of AhR to UGT1A6 promoter.** Cells were incubated with or without  
50 MTX for 24 hours prior to nuclear extracts harvest. Nuclear extracts were incubated with a  
51 radiolabelled probe corresponding to UGT1A6 AhR/ARNT promoter sequence. A) MCF7  
52 sensitive and resistant and MDA-MB sensitive and resistant. B) AhR supershift. Incubation of  
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1 MCF7-S nuclear extracts with AhR antibody. Shifted and supershifted bands are indicated by  
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7 **Figure 7. Binding of ARNT to UGT1A6 promoter.** Cells were incubated with or without  
8 MTX for 24 hours prior to nuclear extracts preparation. Nuclear extracts were incubated with  
9 a radiolabelled probe corresponding to UGT1A6 ARNT promoter sequence. A) MCF7  
10 sensitive and resistant and MDA-MB sensitive and resistant. B) ARNT supershift. Incubation  
11 of MCF7-S nuclear extracts with ARNT antibody. Shifted and supershifted bands are  
12 indicated by arrows.  
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24 **Figure 8. SN-38 cytotoxicity in the presence of MTX in MDA-MB cells.** 30,000 cells were  
25 incubated in –GHT medium either in the absence or in the presence of MTX. The following  
26 day, cells were incubated with the appropriated SN-38 concentration. The vehicle of SN-38,  
27 DMSO was tested alone at the concentrations used. MTT assays were performed a week after  
28 adding MTX. A) MDA-MB-S cells and B) MBA-MB-R cells. Data represent the mean  $\pm$   
29 SEM of four experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$ , or \*\*\*  $p < 0.005$ .  
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41 **Figure 9. SN-38 cytotoxicity in the presence of MTX in MCF7 cells.** A) MCF7-S cells and  
42 B) MCF7-R cells. Data represent the mean  $\pm$  SEM of four experiments. Other conditions as  
43 in Figure 8.  
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51 **Figure 10. Tamoxifen cytotoxicity in the presence of MTX in MCF7 cells.** 30,000 cells  
52 were incubated in –GHT medium in the absence or in the presence of MTX. The following  
53 day, cells were incubated with the appropriated tamoxifen concentration. The vehicle of  
54 tamoxifen, DMSO was tested alone at the concentrations used. A) MCF7-S cells and B)  
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MCF7-R cells. Data represent the mean  $\pm$  SEM of four experiments. Other conditions as in

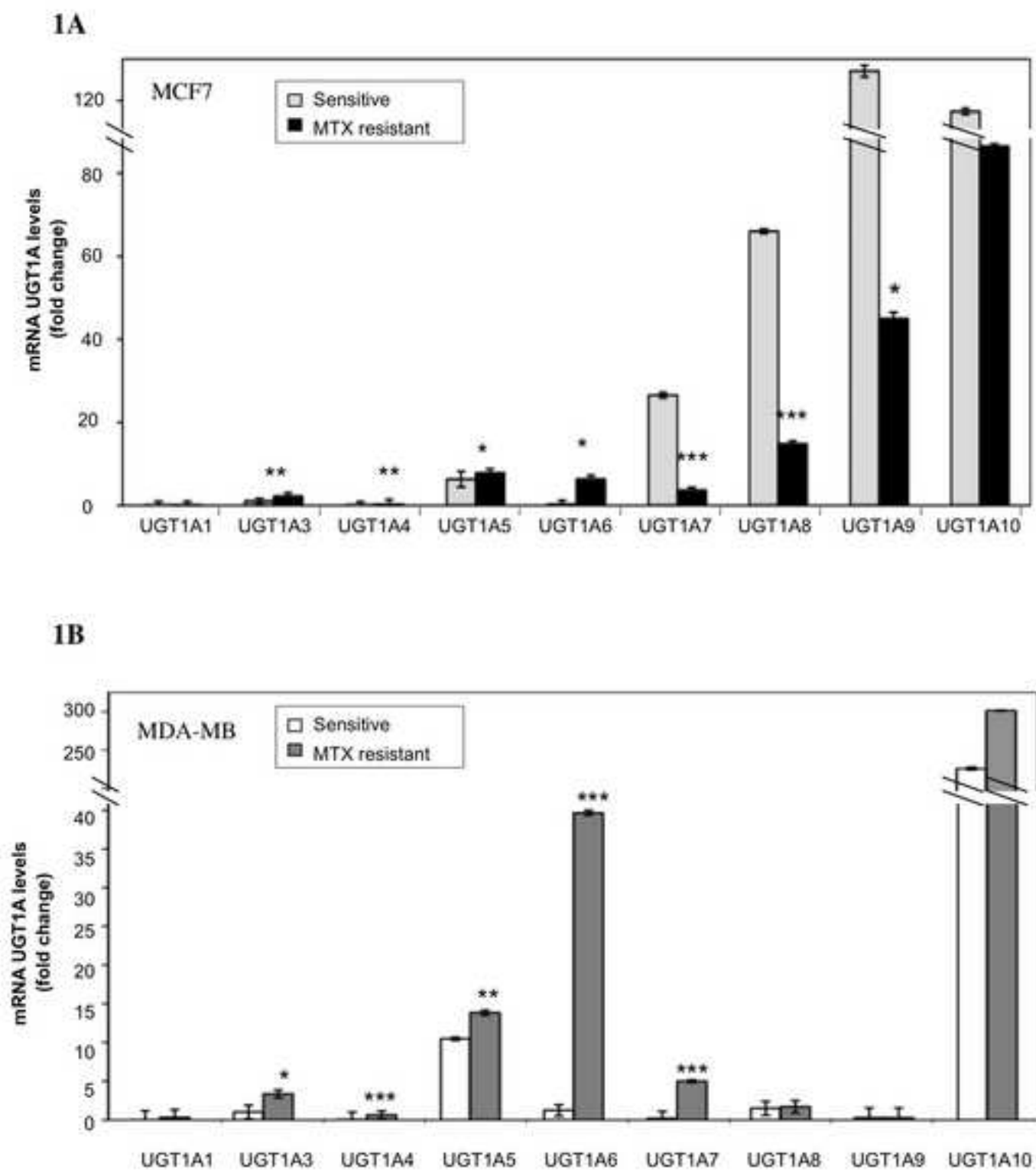
Figure 8.

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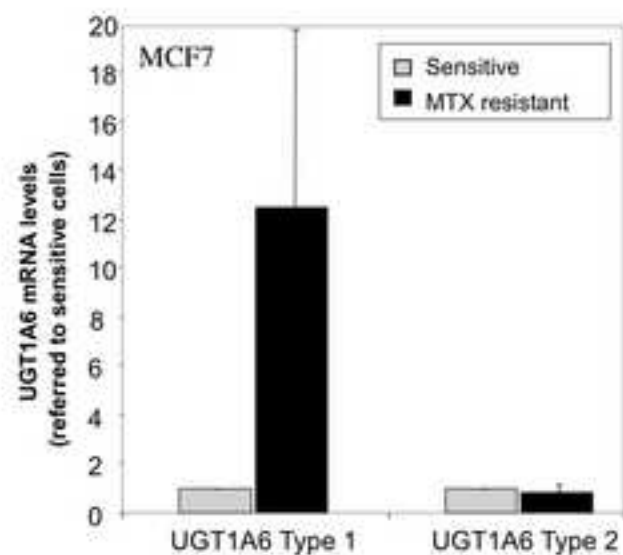
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Additional table 1. Sequences of the primers used in the study

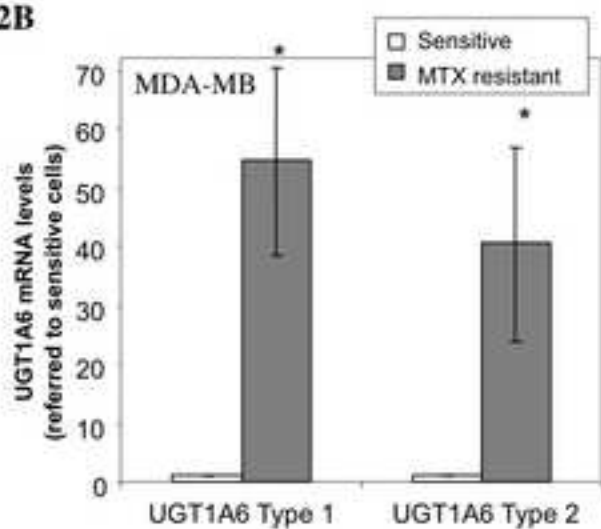
Primers	For	Rev
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<b>UGT1A1</b>	CATCAGAGACGGAGCATTTTACAC	GTCCGTCAGCATGACATCAAAG
<b>UGT1A3</b>	TAACAGACCCCGTTAACCTCTGC	AAAAGCATGGCAAATGTAGGACAG
<b>UGT1A4</b>	CTTCTGCTGAGATGGCCAGAG	CTGGGTCCATGGAACAGCATAG
<b>UGT1A5</b>	GGGAAGGTGCTGGTGGTG	TGTTTCAAAGAACGATTGAGTGTG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



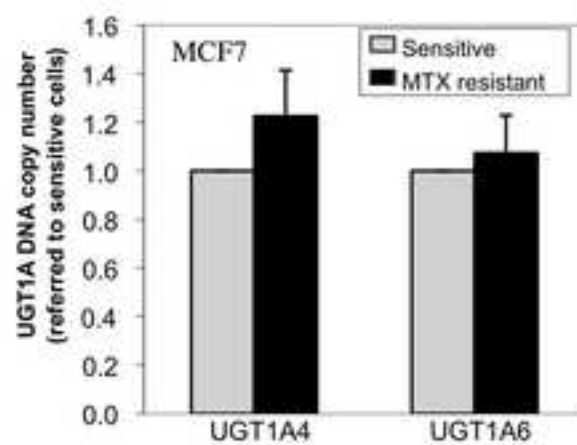
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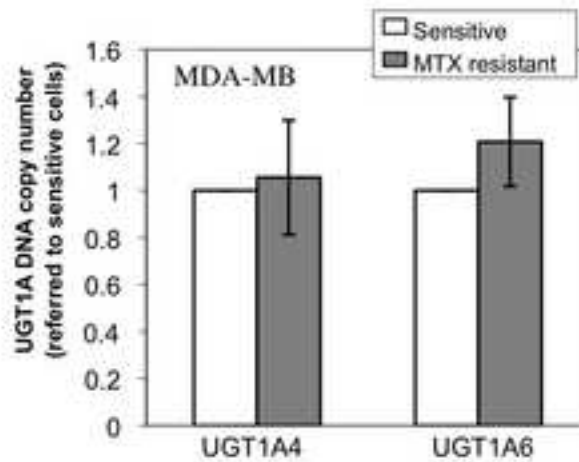
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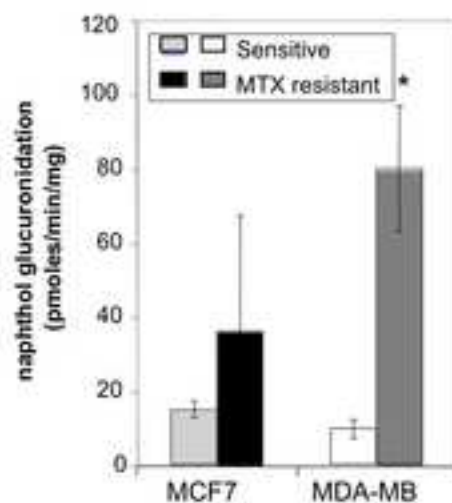
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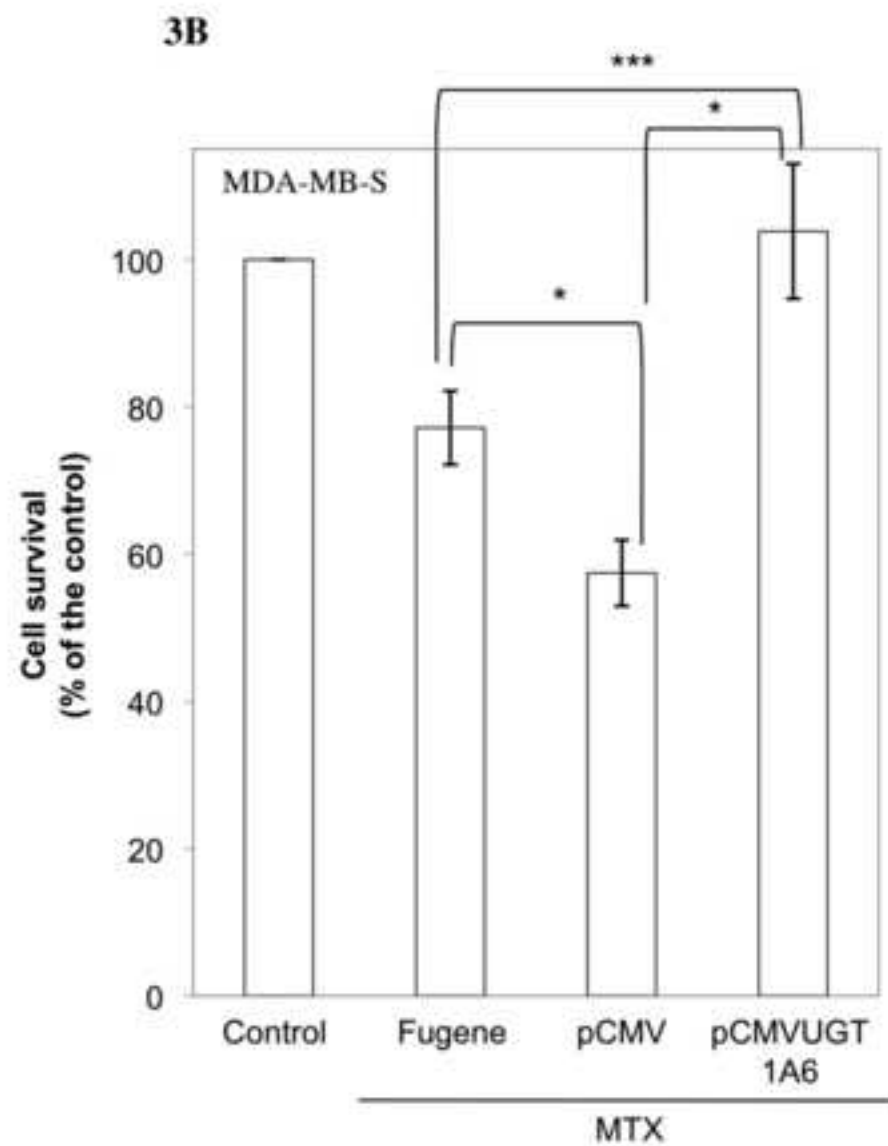
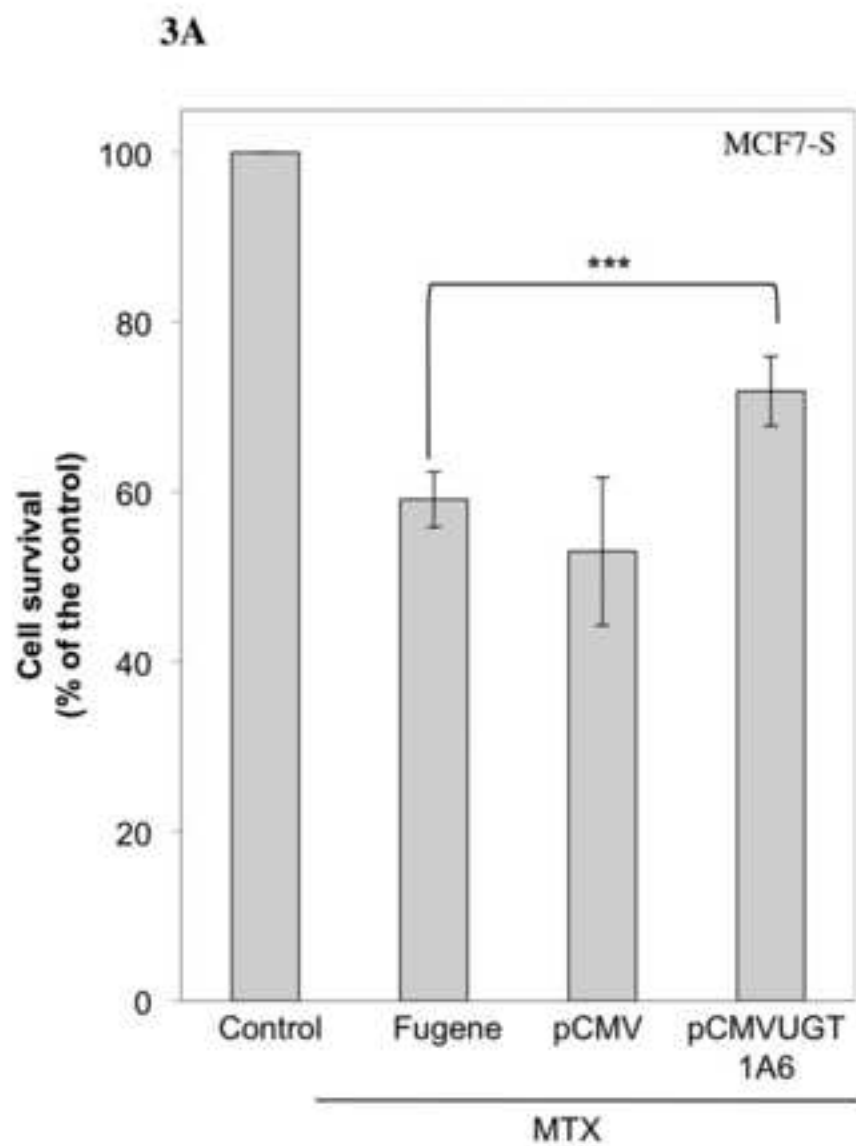


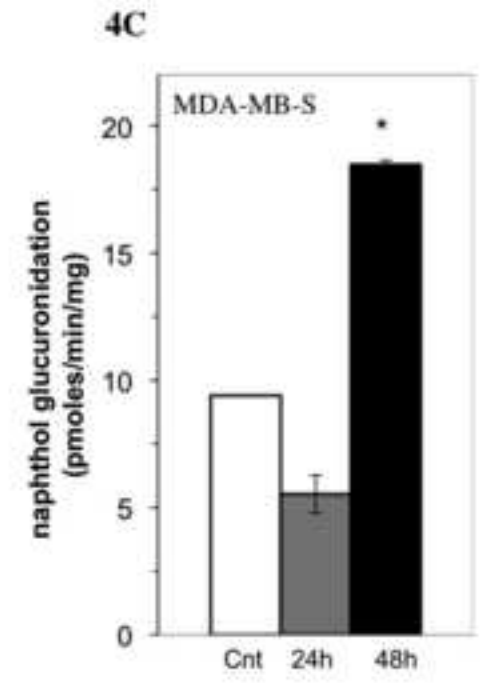
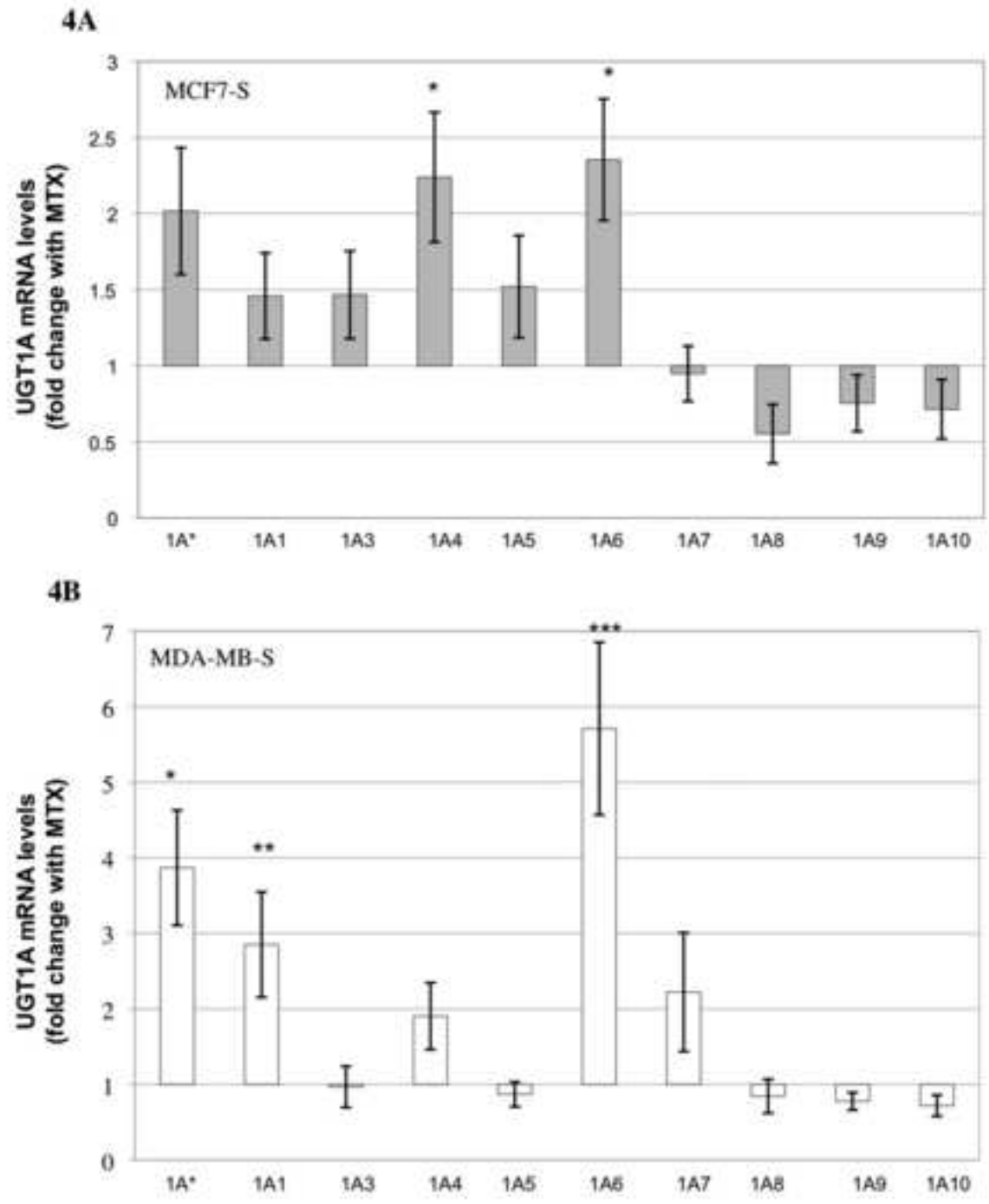
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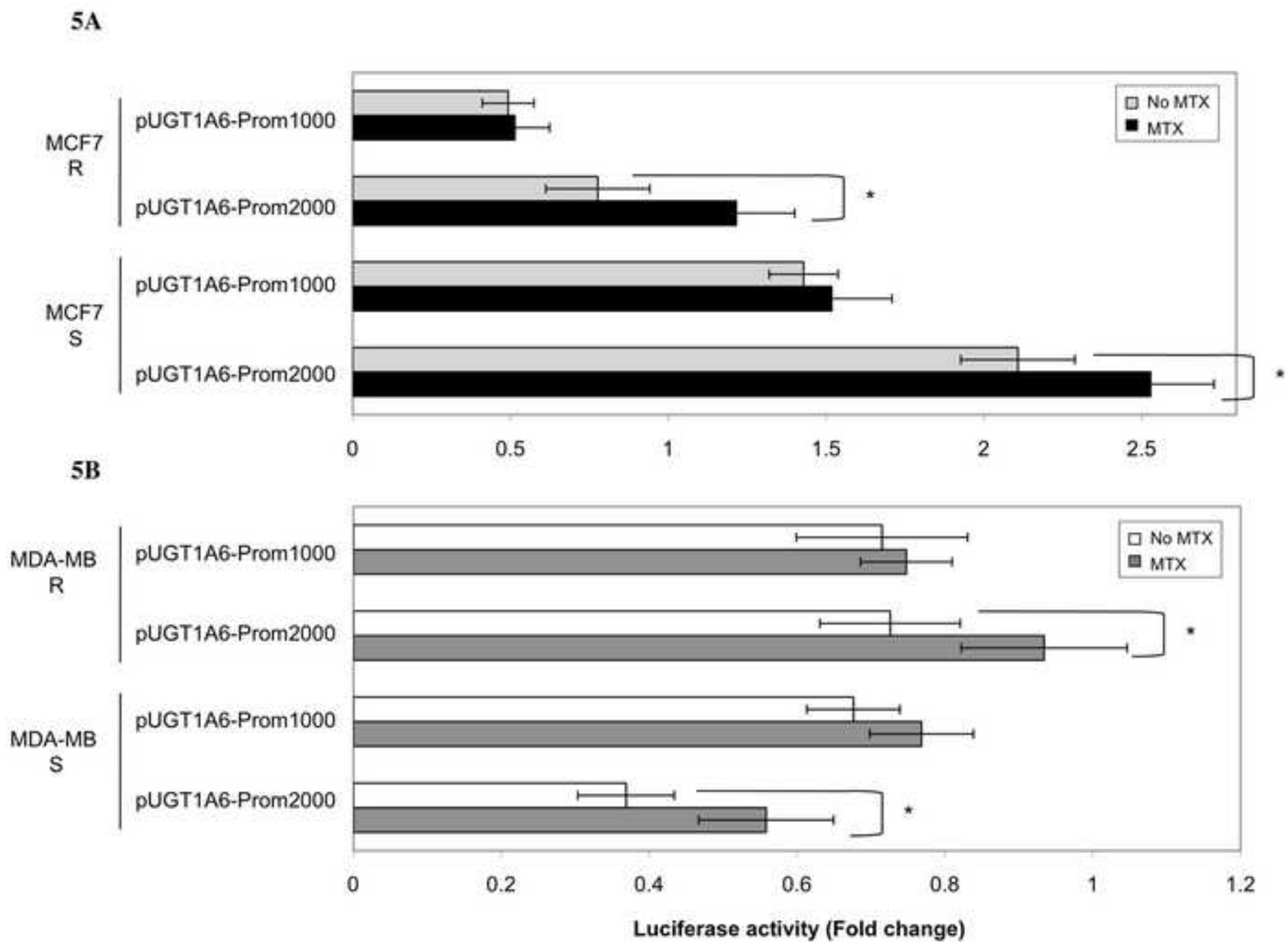


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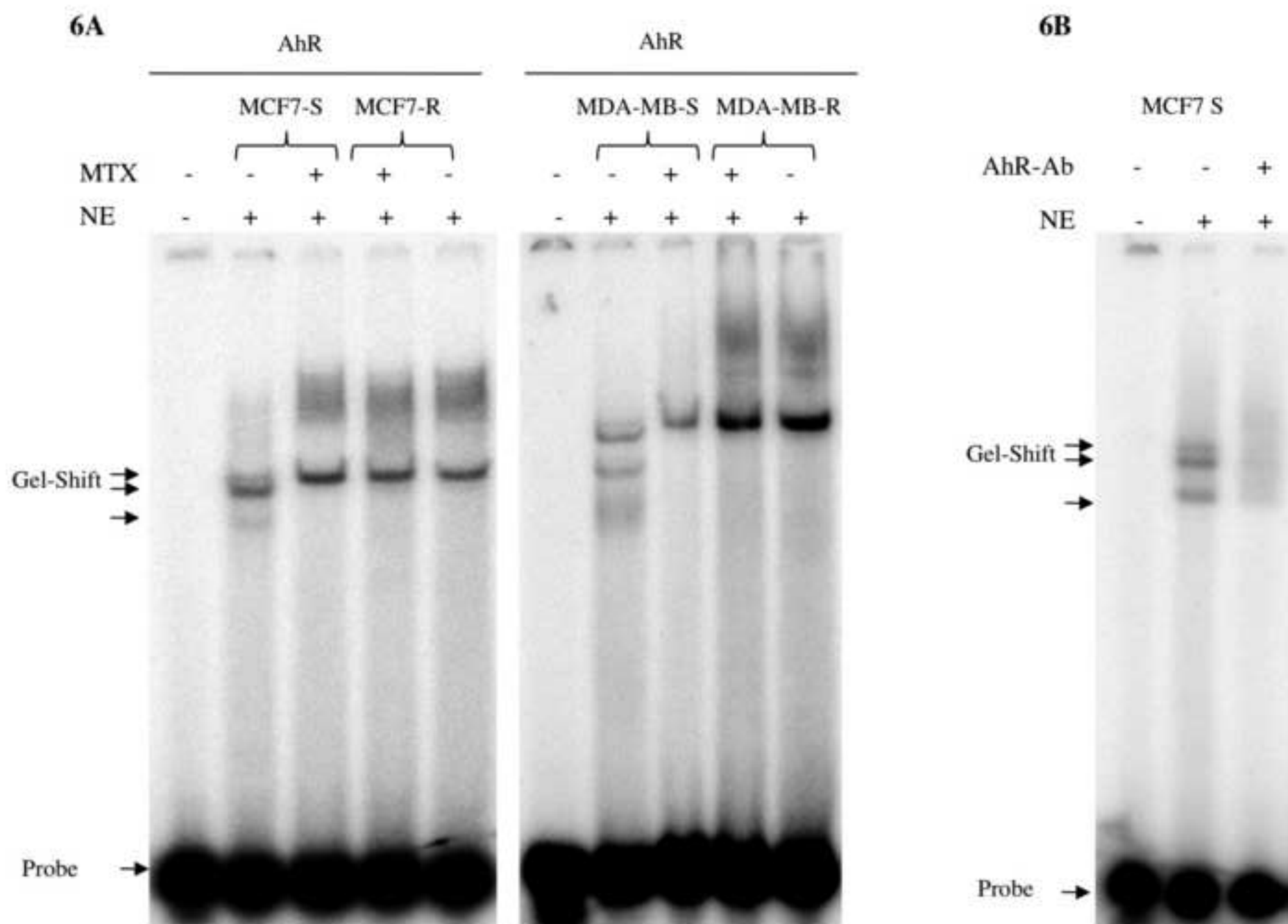




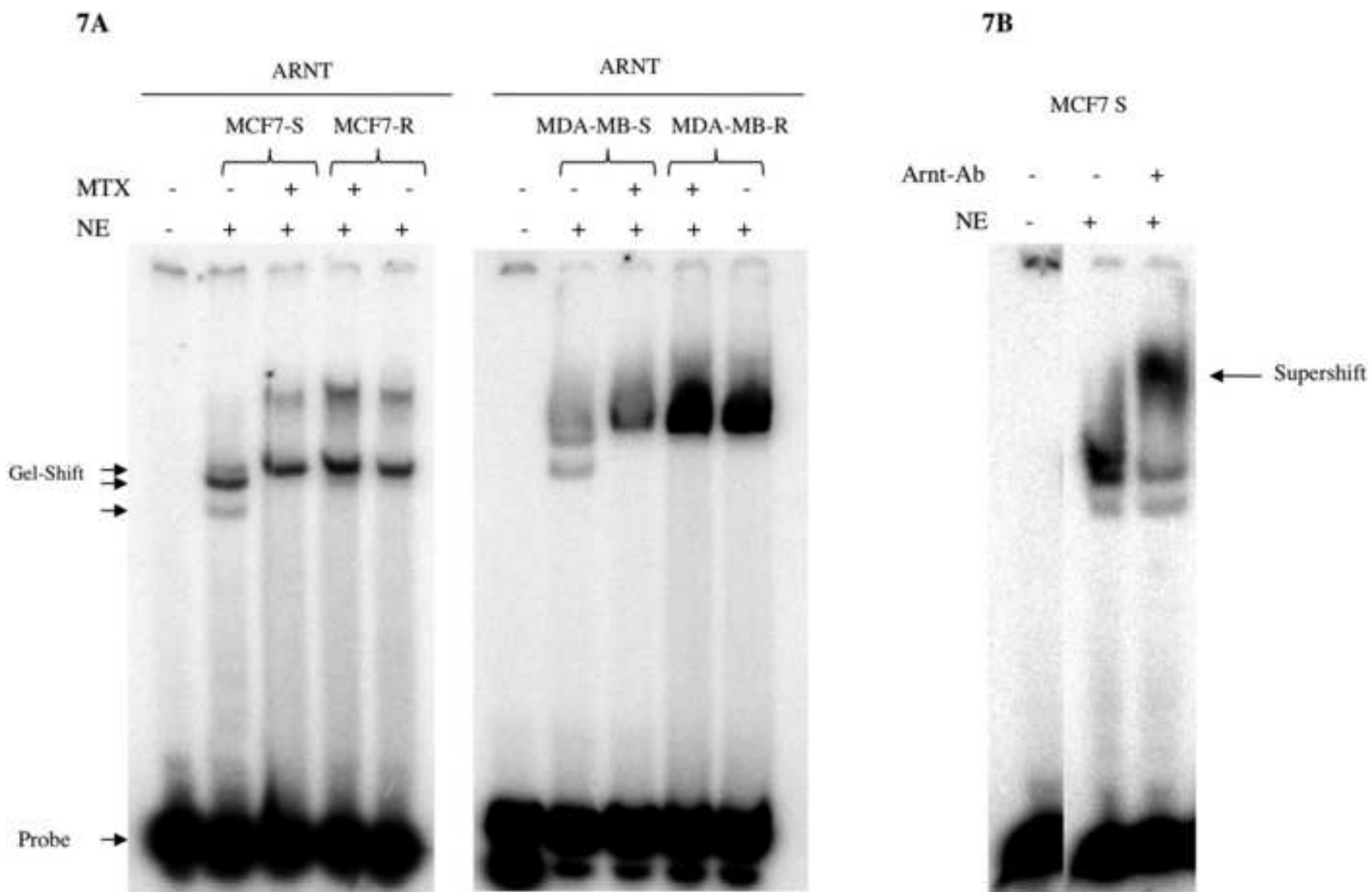


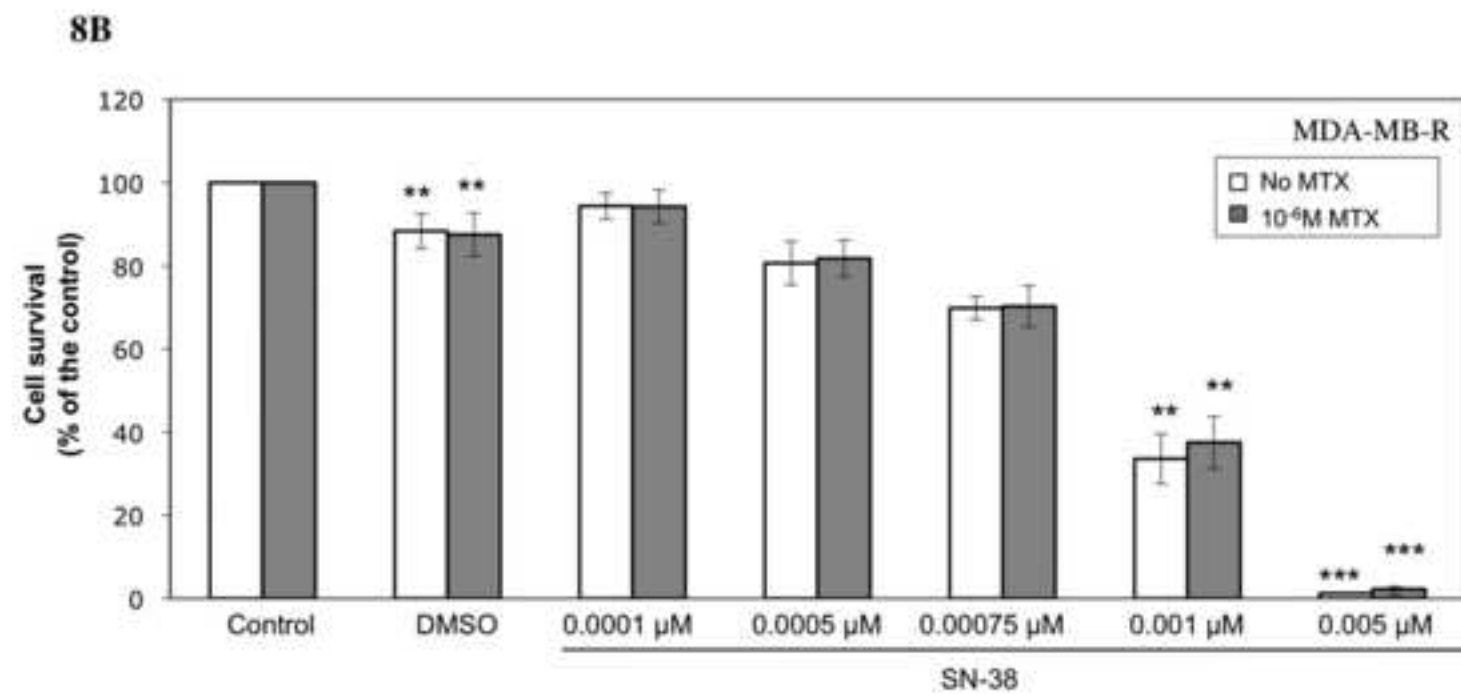
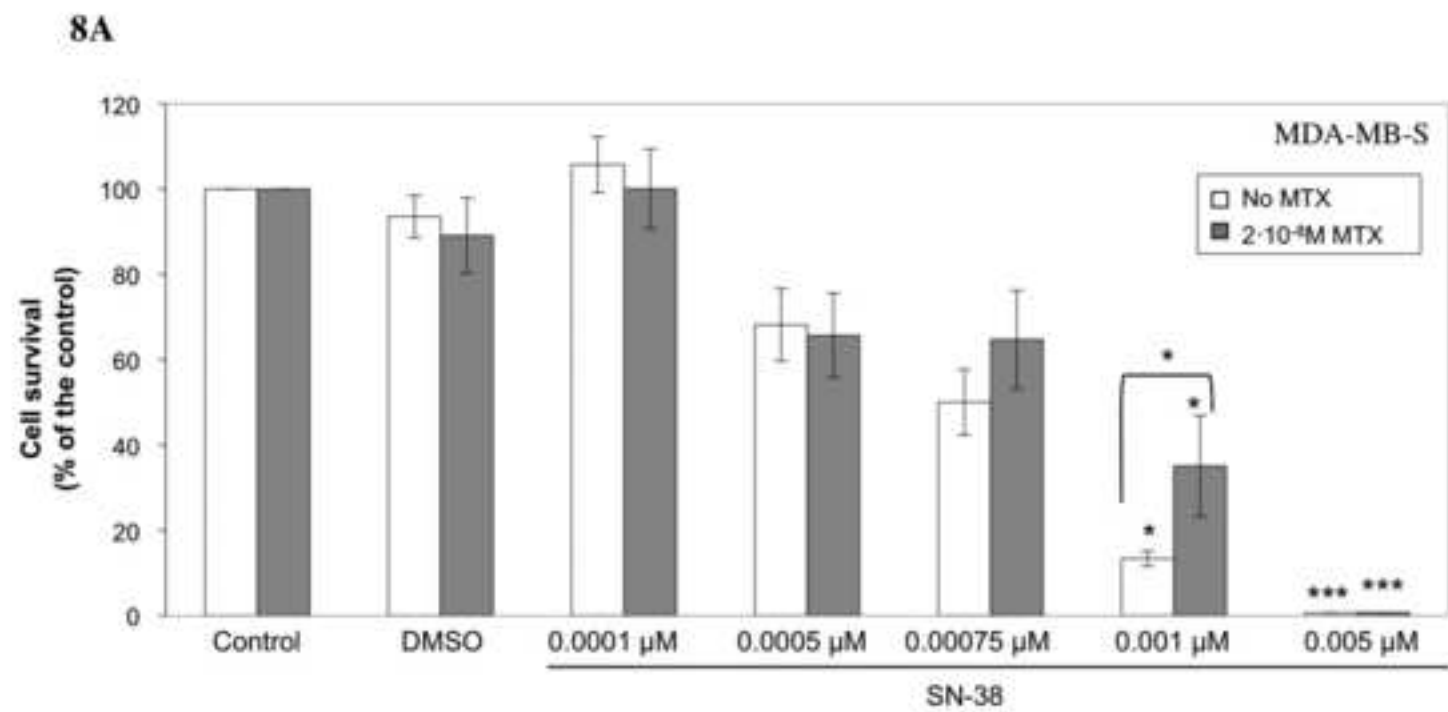


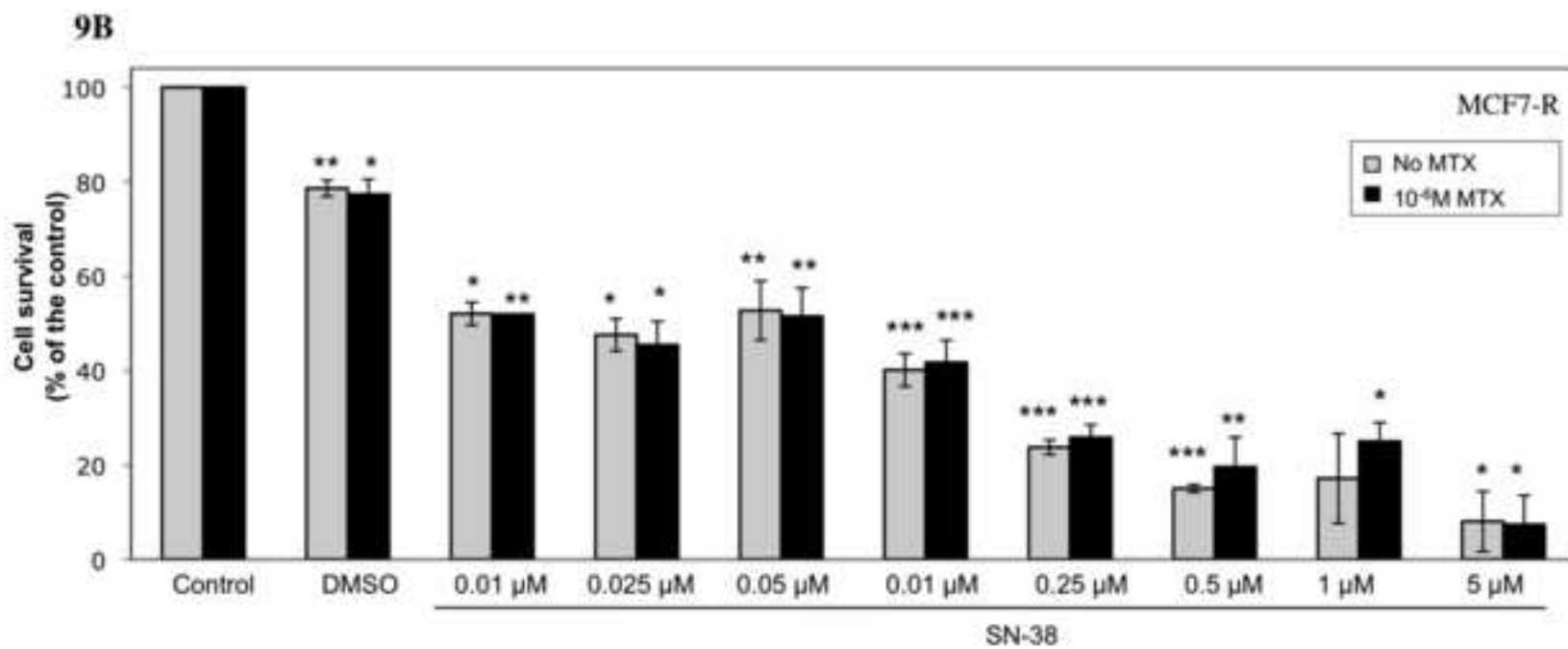
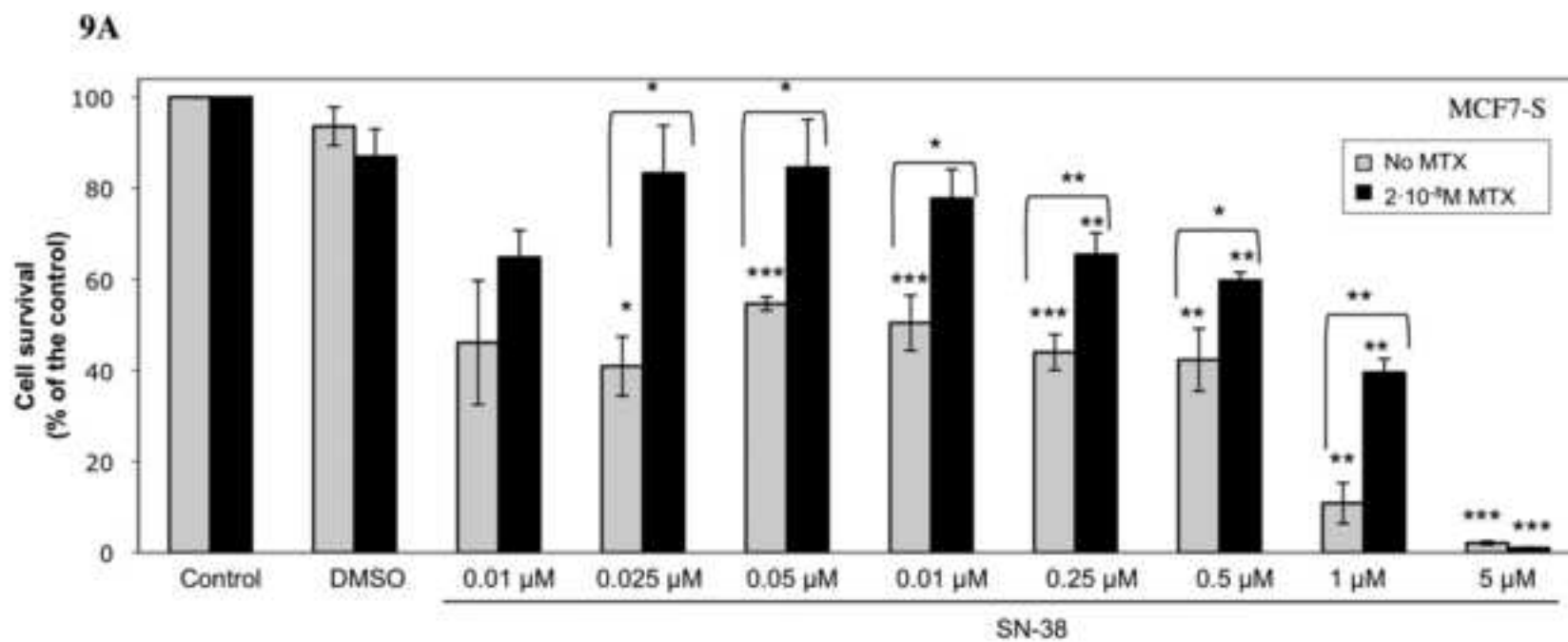


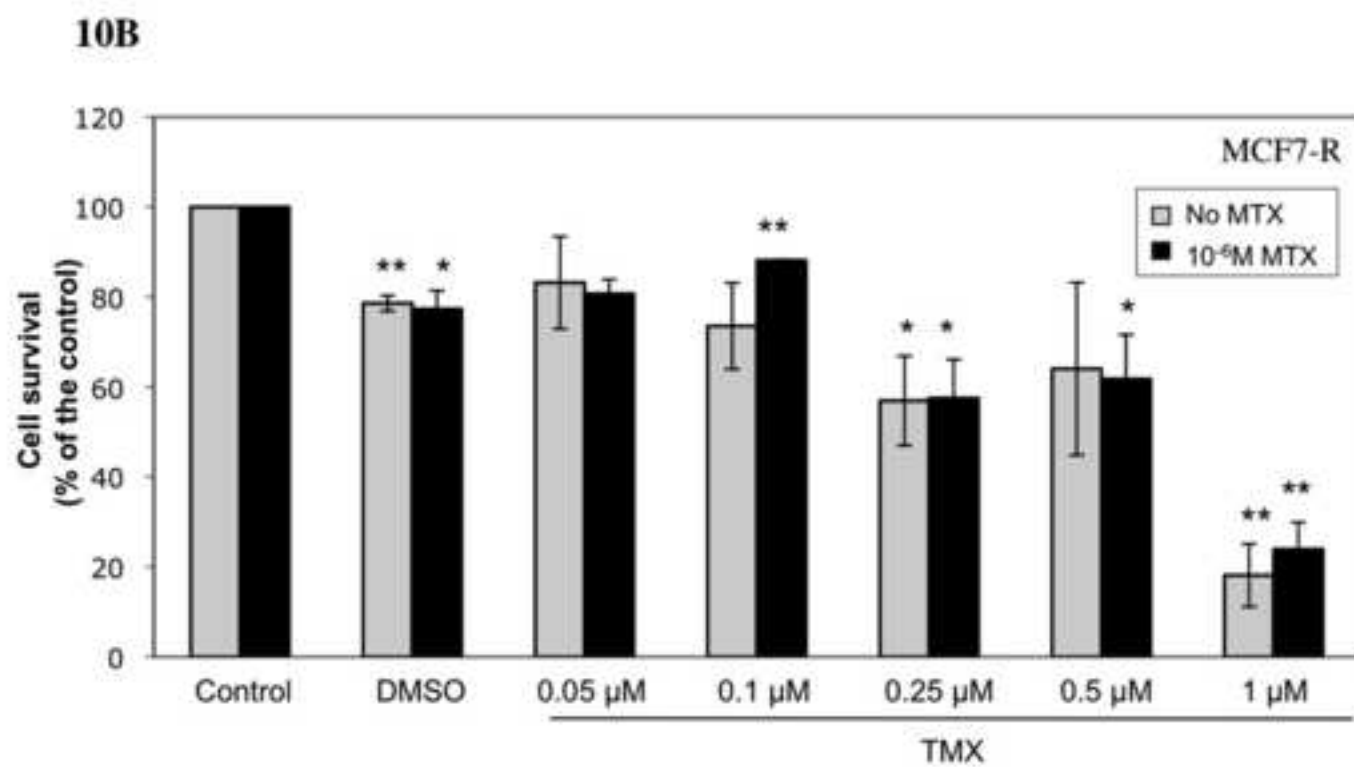
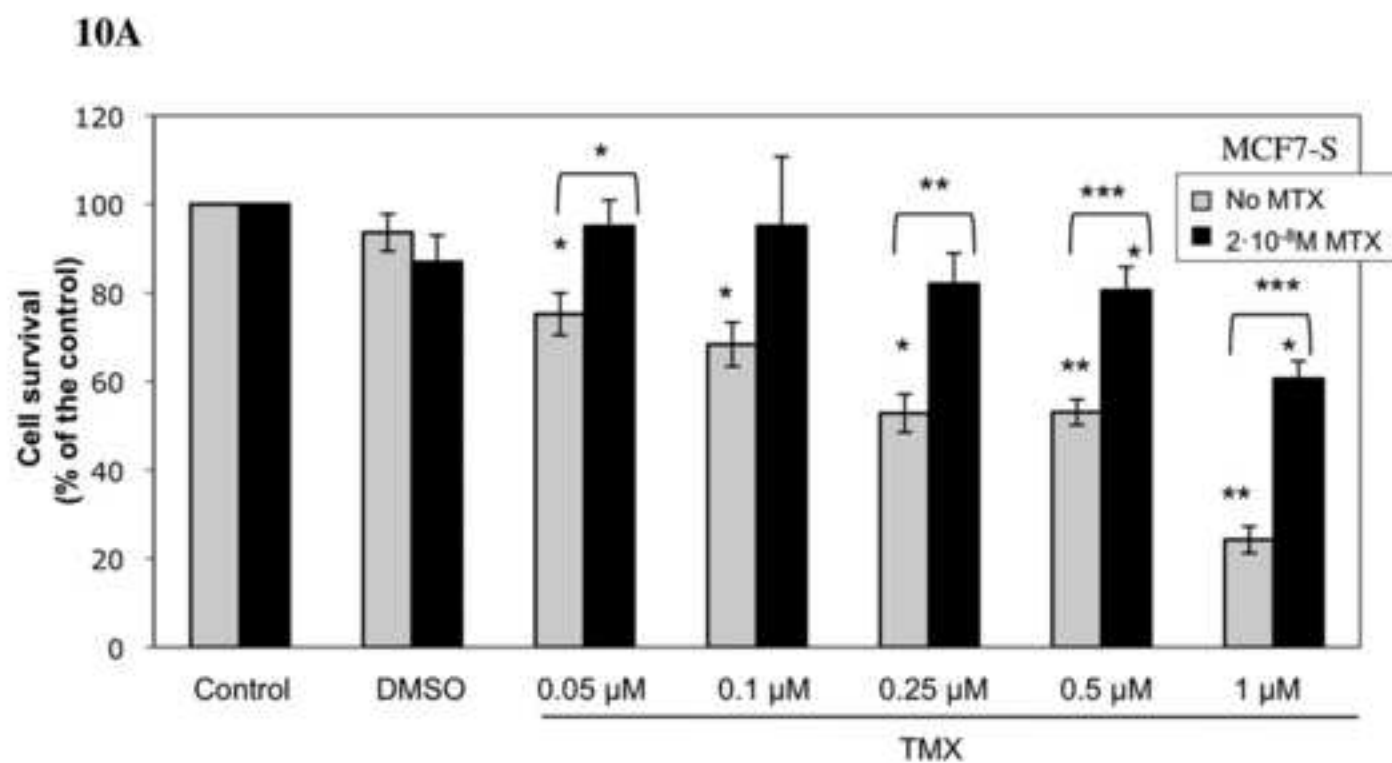


Script











### Induction of UGT1A mRNA and activity caused by methotrexate in breast cancer cells

