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1. Generation of biological association networks: A novel strategy to detect new targets in cancer therapy

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Abstract. The aim of this work was to design a novel strategy to detect new targets for anticancer treatments. The rationale was to build Biological Association Networks from differentially expressed genes in drug-resistant cells to identify important nodes within the Networks. These nodes may represent putative targets to attack in cancer therapy, as a way to destabilize the gene network developed by the resistant cells to escape from the drug pressure. As a model we used cells resistant to methotrexate (MTX), an inhibitor of DHFR. Selected node-genes were analyzed at the transcriptional level and from a genotypic point of view. In colon cancer cells, DHFR, the AKR1 family, PKCα, S100A4, DKK1, and CAV1 were overexpressed while E-cadherin was lost. In breast cancer cells, the UGT1A family was overexpressed, whereas EEF1A1 was

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overexpressed in pancreatic cells. Interference RNAs directed against these targets sensitized cells towards MTX.

Introduction

Cells regulate their functions by timely changing the expression of genes in a complex and precise way. This complexity cannot be explained by a collection of separate parts or pathways [1], but through larger networks, which are more informative and real [2]. Biochemical networks can be constructed at several levels and can represent different types of interactions. Literature mining allows the extraction of meaningful biological information from publications to generate networks [3] that, when built from properly curated interactions, can help to uncover the complete biochemical networks of the cells.

In this work, we used biological association networks (BANs) as a tool to define putative targets for gene therapy in combination with methotrexate (MTX). Basically, human cell lines resistant to MTX were developed by stepwise selection with this drug over a long period of time (0.5-1 year). Using total RNA from sensitive and resistant cells, expression microarrays were performed to obtain lists of differentially expressed genes. These lists were used as the input to build networks using the Pathway Architect software. This software confronts the genes in those lists with the information stored in Interaction Databases extracted from the literature using a Natural Language Processing (NLP) tool to construct novel views as to how the entities (the genes) in a list could be interacting with each other, even including entities not present in the original list.

Each generated network shows interplays of relationships among the differentially expressed genes and also includes highly connected nodes. Those gene-nodes support the infrastructure of the network developed in response to the sustained treatment with the drug responsible for the resistance. A novel strategy to select new targets to be used in cancer therapy is to concentrate on a variety of node-genes and test them, alone or in combination, for their effect when inhibited or overexpressed, on the final sensitivity to the main drug (MTX in this case).

The contribution to the resistance to MTX by some of the genes identified by this novel method were new to our knowledge (DKK1, UGT1A, EEF1A1) and some had already been the subject of study of our group since they appeared as differentially expressed in previous works of our team (DHFR, AKR1C1, SP1).

1. Identification of gene-nodes

1.1. Development of cell lines resistant to methotrexate

Cell lines representative of 5 types of human cancer were used: HT29 and Caco-2 of colon cancer, MCF-7 and MDA-MB-468 of breast cancer, MIA PaCa-2 of pancreatic cancer, K562 of erythroblastic leukemia, and Saos-2 of osteosarcoma. These cell lines are sensitive to MTX, with a IC_{50} of 16.7 nM MTX for HT29, 48.7 nM MTX for MDA-MB-468 and 11.6 nM MTX for MIA PaCa-2 cells. IC_{50} were calculated using GraphPad Prism 5 version 5.0a for Macintosh (GraphPad Software, San Diego, California, USA). Resistant cells were obtained in the laboratory upon incubation with stepwise concentrations of MTX (Lederle) as previously described [4]. HT29, Caco-2 and K562 resistant cells were able to grow in 10 μM MTX; MIA PaCa-2, Saos-2, MCF-7 and MDA-MB-246 were resistant to 1 μM MTX.

1.2. Microarray data analyses

Gene expression analyses were performed using three samples of both sensitive and resistant cells for each of the 7 cell lines studied. These analyses were carried out with the GeneSpring GX software v 7.3.1 (Agilent Technologies), using the latest gene annotations available (March 2009). This software package allows multi-filter comparisons using data from different experiments to perform the normalization, generation of restriction (filtered) lists and functional classifications of the differentially expressed genes. The expression of each gene was reported as the ratio of the value obtained for each condition relative to the control condition after normalization of the data. Additionally, a filter was performed by selecting the genes that displayed a p-value, corrected by false discovery rate (Benjamini and Hochberg FDR), of less than 0.05. The output of this analysis was then filtered by fold expression. Thus, lists of differentially expressed genes by 2-fold were generated for each of the 7 resistant cell lines.

1.3. Common genes between cell lines

The lists of genes differentially expressed by 2-fold with a p-value < 0.05 including multiple testing correction for each cell line were split in overexpressed and underexpressed genes. Lists of overexpressed genes were compared by using Venn Diagrams in GeneSpring GX. Lists of underexpressed genes were also compared among them using the same

approach. All lists were compared in pairs and lists of genes in common between each pair were generated.

1.4. BANs generation

Biological Association Networks (BANs) were constructed with the aid of Pathway Architect software v3.0 (Stratagene-Agilent). Briefly, this software package generates interaction networks starting with the genes in a given list (entities) taking into account the information present in a database of known molecular interactions. The lists correspond to the collection of differentially expressed genes under specific conditions. The database of molecular interactions is composed by millions of interactions divided into different classes (binding, regulation, promoter binding, transport, metabolism, protein metabolism and expression). The interactions are extracted from literature using a Natural Language Processing tool run on Medline Abstracts (NLP references), plus those obtained from external curated databases like BIND [5] and MINT [6]. Interactions in the interaction database are scored into 5 different categories: maximum, high, medium, low and minimum. Curated interactions (BIND and MINT sources) get the maximum quality scores as do any interactions that have at least 3 NLP references. Pathway Architect gathers all that information to construct novel views as to how the entities in a list could be interacting with each other, including entities not present in the original list (neighbours resulting from the expanded interaction). Customized analyses were performed to select relevance interaction networks with an associated high confidence index since such networks are likely to mirror biological significance. One-step expansion (expand network) of the original set of entities with maximum score interaction was then analyzed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation. This procedure gives a final view formed by a collection of nodes, with different degrees of interrelationship. On the other hand, a number of gene products from the original lists were not significantly connected with the other members or neighbours, and therefore were removed from the final view. Finally, overlapping of the expression levels to the members of the network was also performed.

When generating the BAN out of the list of the common genes differentially expressed in HT2929 and CaCo2 colon cancer cells, different nodes including DKK1, AKR1C and DHFR were detected (Fig. 1).

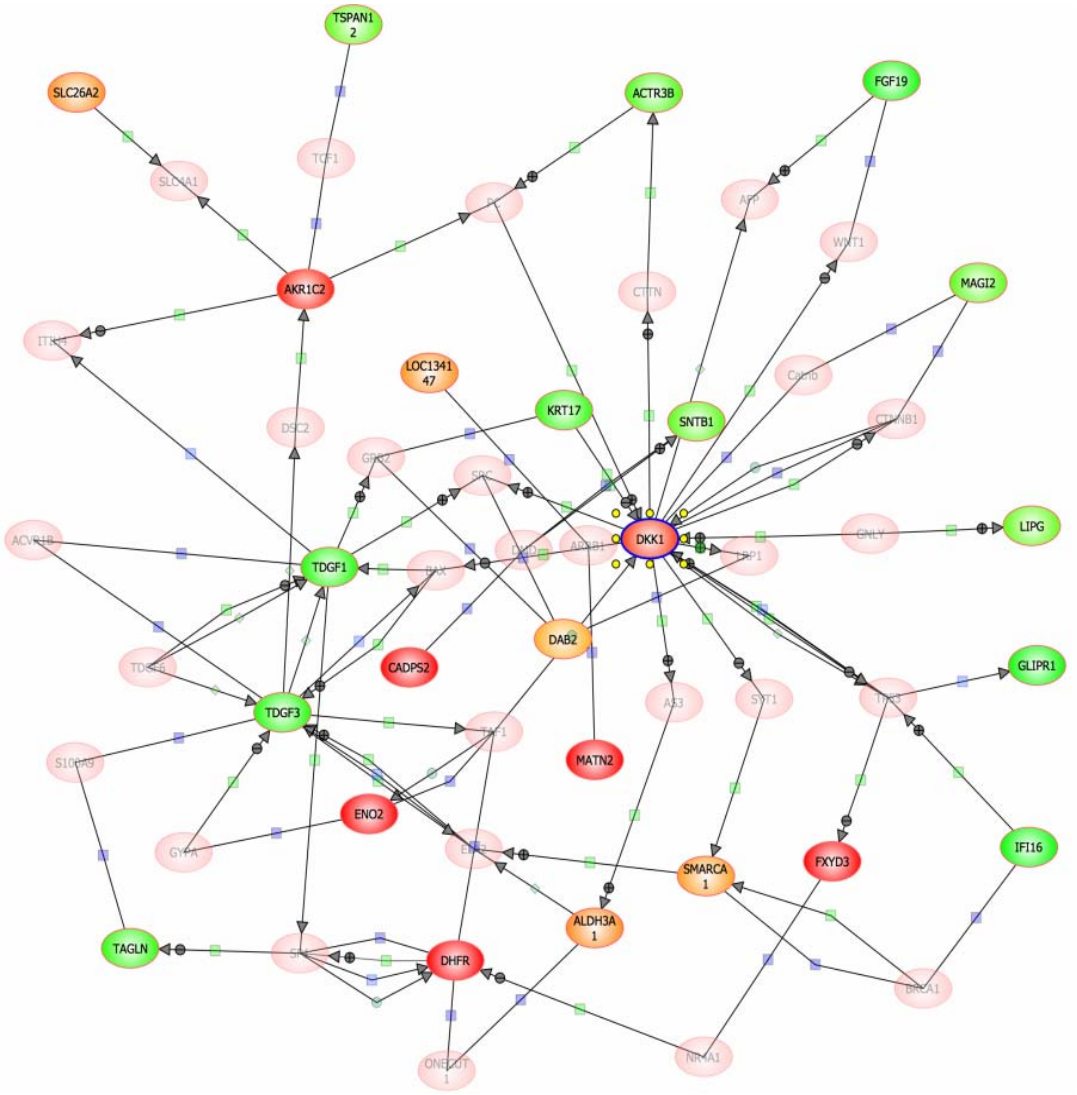


Figure 1. Network constructed with differentially expressed genes in common between HT29 and CaCo2 cells. Overexpressed genes are displayed in red while underexpressed genes are shown in green. Translucent shading represents genes that were not in the list and were added by the program from the interaction database.

When the BAN was generated from the list of differentially expressed genes in common between MCF7 and MDA-MB468 breast cancer cells, a main node including different members of the UGT1A family was formed (Fig. 2).

On the other hand, when the list came from the intersection of the differentially expressed genes in K562, MiaPaCa and SaOs2 cells, a node coresponding to EEF1A1 was obtained (Fig. 3).

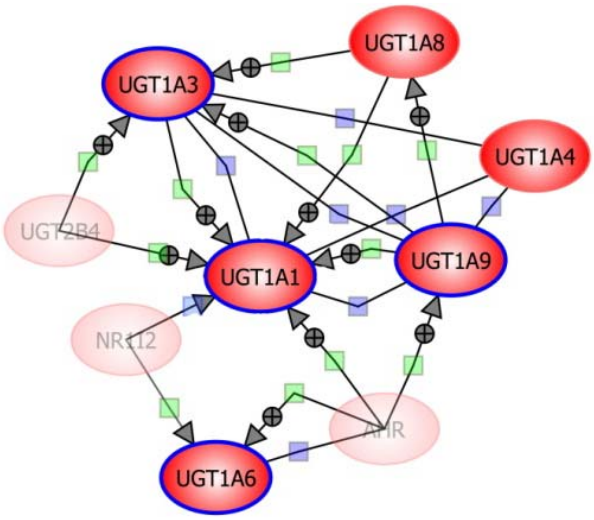


Figure 2. BAN generated using a list of differentially expressed genes in common between MCF7 and MDA-MB-468 breast cancer cells. Overexpressed genes are displayed in red while underexpressed genes are shown in green. Translucent shading represents genes that were not in the list and were added by the program from the interaction database.

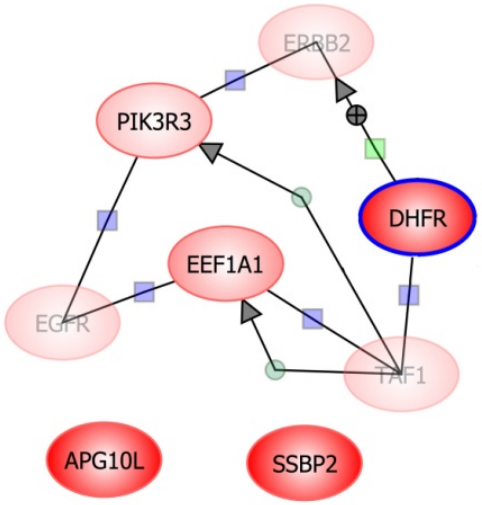


Figure 3. BAN generated using the differentially expressed genes among K562, MiaPaCa and SaOs2 cells. Overexpressed genes are displayed in red while underexpressed genes are shown in green. Translucent shading represents genes that were not in the list and were added by the program from the interaction database.

An additional BAN is presented which was generated with a list including all the entities from the three lists of differentially expressed genes (from colon cancer, breast cancer and among K562, MiaPaCa and SaOs2) (Fig. 4). In this case, DKK1, UGT1A1, EEF1A1 and AKR1C can be seen as

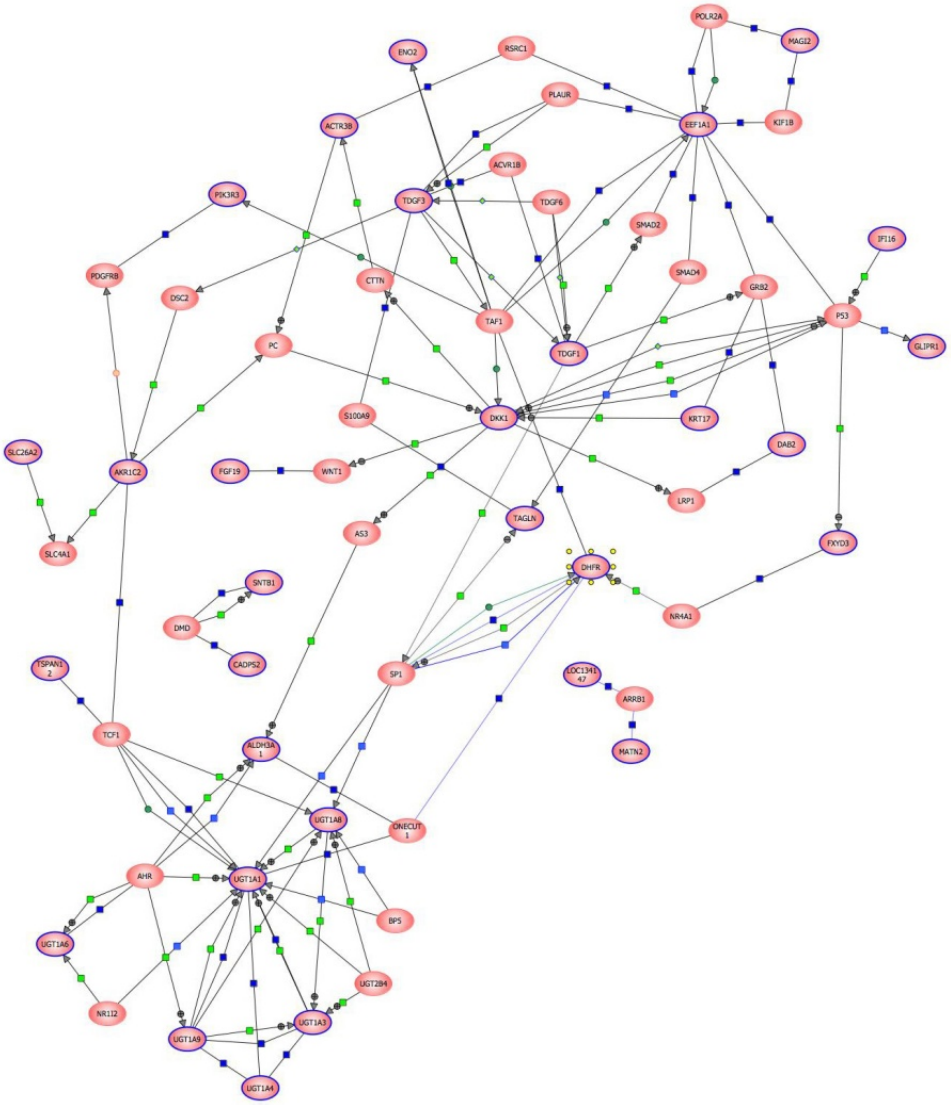


Figure 4. BAN generated using the list of the common differentially expressed genes in cells from colon cancer, breast cancer and among K562, MiaPaCa and SaOs2.

major nodes of the network, although other minor nodes can also be observed, such as Sp1.

The description of the regulation and characteristics of each target gene is given below considering the cell type and from the point of view of their contribution to resistance to the chemotherapeutic agent methotrexate.

2. MTX resistance in colon cancer

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. Colon

cancer causes 655,000 deaths worldwide per year [7]. Therapy is usually through surgery, followed in many cases by chemotherapy, which is used to slow tumor growth, to shrink tumor size and to reduce the likelihood of metastasis development. Chemotherapy effectiveness in colon cancer cells is usually compromised by the achievement of drug resistance. Therefore, gaining insight into the mechanisms underlying drug resistance is basic to develop more effective therapeutic approaches for colon cancer. With this goal in mind, we performed a genome wide analysis of two colon cancer cell lines, namely HT29 and CaCo-2. The expression profiles derived from these microarrays evidenced changes in a wide proportion of genes. We further studied some of these differentially expressed genes and proposed a role for all of them on MTX resistance in HT29 cells.

2.1. Role of DHFR on MTX resistance

MTX resistance can be easily acquired through different ways, although amplification of the target gene (Dihydrofolate reductase, *dhfr*) has been shown to be the most important mechanism of resistance in cultured cells [8].

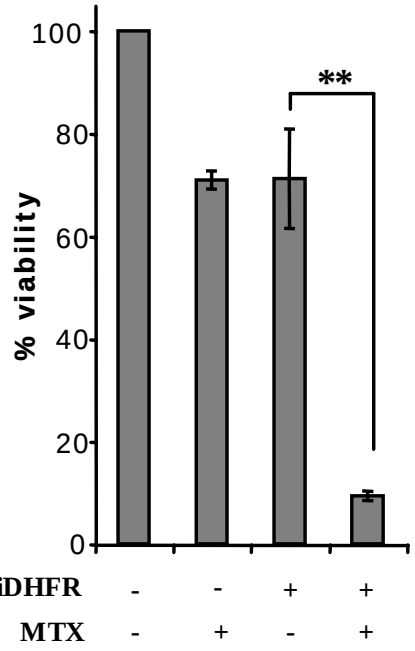


Figure 5. Effects of modulating DHFR mRNA levels on HT29 viability. Cells were treated with a siRNA against DHFR, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. ** $p<0.01$.

The human colon adenocarcinoma cell line HT29 can be adapted to grow in high concentrations of MTX and concomitantly develop amplification of the *dhfr* gene. We detected that DHFR was overexpressed by about 10 fold in HT29 resistant cells, and determined that the *dhfr* gene had undergone an amplification process of about 10-fold compared to the sensitive cells.

Moreover, use of iRNA technology caused a huge sensitization of HT29 cells toward MTX (Fig. 5), thus confirming a role for DHFR in MTX resistance. CaCo-2 MTX-resistant cells showed a 50-fold overexpression of DHFR, which originated from an increased gene copy number (about 80-fold) in the resistant cells.

2.2. Role of genes flanking DHFR on MTX resistance

Amplification of 5q12-14 regions, where *dhfr* is located, has been described in MTX-resistant HT29 cells [9]. A cluster of genes flanking the *dhfr locus* in chromosome 5 were overexpressed and amplified in MTX-resistant HT29 cells (Fig. 6). The genes included in this cluster are known to be involved in DNA repair, in cell signalling, in apoptosis prevention or in cell proliferation.

Despite the confirmation of the co-amplification of all these genes with *dhfr* in HT29 resistant cells, we did not observe a clear sensitization toward MTX when reducing their respective mRNA levels by means of iRNA technology. Our observations indicate that the increase in copy-number and the resulting upregulation of the studied genes in 5q14 may be a consequence of *dhfr* amplification more than an adaptation of the cells to MTX resistance. Indeed, many mammalian species (mouse, rat, bull, cock, dog and chimpanzee) show this set of genes in the same order around *dhfr* as in human chromosome 5 (using the MapViewer at NCBI), indicating a conserved pattern of gene organization. In keeping with this, its overexpression in the resistant cells could have been useful to improve some cellular processes that might facilitate survival. However, the increase in copy number of this set of genes does not directly favour MTX resistance.

2.3. Role of AKR1C1 on MTX resistance

Members of the Aldo-Keto Reductase (AKR) superfamily are monomeric cytoplasmic proteins of about 320 amino acid residues, which catalyze the NAD(P)H- dependent oxido-reduction of a wide range of substrates [10]. AKR1C family members' overexpression has been previously related to some types of cancer, and has been correlated with a poor prognostic outcome and

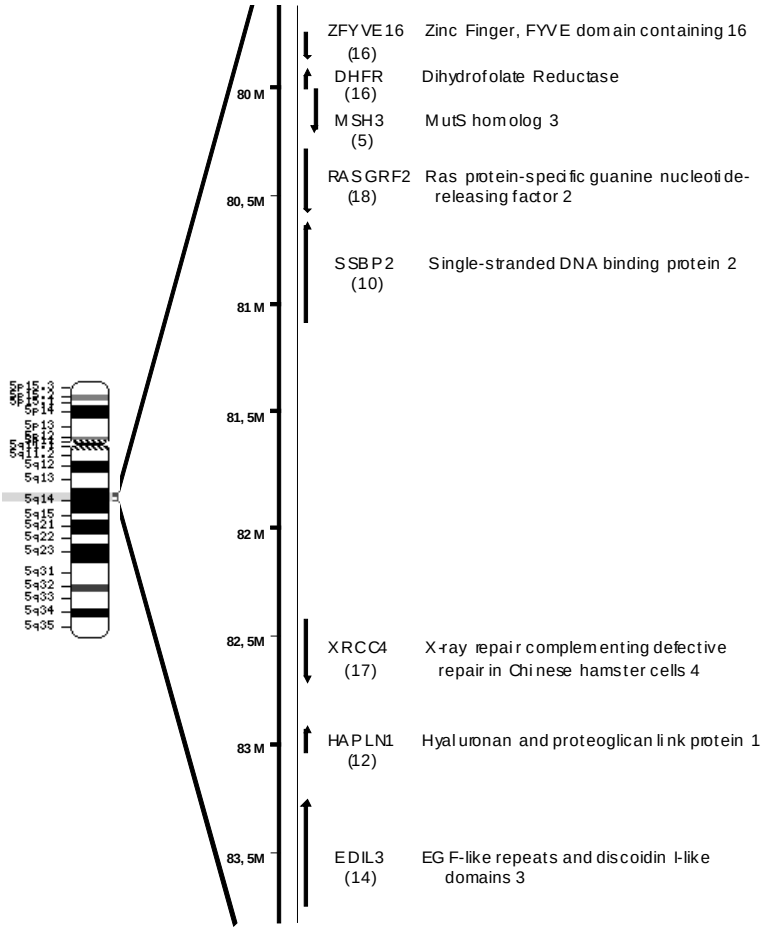


Figure 6. Localization of *dhfr* and other genes in chromosome 5 that are overexpressed in HT29 MTX-resistant cells. It is presented a magnification of the region in chromosome 5 where *dhfr* is located (5q14). The left part is an ideogram of chromosome 5; the right part shows the relative position of all genes studied that are located in this chromosome and that were amplified. The arrows indicate their transcription orientation and the values in parentheses under the names correspond to their respective copy-number validated by Real-Time PCR.

with disease progression [11]. We contributed to the field [4] by demonstrating an association between AKR1C1 expression levels and colon cancer. Indeed, this gene showed a 7-fold overexpression in HT29 colon cancer cells resistant to MTX. AKR1C1 upregulation has been previously related to drug (daunorubicin, cisplatin, adriamycin and ethacrynic acid)-resistance in a wide variety of cancers [12]. It has been suggested that AKR1C1 could metabolize some anticancer drugs since high similarity exists between drug chemical structures and some compounds that can be processed by this enzyme [11]. Thus, AKR1C1 would have a putative role in detoxification processes.

Our results showing an increased AKR1C1 activity in HT29 MTX-resistant cells (Fig. 7) are in agreement with this possibility. AKR1C1 would play a role on the detoxification of MTX in the resistant cells, a process that would have contributed to the establishment of drug resistance in this cell line.

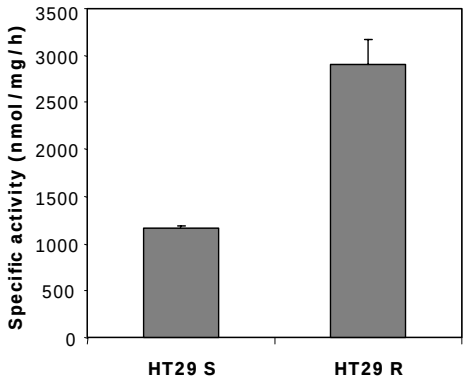


Figure 7. AKR1C specific activity. AKR1C activity was determined in protein extracts from both sensitive and MTX-resistant HT29 cells by measuring the change in absorbance at 340 nm, using 9,10-phenanthrenequinone as a substrate. To distinguish between unspecific and AKR1C specific activity, the AKR1C inhibitor flufenamic acid (FA) was added in the activity assay. The remaining activity upon FA inhibition was considered as non-AKR1C activity and was subtracted from the total activity. Results are shown as means ± SE from three independent experiments.

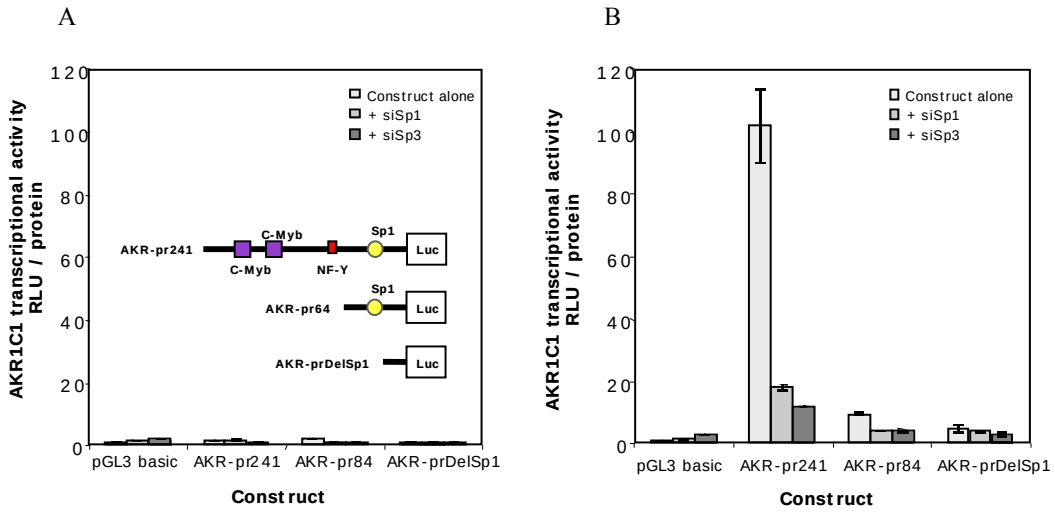


Figure 8. AKR1C1 transcriptional activity. HT29 sensitive (A) and resistant cells (B) were incubated with siRNAs against either Sp1 or Sp3. Twenty-four hours later, cells were transfected with one of the three different AKR1C1 promoter deletion constructs (see inset), and luciferase (Luc) activity (RLU, relative light units) was assayed after 30h. The protein content was used to normalize the Luc activity for each sample, which is expressed relative to that of pGL3 basic vector (means ± SE for triplicate wells).

It is of note that the increase in RNA and AKR1C1 protein in the resistant cells were at the same level, about 7-fold, which suggests that the overexpression is caused mainly by an increase in mRNA abundance rather than by a translational control. The increase in mRNA was not due to amplification of the *akr1c1 locus* but to transcriptional regulation. In this regard, we could conclude, from luciferase experiments (Fig. 8), that: i) there is a vast increase in transcriptional activity originating from the AKR1C1 promoter in the resistant cells compared to the sensitive cells; and ii) AKR1C1 transcriptional activation in the resistant but not in the sensitive cells depends at least in part on Sp1 and also Sp3. Gel-shift experiments (Fig. 9) also corroborate the role of Sp1 and Sp3 in regulating AKR1C1 in the resistant cells.

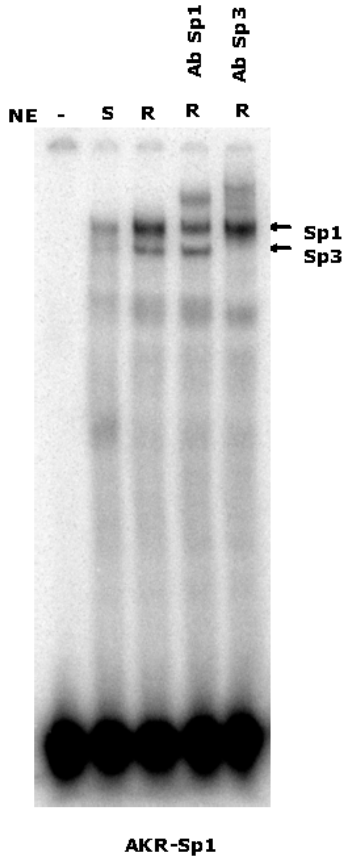


Figure 9. Characterization of Sp1 and Sp3 binding to AKR1C1 promoter. Binding reactions were performed with 20,000 cpm of each ds probe, 2 μg nuclear extracts (NE) from exponentially growing HT29 sensitive (S) or MTX-resistant (R) cells and 1 μg poly [d(I-C)] as the nonspecific competitor. Supershift mobility assays were performed in the presence of specific antibodies against either Sp1 or Sp3 (Ab Sp1 or Ab Sp3, respectively). Shifted and supershifted bands are indicated by arrows.

As there was no difference in the levels of Sp1 protein between resistant and sensitive cells, we explored the phosphorylation status of this transcription factor in the two types of cells, taking into account that phosphorylated Sp1 shows increased binding compared to the non-phosphorylated form [13]. Indeed, the increase in Sp1 binding in the resistant condition could be due to a higher phosphorylation state of Sp1 as dephosphorylation of the extracts coursed with a decrease in its binding. In this direction, we searched for the differential expression, in the microarray data, of those kinases and phosphatases known to control the phosphorylation state of Sp1 [14]. Within the kinases, PKC alpha was overexpressed. The role of protein kinase C alpha (PKCα) on MTX resistance in HT29 cells is discussed below.

Trying to mimic the overexpression of AKR1C1 present in the resistant cells, we transiently transfected an expression vector for this gene into HT29 sensitive cells, which resulted in a decrease in sensitivity toward methotrexate. We also decreased AKR1C1 mRNA levels by means of iRNA technology, and observed an increase in sensitivity toward MTX. These results supported the idea that the overexpression of AKR1C1 in the resistant cells may contribute, at least partially, to the resistant phenotype.

A relationship between AKR and proliferation has been reported [15]. We demonstrated that AKR1C1 overexpression counteracts the cell cycle S-phase arrest caused by MTX (Fig. 10A), suggesting that AKR1C1 activity may be needed for the cells to progress throughout the cell cycle. This could be a strategy, concomitant with the amplification of the *dhfr locus*, for the resistant cells to bypass the metabolic pressure exerted by MTX on nucleotide synthesis. Since AKR1C1 is functionally involved in the metabolism of xenobiotics, its activity might be acting either on MTX directly or on one of its metabolites to decrease the concentration of a DHFR inhibitor. In addition, AKR activity inversely correlates with apoptosis [16] and has been proposed to lead to apoptosis-related development of drug resistance. Our results are in keeping with this possibility since overexpression of AKR1C1 counteracts the apoptosis induced by MTX (Fig. 10B).

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

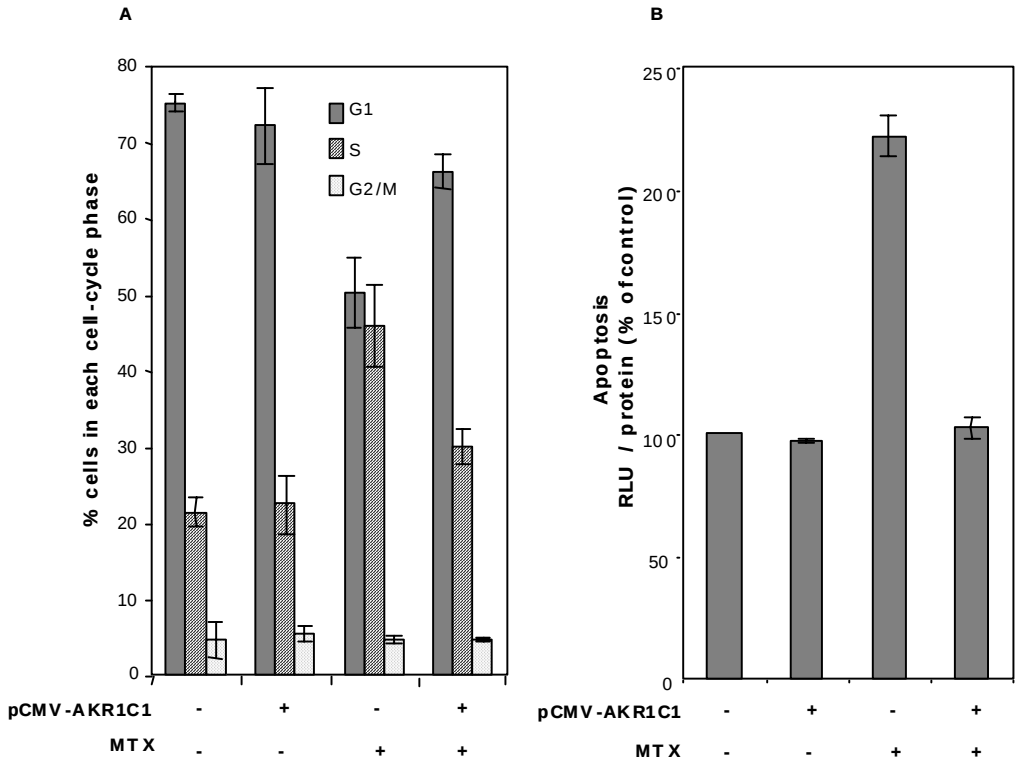


Figure 10. Changes in the cell cycle and in the apoptosis caused by AKR1C1 overexpression. A) Changes in cell cycle distribution. AKR1C1 was transiently overexpressed by means of an expression vector (pCMV6-XL5-AKR1C1). MTX was added 1 hour after plasmid treatment. After forty-eight hours of drug incubation, cells were collected, stained with propidium iodide and analyzed for DNA content using flow cytometry. Results are shown as means ± SE from at least three independent experiments. B) Changes in apoptosis. Overexpression of AKR1C1 was performed by transfecting its expression plasmid, either alone or in combination with MTX, for 24 hours in HT29 sensitive cells. MTX was added during the last six hours of treatment. Caspase 3/7 activation was finally measured and expressed as percentage of relative light units (RLU) referred to the control and normalized by protein content (means ± SE from at least three independent experiments).

2.4. Role of PKCα on MTX resistance

PKCα-mediated protein phosphorylation triggers a wide variety of cellular responses including proliferation, differentiation, membrane transport, gene expression and tumor promotion [17]. Chemical inhibitors of PKC activity have been proposed as resistance modulators in MTX chemotherapy [18]. Furthermore, decreasing PKCα mRNA levels attenuates the MDR phenotype in tumor cells and increases the sensitivity to anticancer drugs, both *in vitro* and *in vivo*. These observations are in accordance with our results [19]

showing that the decrease of PKCα mRNA levels by means of iRNA technology causes a sensitization of the cells toward MTX (Fig. 11A). It is interesting to note that this kinase has already been related to MTX resistance through a mechanism involving transcription factor Sp1 [13]. In HT29 resistant cells, this relation could be explained by the regulation of AKR1C1 transcriptional activity through Sp1 (see above).

2.5. Role of CAV1 on MTX resistance

Caveolin 1 (CAV1), the principal component of *caveolae*, has been associated with progression of colon and breast carcinomas and with enhanced invasiveness in lung adenocarcinoma cells [20]. Although suggested as tumor suppressor gene, and downregulated in some oncogene-transformed and tumor-derived cells [21], overexpression of CAV1 has been found in prostate and esophageal cancer [22]. Moreover, re-expression of CAV1 at latter stages of tumor development has been described in human and mouse prostate adenocarcinomas, a scenario that could resemble chemotherapy resistance. Indeed, Bender *et al.* [23] found significantly higher levels of CAV1 in MTX resistant HT29 clones. Our experiments using iRNA technology against CAV1 (Fig. 11B) allowed us to confirm the implication of this gene in MTX resistance in our HT29 cell line.

2.6. Role of E-cadherin on MTX resistance

Loss of E-cadherin, frequently observed in epithelial tumors, has been associated with tumor progression and is considered a crucial event that favours metastasis and invasiveness [24]. There is a functional correlation between E-cadherin levels and malignancy. Our experiments show a decrease of 3-fold in E-cadherin levels in resistant cells, and also that a mild overexpression of E-cadherin causes a higher sensitivity toward MTX (Fig. 11C).

2.7. Role of S100A4 on MTX resistance

S100A4 is a member of the S100 calcium binding protein family. S100A4 is involved in a wide variety of intra- and extracellular processes, such as cell cycle progression, cell motility and as a modulator of intercellular adhesion and of the invasive properties of cells [25]. Overexpression of S100A4 has been associated with tumor malignancy as well as to metastasis, angiogenesis and chemoresistance [26]. A correlation between S100A4 expression levels

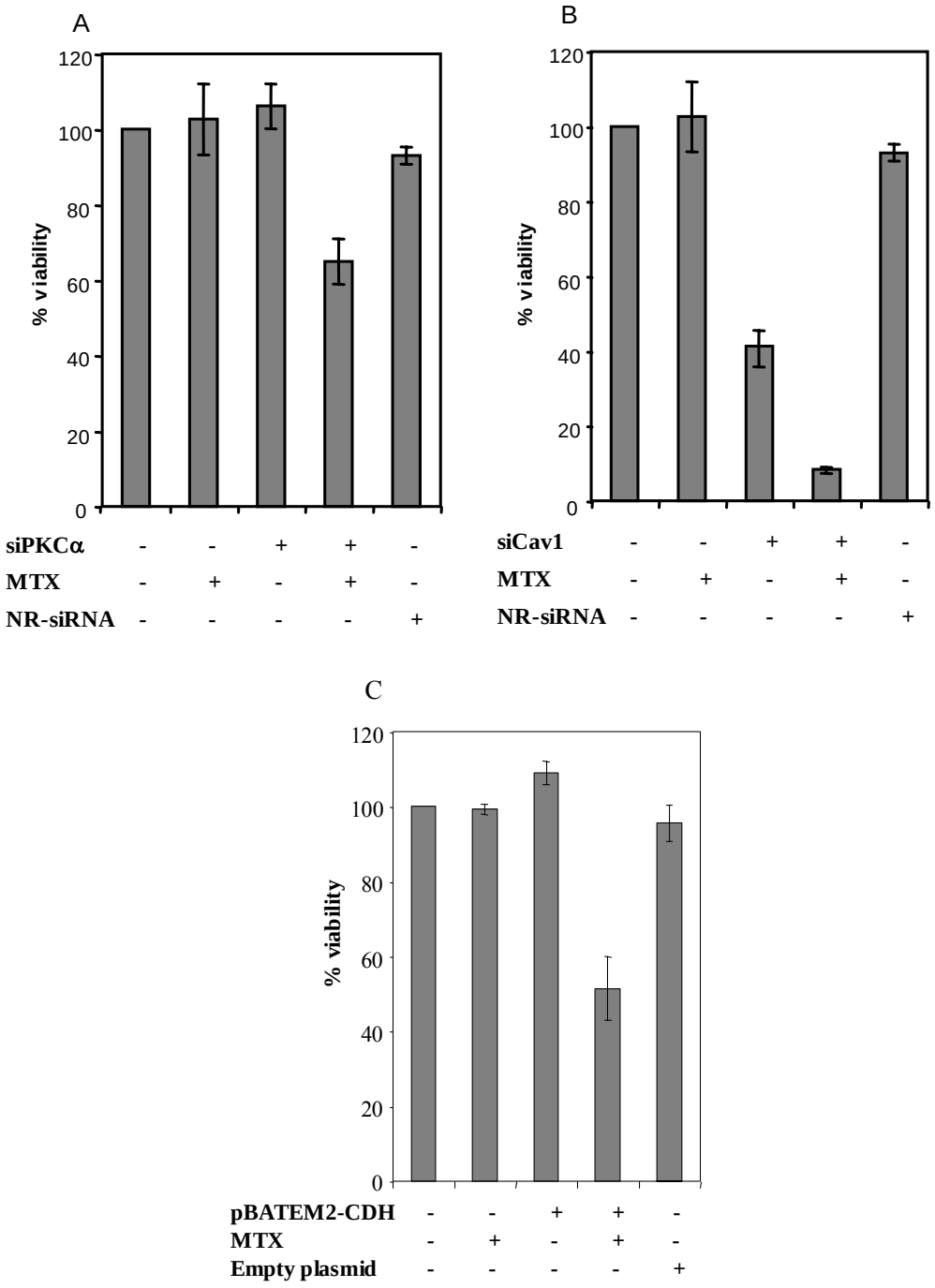


Figure 11. Effects of modulating PKCα, Cav1 or E-Cadherin mRNA levels on cell viability. Cells were treated with a siRNA against PKCα (A) or against Cav1 (B), or with an expression plasmid for E-Cadherin (pBATEM2-CDH; C). MTX was added after 48h, and cell viability was assessed after 5 days from the beginning of the treatment. All results are expressed as percentages referred to untreated cells. A non-related (NR) siRNA or an empty plasmid was used as negative control. The mean values ± SE of three independent experiments are depicted.

and the invasive potential of HT29 cells has been suggested. Importantly, we could observe a reversion of the cytotoxicity caused by MTX upon transfection with S100A4 expression vector, and transfection of a siRNA against S100A4 in HT29 sensitive cells caused chemosensitization of these cells toward MTX (Fig. 12) [27].

The present results are in agreement with Mahon *et al.* [28] that showed that inhibition of S100A4 expression results in an increased sensitivity of pancreatic ductal adenocarcinoma cell lines to gemcitabine treatment and induced apoptosis. Our results provide evidence that S100A4 acts as a pro-survival factor that contributes to chemoresistance in HT29 MTX-resistant cells.

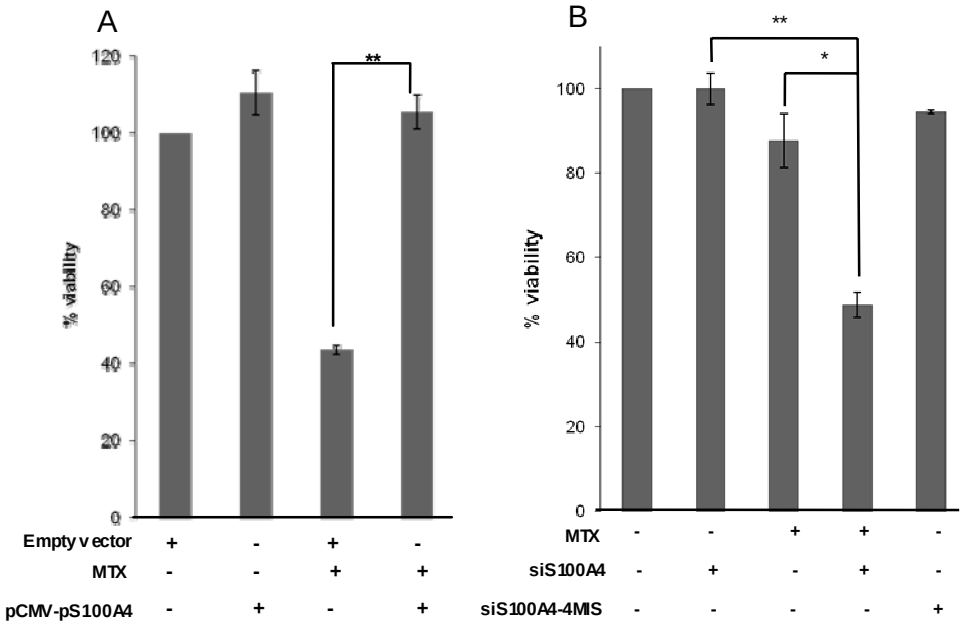


Figure 12. Effects of modulating S100A4 mRNA levels on cell viability. HT29 cells were treated with either the expression plasmid for S100A4 (pCMV-S100A4; A) or with a siRNA against its RNA (siS100A4; B), and MTX was added 48h later. Cell viability was assessed by the MTT assay 3 days after MTX treatment. Results are expressed as percentages referred to the untreated cells. Values are the mean of three independent experiments ± SE. *$p<0.05$, **$p<0.01$.

2.8. Role of DKK1 on MTX resistance

Dikkopf homolog 1 (DKK1) is a secreted protein involved in embryonic development and it is classically considered to function as an inhibitor of the canonical Wnt signaling pathway (see [29] for a review). However, DKK1 does not take an active part in Wnt / β-catenin pathway in colon cancer cells,

as mutation of adenomatous polyposis coli (APC, one of the components of Wnt pathway) occurs in most human colon cancers, thus disconnecting the effector part of the signaling cascade from the Wnt receptors, where DKK1 plays its inhibitory effect [30]. This situation led us to the hypothesis that DKK1 could have other cellular functions aside from its role in Wnt pathway. Indeed, a role for DKK1 overexpression in cancer and proliferation has been previously stated, although its precise mechanism of action has not yet been elucidated. In the case of HT29 MTX-resistant cells, the role of DKK1 is unclear, although it seems to be related to the resistant phenotype, since treatment with a siRNA against DKK1 mRNA showed a chemosensitization toward MTX (Fig. 13). In keeping with this, Katula and collaborators showed that folate deficiency led to the downregulation of DKK1, and that MTX inhibited DKK1 transcription [31]. Thus, DKK1 overexpression in HT29 MTX-resistant cells could constitute a mechanism to overcome the transcriptional repression exerted by MTX [32].

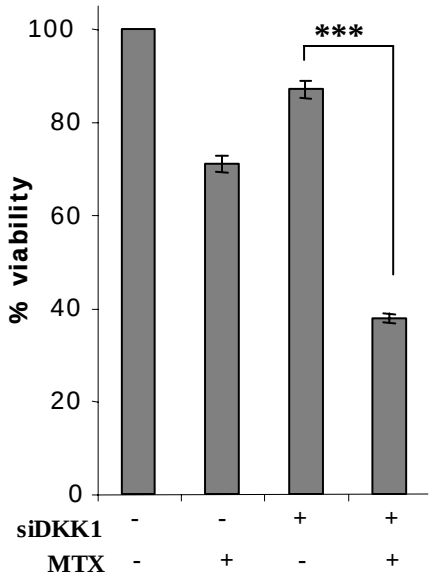


Figure 13. Effects of modulating DKK1 mRNA levels on cell viability. Cells were treated with a siRNA against DKK1, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. *** $p<0.001$.

2.9. Integrative view of MTX resistance in HT29 colon cancer cells

It has been shown that activated PKCα translocates from the nucleus to the membrane, where it associates with *caveolae*, and regulates the function

and formation of such biological structures [33]. PKCα has been described to directly interact with Cav1. Further, activation of PKCα by phorbol esters dislocates the enzyme from *caveolae*. These observations indicate that PKCα interacts functionally with these membrane structures. Moreover, PKCα has been proposed to be involved in the rearrangement of the cytoskeleton. Masur *et al.* showed that a high level of PKCα expression plus a low E-cadherin level predicts an elevated migratory activity of colon carcinoma cells, which could be derived more easily to metastasis [34]. Lahn *et al.* speculated that PKCα overexpression may represent an important cellular event leading to enhanced tumor progression, as they showed that MCF-7 breast cancer cells transfected with PKCα had reduced expression of E-cadherin and β-catenin, resulting in a loss of cell-cell adhesion and thus in a more aggressive tumor phenotype [35].

Specific protein-protein interactions between CAV1 and other proteins have been proposed to regulate cell signalling. Indeed, CAV1 is known to control cell proliferation and viability via a transcriptional mechanism involving the Wnt/β-catenin-Tcf/Lef-1 pathway [36]. One of the possible locations of β-catenin is within a complex with E-cadherin in the adherence junctions, specialized cell-cell adhesion sites that link the cadherin molecules to the actin microfilaments. E-cadherin promotes co-localization and co-immunoprecipitation of CAV1 with β-catenin, as well as inhibition of β-catenin-Tcf/Lef-1 dependent transcription of a wide variety of genes regulated by this pathway. However, the ability of CAV1 to regulate gene expression and cell proliferation is severely impaired in metastasic cancer cells lacking E-cadherin. If E-cadherin is lost, β-catenin is not retained in the plasma membrane and can be then translocated into the nucleus [37], thus activating Tcf/Lef-1 transcription factors-mediated expression of genes implicated in cell proliferation and tumor progression [38]. E-cadherin has been shown to be an important permissive element in defining the functions of CAV1, since several characteristics potentially relevant to CAV1 function as a tumor suppressor are compromised in E-cadherin-deficient HT29 cells. In this sense, we demonstrated that combination treatments to simultaneously decrease the expression levels of CAV1 and overexpress E-Cadherin were able to reduce cell viability by about 85% in the sensitive cells, and importantly, also in HT29 resistant cells (Fig. 14). These results give evidence of CAV1 and E-Cadherin interrelations in HT29 cells.

In order to shed some light on the possible role of Wnt/β-catenin-Tcf/Lef-1 pathway in HT29 resistant cells, we performed transient transfection experiments with a luciferase reporter of β-catenin-mediated transcriptional activation. These experiments showed that Wnt / β-catenin-Tcf/Lef-1 pathway

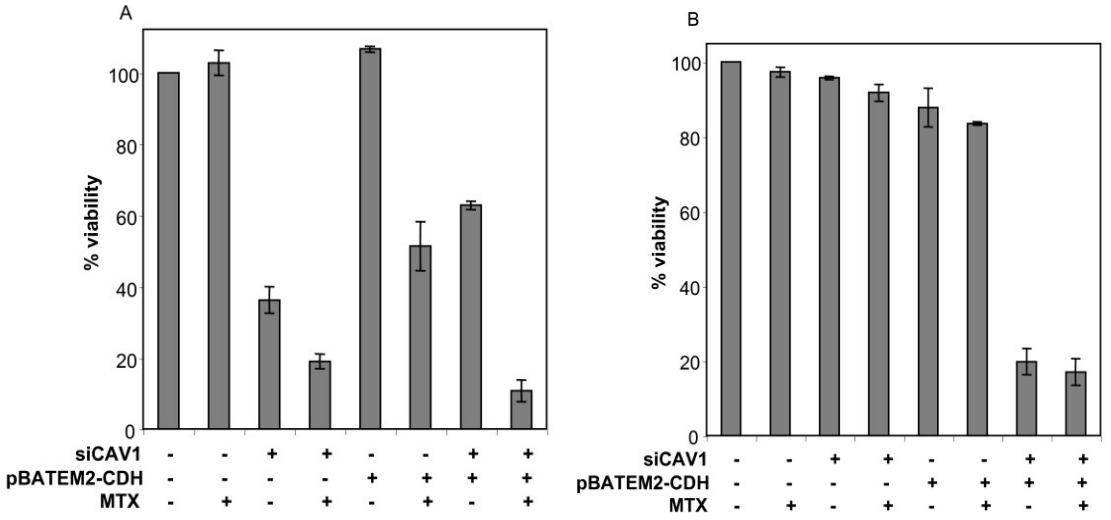


Figure 14. Effect of combining the siRNA against CAV1 and the expression plasmid for E-cadherin. Simultaneous treatments with a siRNA against CAV1 (siCAV1) and an expression plasmid for E-cadherin (pBATEM2-CDH) were performed in both sensitive (A) and resistant (B) HT29 cells. MTX was added 48 hours after transfection and the MTT assay was used to determine cell viability. Results are expressed as percentages referred to untreated cells. Values are the mean of three independent experiments ± SE.

was more active in the HT29 resistant cells than in HT29 sensitive cells, and that re-expression of E-Cadherin in the resistant cells results in a lower β-catenin-mediated transcriptional activation probably due to recruitment of β-Catenin to the adherent junctions (Fig. 15). Interestingly, DKK1 is transcriptionally regulated by Wnt / β-catenin signaling. Thus, constitutive activation of this signaling pathway through β-catenin, downstream of APC, could represent a mechanism for DKK1 overexpression in HT29 MTX-resistant cells.

It has been shown that S100A4 is also a target of Wnt signaling pathway in colon cancer, and a functional TCF binding site has been identified in its promoter sequence [39]. In this direction, an inverse correlation has been established between the expression levels of S100A4 and E-Cadherin [40] and has been associated with poor differentiation of cancer cells. Moreover, transfection of an E-Cadherin expression vector has been reported to cause a decrease in S100A4 expression levels. We showed a two-fold increase in S100A4 mRNA expression upon β-catenin overexpression (Fig. 16), thus giving support to a Wnt/β-catenin pathway-mediated S100A4 transcription.

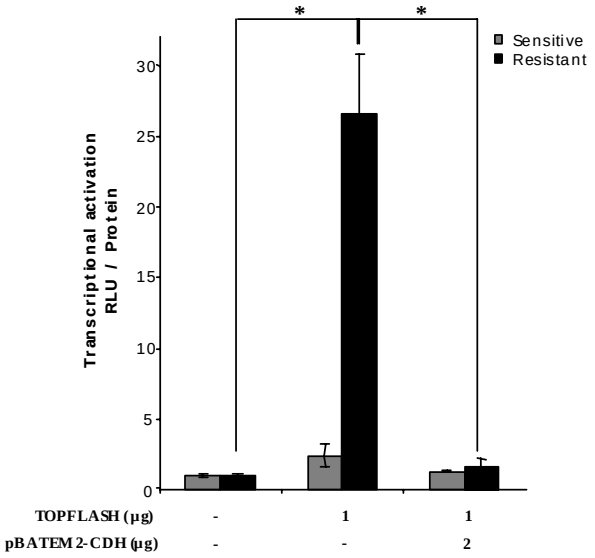


Figure 15. Transcriptional activation of Wnt pathway in MTX-resistant cells. HT29 cells were transiently transfected with a luciferase reporter of β-catenin-medi-ated transcriptional activation (TOPFLASH) in the presence or in the absence of E-Cadherin expression vector (pBATEM2-CDH). Thirty hours after transfection, luci-ferase activity (relative light units, RLU) was assayed. The protein content was used to normalize the luciferase acti-vity for each sample and is expressed relative to that of pGL3 basic vector (mean ± S.E.M. for triplicate wells). * $p<0.05$.

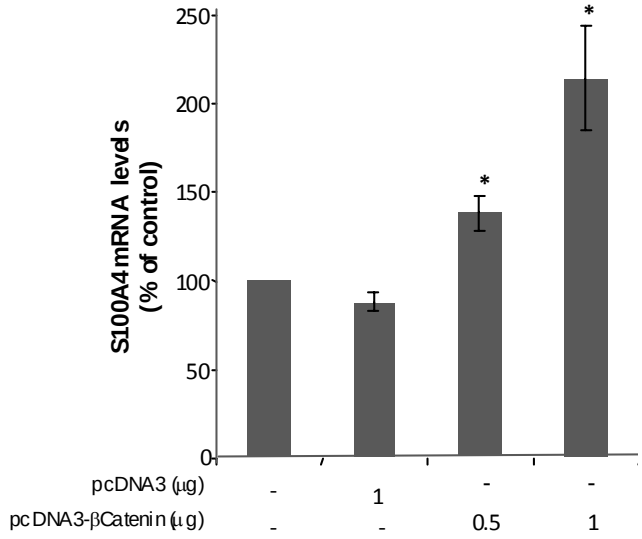
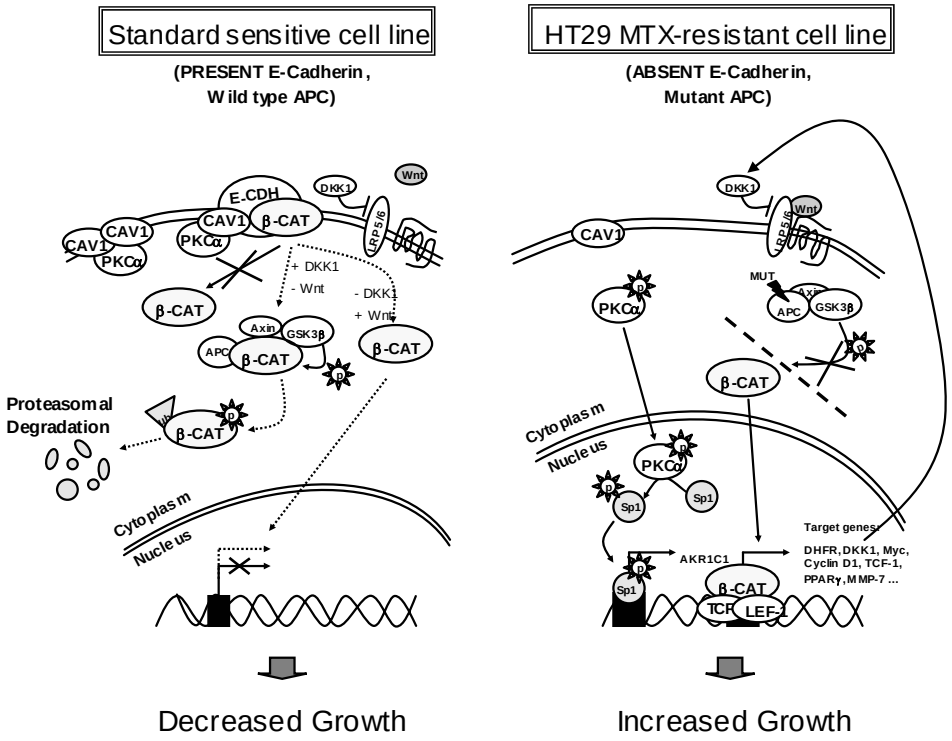


Figure 16. Effects of transfecting an expression vector encoding for β-Catenin on S100A4 mRNA levels. Transfection with β-Catenin expres-sion vector (pcDNA3-β-Catenin) was performed in HT29 cells. S100A4 mRNA levels were determined by RT-Real-Time PCR 48h after trans-fection. All results are expressed as percentages referred to untreated cells. Values are the mean of three independent experiments ± SE. * $p<0.05$.



Scheme 1. Regulation of the β-catenin-Tcf/Lef-1 pathway in sensitive and MTX-resistant cells.

The interrelationship of AKR1C1 with the scenario described until now would be mediated trough PKCα. This kinase could be responsible for Sp1 phosphoryaltion in HT29 MTX-resistant cells, thus being important for AKR1C1 transcriptional regulation in this cell line. No reports exist on a possible regulation of AKR1C1 promoter through the β-catenin-Tcf/Lef-1 signaling pathway. However, our results show the interrelationship between AKR1C1, cell proliferation, and apoptosis, two processes that can be regulated trough the β-catenin-Tcf/Lef-1 pathway. One could hypothesize that AKR1C1 may favour or help the proteins from genes regulated throug the β-catenin-Tcf/Lef-1 pathway exhert their functions, maybe metabolizing a compound that could be interrfering in its pro-proliferative or anti-apoptotic functions. Thus, AKR1C1 overexpression in HT29 MTX-resistant cells would represent a parallel mechanism that could positvely contribute to the effects of the β-catenin-Tcf/Lef-1 signaling pathway.

In summary, HT29 MTX-resistant colon cancer cells bear many expression changes with respect to their sensitive counterparts. This scenario leads to changes in proteins that play a role in many processes, that are somehow interconnected (see scheme 1), and finally lead to increased growth, and thus to drug resistance.

3. MTX resistance in breast cancer

Breast cancer is the most common cancer in women in developed countries, and after lung cancer, the tumor that causes more deaths among females [41]. One of the possible treatments for this type of cancer is MTX, usually given in combination with cyclophosphamide and 5-fluorouracil. With the aim to get further insight into the mechanisms of MTX resistance, functional genomics analysis using microarrays were performed in two breast cancer cell lines sensitive and resistant to MTX. We identified DHFR and UGT1A as differentially expressed genes in common between two breast cancer cell lines, namely MCF-7 and MDA-MB-468, resistant to MTX [42].

3.1. Role of DHFR on MTX resistance

DHFR was overexpressed in both breast cancer cell lines studied, by about 30 fold in MCF-7 resistant cells and about 3 fold in MDA-MB-468 resistant cells. Moreover, use of iRNA technology caused a sensitization of

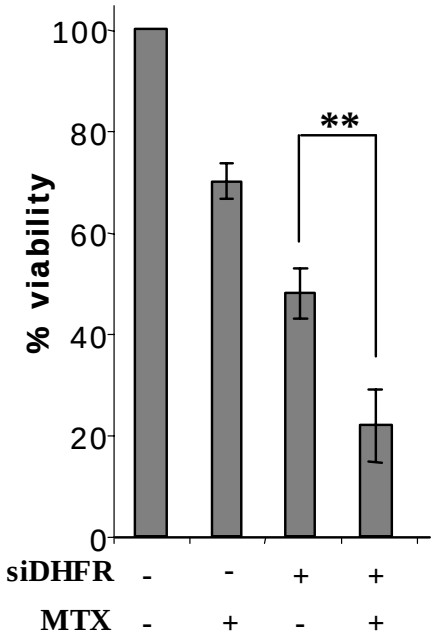


Figure 17. Effects of modulating DHFR mRNA levels on MCF-7 viability. Cells were treated with a siRNA against DHFR, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. ** $p<0.01$.

MCF-7 cells toward MTX (Fig. 17), thus confirming a role for DHFR in MTX resistance in this cell line.

We also determined DHFR copy number in both cell lines. While 10-fold amplification was observed in MCF-7 resistant cells, no changes in gene copy number were detected in MDA-MB-468 resistant cells. Thus, gene amplification of the *dhfr locus* could represent a mechanism for MTX resistance in MCF-7 cells, while drug resistance in MDA-MB-468 cells may obey to any of the other known mechanisms for MTX resistance [32]. Additionally, one has to take into account that MTX causes the differential expression of many genes that may be direct or indirect regulators of cell proliferation, survival or apoptosis, and that this expression pattern can contribute to modulate the resistance phenotype. In breast cancer cells, UGTs play a role in MTX resistance (see below).

3.2. Role of the UGT1A family on MTX resistance

UDP-glucuronosyltransferases (UGTs) are a family of enzymes involved in phase II metabolism. The addition of a glycosyl group from uridine

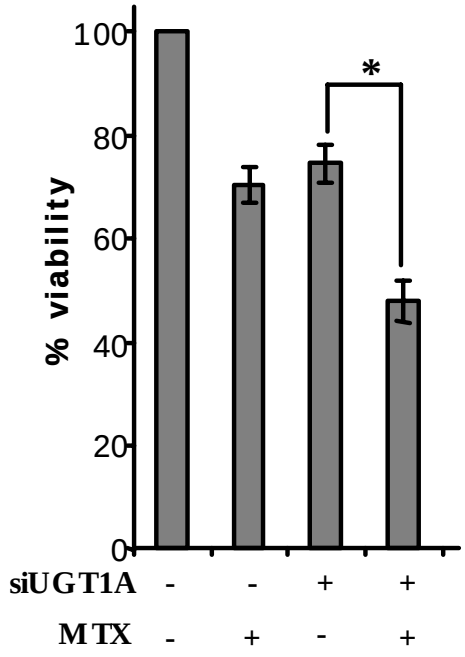


Figure 18. Effects of modulating UGT1A mRNA levels on cell viability. Cells were treated with a siRNA against UGT1A, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. * $p<0.05$.

diphosphoglucuronic acid (UDPGA) renders hydrophobic compounds more soluble for their elimination via bile and urine. UGTs catalyze 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 glucuronidated, contributing significantly to the overall availability and pharmacological effect of these xenobiotics [43]. The UGT1 gene family expresses nine functional UGT1A proteins by alternative splicing of 13 different tandem exons 1 with the common exons 2–5 [44]. Thus, all the UGT1A isoenzymes have a highly conserved "common" region, which is believed to contain the cofactor-binding site, and a variable region containing unique substrate-binding sites. Treatments that combine a siRNA designed to decrease the mRNA levels of some members of the UGT1A family and MTX showed an increased sensitivity toward the drug (Fig. 18), thus demonstrating a role for this gene family in MTX resistance in breast cancer.

3.3. Role of UGT1A6 on MTX resistance

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 and is responsible for the metabolism of some drugs, carcinogens, and endogenous substrates [45]. UGT1A6 could be responsible for metabolising MTX in a certain degree, as it shares a phenolic structure common to other UGT1A6 substrates. A detoxification role can be hypothesized for UGT1A6 since its ectopic overexpression in sensitive breast cancer cells counteracts the cytotoxic effect of MTX (Fig. 19).

UGT1A family members' 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 characterized by its induction by a wide range of compounds. MTX could be an UGT1A6 inducer according to our results of increased mRNA levels and UGT1A enzymatic activity (Fig. 20).

Some transcription factors have been described to be involved in UGT1A induction. AhR has been described to provoke moderate UGT1A6 induction in humans and rodents [46]. 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

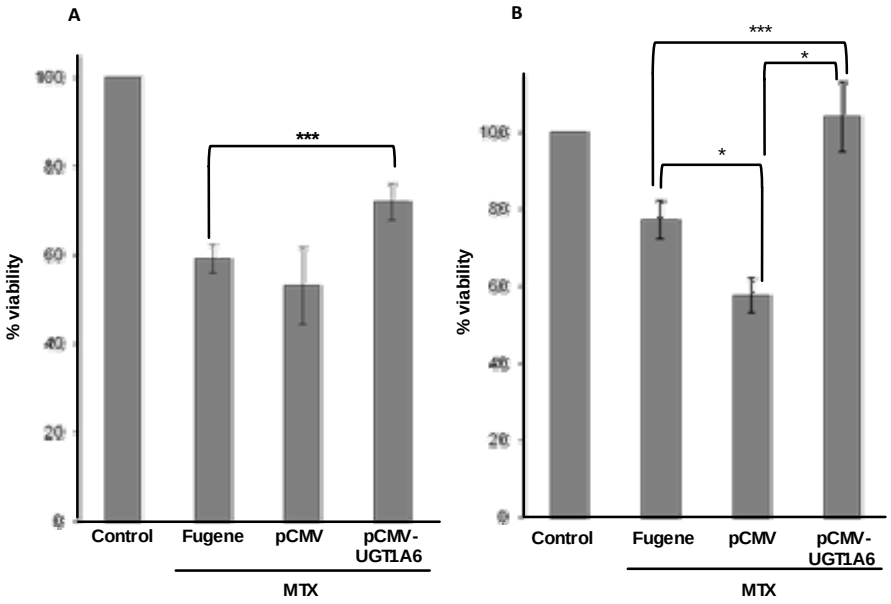


Figure 19. Methotrexate cytotoxicity upon UGT1A6 overexpression. Cells were transfected with either the expression plasmid for UGT1A6 (pCMV-UGT1A6) or with the empty vector pCMV, which was used as a negative control. MTX was added 48 hours after transfection and cell viability was determined a week after transfection. A) MCF-7 cells. B) MDA-MB-468 cells. Results are presented as percentages referred to control cells. Values are the mean ± SE of at least five experiments. * $p < 0.05$; *** $p < 0.005$.

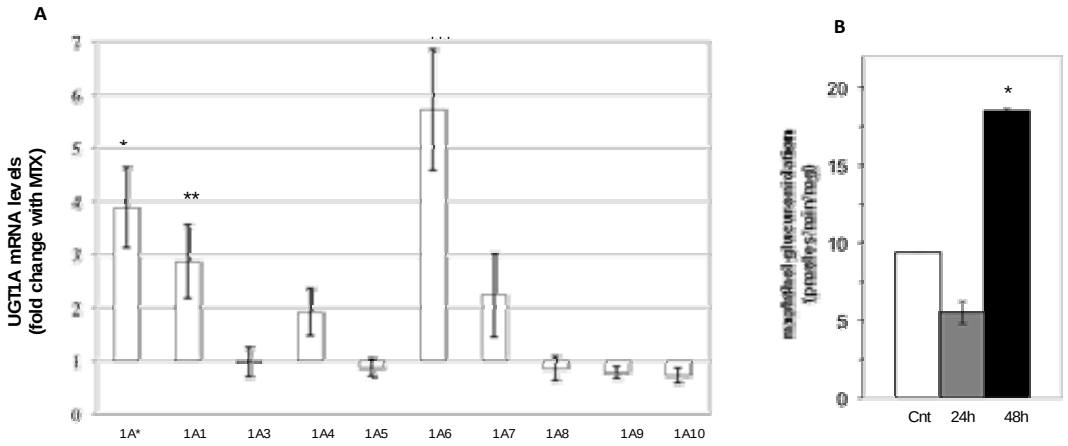


Figure 20. UGT1A mRNA and activity after MTX incubation. A) MDA-MB-468 cells were incubated for 24 hours with MTX. Total mRNA was obtained and subjected to DNAase treatment prior to RT-Real Time PCR. Data represent the fold change referred to control cells. Values are the mean ± SE of at least three experiments. B) MDA-MB-468 cells were incubated with MTX for 24 and 48 hours. The microsomal fraction was obtained and UGT1A activity was analysed through [1-14C]-α-naphthol glucuronidation. Values are the mean ± SE of two experiments. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.005$.

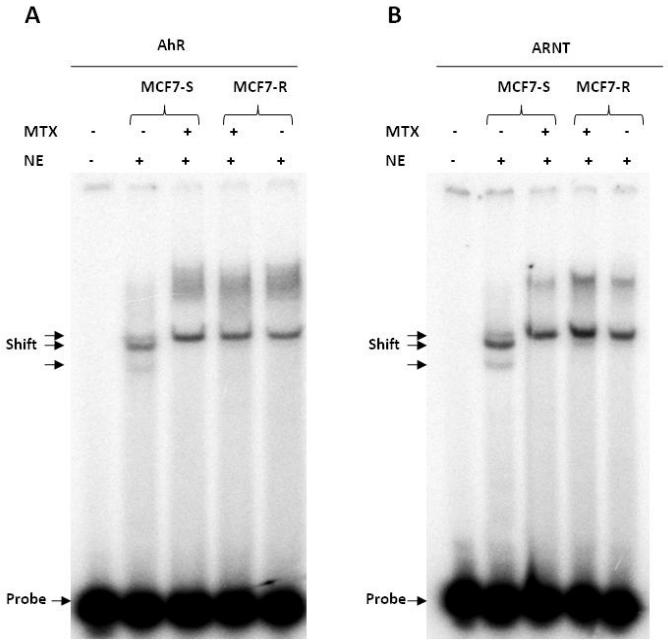


Figure 21. Binding of AhR and ARNT to UGT1A6 promoter. Cells were incubated with or without MTX for 24 hours prior to nuclear extracts harvest. Nuclear extracts were incubated with a radiolabelled probe corresponding to: (A) UGT1A6 AhR/ARNT promoter sequence. (B) UGT1A6 ARNT promoter sequence. Shifted bands are indicated by arrows.

xenobiotic response element (XRE, 5'-GCGTG-3'). AhR/ARNT binding to XRE activates the expression of a battery of genes involved in drug and hormone metabolism, among which UGT1A6 is found [47]. 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. Our gel-shift results (Fig. 21) support the idea that MTX could be inducing UGT1A6 expression through AhR/ARNT heterodimers and ARNT itself. Another possibility would be that MTX, instead of directly activating AhR or ARNT, could bind to an ARNT inhibitor or activate ARNT through coactivators.

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 and mycophenolic acid, 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 tumor treatment, since MTX 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 MTX, 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 MTX with other drugs susceptible to glucuronidation.

4. MTX resistance in other cancer cells

4.1. Role of DHFR on MTX resistance in pancreatic cancer, erythroblastic leukemia and osteosarcoma

We also studied DHFR expression and copy number in three other cell lines representative of pancreatic cancer (MIA PaCa-2), erythroblastic leukemia (K562) and osteosarcoma (SaOs-2). DHFR is overexpressed in all three cell lines, but only displays clear gene amplification in MIA PaCa-2 cells (Table 1).

DHFR expression levels are presented both as the values found in the microarrays and as validated by RT-PCR. DHFR copy number was determined by Real-Time PCR. Values are the mean (in fold change relative to the sensitive cells) of three independent experiments ± SE.

We further studied the role of DHFR on MTX resistance in MIA PaCa-2 cells. Transfection of a siRNA designed against DHFR RNA caused a sensitization toward MTX (Fig. 22).

Table 1. Validation of DHFR overexpression and copy number determination in the different cell lines.

Cell Line	Expression		Copy-number
	Microarray	RT-PCR Validation	
MIA PaCa-2	9.5	8.2 ± 1.1	32.2 ± 2.2
K562	9.4	9.8 ± 0.2	1.9 ± 0.1
Saos-2	4.1	4.1 ± 1.1	0.6 ± 0.1

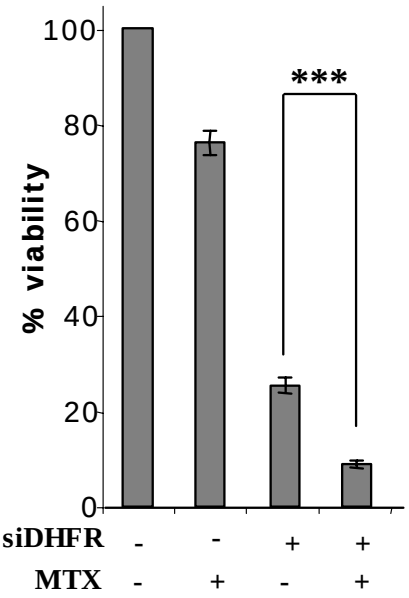


Figure 22. Effects of modulating DHFR mRNA levels on Mia PaCa-2 viability. Cells were treated with a siRNA against DHFR, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. *** $p<0.001$.

4.2. Role of EEF1A1 on MTX resistance in pancreatic cancer

Eukaryotic translation elongation factor 1A1 (EEF1A1) was a gene overexpressed in common among MIA PaCa-2, K562 and Saos-2 resistant cell lines. EEF1A1 is a ubiquitously expressed protein elongation factor that recruits amino-acetylated tRNAs to the A site of the ribosome (see [48] for a review). Although it has been traditionally described as a cellular housekeeper enzyme, overexpression of EEF1A1 is found in melanomas and in some tumors [49]. It has been demonstrated that EEF1A expression is related to increased cell proliferation, oncogenic transformation, delayed cell senescense and metastasis. Moreover, increased EEF1A1 expression has been related to cisplatin, doxorubicin and MTX resistance, maybe due to its ability to inhibit apoptosis. It has been proposed that EEF1A overexpression promotes cell growth and replication by contributing to an overall increase in protein translation. Antisense-mediated abrogation of EEF1A1 expression inhibits tumorigenesis and anchorage-independent cell replication in prostate tumor cells [50]. Our functional analyses using siRNA technology against EEF1A1 (Fig. 23) are in keeping with these results, and show chemosensitization of MIA PaCa-2 cells. Thus, we could state a role for EEF1A1 in MTX resistance in this cell line.

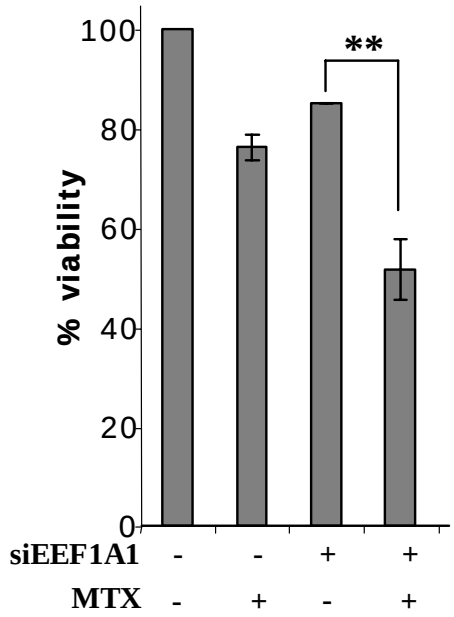


Figure 23. Effects of modulating EEF1A1 mRNA levels on cell viability. Cells were treated with a siRNA against EEF1A1, MTX was added after 48h, and cell viability was assessed after 3 days from the beginning of the treatment. All results are expressed as percentages referred to a non-related negative control siRNA (NR-siRNA). Values are the mean of three independent experiments ± SE. ** $p<0.01$.

5. Conclusions

- Gene Networks can be constructed, using specific software, from lists of differentially expressed genes by correlating them with the information already available in interaction databases. Then, significant node-genes within the networks can be selected and explored as putative targets for different types of therapy (e.g. cancer).
- We have applied this methodology to detect genes that could be used as novel targets in cancer chemotherapy. We used as a model cells resistant to methotrexate representative of 5 types of human cancer: colon, breast, pancreas, osteosarcoma and leukemia.
- In human colon cancer cells, DKK1, AKR1C and DHFR were detected as node-genes. The appearance of DHFR in the network was obvious and served as positive control; AKR1C had already been studied by our group since it appeared as a clear overexpressed gene in the lists of differentially expressed genes; but DKK1 had been missed in our previous studies. The methodology described in this chapter was able to detect it and subsequently its relationship with E-Cadherin, ß-Catenin, Caveolin 1 and PKCα.

- When analyzing breast cancer cells, network generation enabled the detection of the UGT1A family; and EEF1A1 as well as DHFR were common overexpressed nodes in the BAN constructed for pancreatic cancer, erythroblastic leukemia and osteosarcoma.
- In all cases, functional validation of the detected node-genes in the corresponding cell lines were performed using siRNA technology, obtaining an increased sensitivity toward methotrexate when combining this drug with the antisense molecule.

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