Molecular Pharmacology Fast Forward. Published on May 18, 2005 as doi: 10.1124/mol.105.011015
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MOL (2005/011015)

Expression profiling of ABC transporters in a drug resistant breast cancer cell line using AmpArray

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MOL (2005/011015)

Running title: ABC transporters and drug resistance

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Number of text pages: 26

Number of tables: 4

Number of figures: 4

Number of references: 37

Number *of words in the* Abstract: 237

Number of words in the Introduction: *693*

Number of words in the Discussion: 2076

Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; MXR, mitoxantrone resistance; SUR, sulfonylurea receptor; RT-PCR, reverse-transcription polymerase chain reaction; SRB, sulforhodamine B; ECL, chemiluminenscence; FTC, Fumitremorgin C; Ct, threshold cycle.

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Abstract

ATP-binding cassette (ABC) membrane proteins comprise a superfamily of transporters with a wide variety of substrates. Human has 49 members in this superfamily. Several human ABC transporters such as ABCB1 and ABCC1 have been attributed to cause multidrug resistance (MDR) in cancer treatment when over-expressed. Previously, a MDR cancer cell line MCF7/AdVp3000 has been selected and over-expression of ABCG2 was thought to cause MDR in this cell line. However, ectopic over-expression of ABCG2 in MCF7 cells could not explain the high drug resistance level observed with the selected cell line. In this study, we designed an AmpArray analysis to profile whether other ABC transporters were also selected to contribute to the increased drug resistance in MCF7/AdVp3000 cells. We found that 16 ABC transporters including ABCG2 had ≥1.5-fold altered expression in MCF7/AdVp3000 compared with the parental MCF7 cells. Particularly, the expression of ABCA4 and ABCC3 was increased 132 and 459 fold, respectively, while ABCG2 was increased ~3000 fold. Furthermore, the elevated expression of these three transporters reversed with the reversed drug resistance phenotype and silencing ABCC3 expression in MCF7/AdVp3000 cells significantly reduced Adriamycin resistance. Thus, other ABC transporters in addition to ABCG2 likely contribute to the MDR selected in MCF7/AdVp3000 cells. This study also shows that AmpArray can be used as a quick and easy tool to profile the expression of ABC transporters in resistant cell lines and tumor samples for potential use in individualized design of therapy.

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Introduction

Drug resistance to chemotherapy frequently occurs in cancers and is a major obstacle to successful cancer treatment. Studies with tumor cell lines have revealed that multidrug resistance (MDR) can develop and, thus, cause chemotherapy failure. Advances in elucidating the molecular basis of the MDR phenotype indicate that elevated membrane expression of several drug efflux pumps such as P-glycoprotein (Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1), and ABCG2 is a frequent cause of MDR in human cancers (Borst and Elferink, 2002; Doyle and Ross, 2003; Gottesman et al., 2002; Haimeur et al., 2004; Han and Zhang, 2004; Kruh and Belinsky, 2003; Sarkadi et al., 2004).

These drug efflux pumps belong to the superfamily of ABC (ATP-binding cassette) transporters with a wide variety of substrates (Borst and Elferink, 2002). These ABC transporters share a common structural feature consisting of transmembrane domains and cytoplasmic nucleotide binding domains with walker motifs. The nucleotide binding domains appear to function as an engine which provides the energy required for transport activities by hydrolyzing ATP (Altenberg, 2004). In human, there are 49 members in this ABC transporter superfamily which are divided into seven subfamilies (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG) (see the websites http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html and http://nutrigene.4t.com/humanabc.htm for complete listing and nomenclature). Of these ABC transporter members, several are known to efflux anticancer drugs and, thus, cause drug resistance when over-expressed in model cancer cell lines. On the other hand, two of the 49 members of the human ABC transporter family, ABCE1 (also known as RNase Li and OABP) and ABCF1 (also known as ABC50), are known to lack transmembrane domains and localized in cytosolic fractions (Allikmets et al., 1996; Dean et al., 2001). Therefore, they are unlikely to be membrane transporters.

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Previously, a drug resistant human breast cancer cell line MCF7/AdVp3000 was generated from parental MCF7 cells by stepwise selection using anticancer drug Adriamycin in the presence of verapamil, an inhibitor for ABCB1 (Pgp) and ABCC1 (MRP1) (Doyle et al., 1995; Lee et al., 1997). Characterization of this atypical drug resistant subline revealed that the resistance is mainly due to the reduced cellular accumulation of anticancer drugs, which led to the discovery of the human ABC transporter ABCG2 (also known as BCRP, MXR, and ABCP) that was thought to cause reduced cellular accumulation of anticancer drugs and, thus, drug resistance phenotype in the selected MCF7/AdVp3000 cells (Doyle et al., 1998; Miyake et al., 1999). Although it has been shown that transfection of ABCG2 cDNA into the parental breast cancer cell line MCF7 caused drug resistance, it has also been found that the drug resistance level of the transfected cells was much lower than that of the drug-selected MCF7 cells (Doyle et al., 1998). The relative resistance factor of ABCG2-transfected MCF7 cells to mitoxantrone is 32 times whereas that of the drug-selected MCF7/AdVp3000 cells is 3902 times compared with parental MCF7 cells (Dean et al., 2001). There is a 122-fold difference in mitoxantrone resistance between the drug-selected and ABCG2-transfected MCF7 cells although both express similar levels of ABCG2 and do not differentially express ABCB1 or ABCC1. Similar differences to other anticancer drugs such as Adriamycin were also observed with these cells. The difference in drug resistance level between ABCG2-transfected MCF7 and drug-selected MCF7/AdVp3000 cells must be due to other mechanisms which were selected in the MCF7/AdVp3000 cells.

In this study, we tested the hypothesis that over-expression of other ABC transporters may also be selected in addition to ABCG2 in the MCF7/AdVp3000 cells using a newly designed AmpArray of human ABC transporters. We found that 16 ABC transporters including ABCG2 had ≥1.5 fold of altered expression in MCF7/AdVp3000 cells compared with the parental sensitive MCF7 cells. Particularly, the expression of ABCA4 and ABCC3 was increased 132 and 459 fold,

respectively, at the RNA level in the resistant MCF7/AdVp3000 compared to the sensitive MCF7 cells while ABCG2 was increased for ~3000 fold. Furthermore, the elevated expression of these three transporters vanished with the reversed drug resistance phenotype and silencing ABCC3 expression using siRNA significantly reduced Adriamycin resistance. Therefore, other ABC transporters may also play a role in reducing drug accumulation and causing resistance in the selected MCF7/AdVp3000 cells.

Materials and Methods

Materials. PVDF membranes were purchased from BioRad. SYBR Green PCR Master Mix for real-time PCR was purchased from Applied Biosystems. IMEM and OPTI-MEM were purchased from BioSource International and Invitrogen, respectively. Monoclonal antibody BXP-21 against ABCG2 and anti-ABCC3 antibody were from ID Labs and Chemicon International, respectively. Polyclonal anti-ABCA4 antibody was from Santa Cruz Biotechnology. The monoclonal anti-ABCA4 antibody was a gift from Robert S. Molday (University of British Columbia, Canada). Fumitremorgin C (FTC) was a gift from Susan E. Bates (National Cancer Institute). GF120918 was obtained from GlaxoSmithKline. All other chemicals were of molecular biology grade from Sigma or Fisher Scientific.

Cell lines. Human breast cancer cell line MCF-7 and its derivative line MCF-7/AdVp3000 and MCF-7/AdVpRev are gifts from Dr. Susan E. Bates. MCF-7 and its derivate cell lines were grown at 37^{o}C with 5% CO2 in IMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. To maintain the drug-resistance phenotype of MCF7/AdVp3000 cells, 3000 ng/ml Adriamycin and 5 μg/ml verapamil were included in the medium.

RNA preparation and AmpArray. Total RNAs were isolated from both MCF7 and MCF7/AdVp3000 cells by RNeasy mini kit according to the manufacturer's instruction (Qiagen)

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and treated with RQ1 RNase-free DNase I. Reverse transcription and PCR were performed as described online (http://www.genecopoeia.com). Briefly, cDNAs were reverse transcribed from total RNAs using MMLV reverse transcriptase (Promega) and oligo dT as a primer. The cDNAs from both MCF7 and MCF7/AdVp3000 cells were semi quantified based on GAPDH level using PCR. Normalized levels of cDNAs were then used for amplification using PCR on a 96 well plates with appropriate pairs of primers for 47 human ABC transporters. The PCR products were then separated by agarose gel electrophoresis and the intensity of each band was quantified using GelPicAnalyzer software (GeneCopoeia). The relative quantitative data are consistent between two independent experiments, suggesting that the method is reproducible. The relative level of each gene presented was calculated based on two independent experiments.

Real-time quantitative RT-PCR. Four μg total RNAs from each cell type were reverse transcribed using AMV Reverse Transcriptase and Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen). Primers for real-time PCR were designed using Primer Express software version 2.0 (Applied Biosystems) and synthesized by Invitrogen. The sequences of primers used are shown in Table 1. Real-time quantitative PCR was carried out in ABI Prism@7000 Sequence Detection System (Applied Biosystems) using SYBR Green according to the manufacturer's instruction. The threshold cycle (Ct) was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold reflecting a statistically significant point above the calculated baseline. The Ct of each target product was determined, and normalized against that of the housekeeping gene GAPDH (forward, 5'-AAGGACTCATGACCACAGTCCAT–3', reverse, 5'-CCATCACGCCACAGTTT CC–3'). Fold difference = $2^{\Delta Ct}$

Cell lysate and plasma membrane preparation and western blot analysis. Confluent cells were washed with PBS and lysed in a buffer (1% Triton X-100, l50mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium orthovanadate, 0.2mM PMSF, 0.5% NP-

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40, 0.1% SDS) for 30 minutes at 4°C with constant agitation. The cell lysates were then sonicated briefly and followed by centrifugation (16,000 × g, 4°C) for 15 minutes to remove insoluble materials. Plasma membranes were prepared from confluent cells, as described previously (Yang et al., 2002; Zhang et al., 1993). The protein concentration of cell lysates and membranes was determined using BioRad protein assay kit.

Western blot analysis was performed as previously described (Pincheira et al., 2001; Yang et al., 2002; Zhang et al., 1993). Briefly, cell lysates or plasma membranes were solubilized in SDS sample buffer and separated by SDS-PAGE followed by transfer to a PVDF membrane. The blot was then probed with antibodies to ABCA4, ABCC3, and ABCG2 followed by reaction with HRP-conjugated secondary antibodies. The signal was captured by x-ray films using enhanced chemiluminenscence (ECL).

siRNA preparation and transfection. Two siRNAs targeting ABCC3 (ABCC3_121: 5'-CCUGUCUGUGCACACAGAA-3' and ABCC3_2459: 5'-GGCGUGCUGGCAGGCAAGA-3') were designed using an online software (http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx) and synthesized by Qiagen. Silencer negative control siRNA (scrambled siRNA) was purchased from Ambion. For siRNA transfection, 2×10^6 MCF7/AdVp3000 cells were plated in 100 mm dish and grown for 24 hrs followed by transfection with siRNAs using Lipofectamine 2000 reagent (Invitrogen) according to the supplier's instructions. Briefly, 30 µl of Lipofectamine 2000 was diluted with 750 µl OPTI-MEM medium and incubated at room temperature for 5 min. 500 pmol siRNAs were added to 750 µl OPTI-MEM medium and then mixed with the diluted Lipofectamine 2000 reagent followed by incubation at room temperature for 20 min. The siRNA-Lipofectamine 2000 reagent complex was added drop wisely onto the culture containing

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7.5 ml fresh culture medium. Cell lysates were then prepared for detection of ABCC3 at 2, 3, or 9 days after siRNA transfection.

Cytotoxicity Assay. The cytotoxicity of the siRNA-transfected MCF7/AdVp3000 cells to Adriamycin was determined using the sulforhodamine B (SRB) colorimetric assay (Papazisis et al., 1997). Briefly, 24 hrs after transfection with ABCC3 or control siRNAs, the cells were trypsinized and seeded in 96-well plate at 3000 cells/well and cultured for 24 hrs. Adriamycin was then added to the cells in the presence of FTC (5 μM) or GF120918 (2 μM) and the cells were cultured continuously for 7 days before SRB assay. For SRB assay, the culture medium was aspirated, and the cells were fixed and stained by adding 70 μl 0.4% (w/v) sulforhodamine B (Sigma) in 1% acetic acid solution to each well and incubated at room temperature for 20 min. The plates were then washed 3-5 times with 150 μl of 1% acetic acid to remove the unbound SRB and air dried at room temperature. The bound SRB was then solubilized with 200 μl 10 m*M* unbuffered Tris-base, and the OD_{570nm} was determined using a 96-well plate reader (MRX, Dynex Technologies).

Results

To profile the expression of human ABC transporters in the drug resistant MCF7/AdVp3000 cells in comparison with the parental sensitive MCF7 cells, we designed an AmpArray assay in collaboration with GeneCopoeia, Inc (http://www.genecopoeia.com/). In this AmpArray analysis, a 96-well PCR plate is arrayed to amplify specific fragments of human ABC transporter genes using duplicated 48 pairs of primers which were designed by using a highly reliable primer designing algorithm which provides an uniform annealing temperature and similar sizes of PCR products so that one set of PCR condition works for all primer pairs. In the current design, two of the 49 human ABC transporter genes, ABCE1 and ABCF1, are known to

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lack transmembrane domains and, thus, were not included in the array. As a control, primers for GAPDH were included.

Following RT-PCR using total RNAs isolated from the parental cell line MCF7 and its derivative drug resistant cell line MCF7/AdVp3000 in the 96-well plate, the reaction mixtures were separated by agarose gel electrophoresis and stained with EtBr. As shown in Fig. 1A, 18 ABC transporter genes were not detectable in either cell line, suggesting that they are not expressed at a detectable level in these cells (see also Table 2A). The expression of 13 ABC transporters had less than 1.5-fold changes in expression level between the sensitive and resistant MCF7 cells (Table 2B). The remaining 16 genes had an altered expression of ≥ 1.5 fold in the drug resistant MCF7/AdVp3000 cells compared to the parental sensitive MCF7 cells (Table 3). Of these genes, the expression of ABCA4, ABCA5, ABCA7, ABCB3, ABCB10, ABCC2, ABCC3, ABCC5, ABCC8, and ABCG2 was increased whereas that of ABCA3, ABCA10, ABCA12, ABCB4, ABCC4, and ABCC11 was decreased in the MCF7/AdVp3000 cells. The findings that the expression of ABCB1 (Pgp) and ABCC1 (MRP1) was not different between the drug sensitive and resistant cell lines and that the expression of ABCG2 is elevated in the resistant MCF7/AdVp3000 cells are consistent with the previous studies (Doyle et al., 1998; Lee et al., 1997; Miyake et al., 1999).

To further validate the above findings, we performed a real-time PCR analysis on genes with ≥ 1.5-fold changes detected by AmpArray. We used a set of primers (Table 1) different from that used in AmpArray to rule out the potential problems generated from primers. To determine if the altered expression of these genes is potentially important for drug resistance selected in the MCF7/AdVp3000 cells, we also included in the real-time PCR analysis a revertant cell line MCF7/AdVpRev which has lost ABCG2 over-expression (Lee et al., 1997; Miyake et al., 1999). We argue that if the altered expression of ABC transporters does not reverse with the

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reversed drug-resistance phenotype as ABCG2 does, the transporter is less likely responsible for the resistance phenotype in MCF7/AdVp3000 cells.

As shown in Fig. 2, except ABCA7 and ABCB3 the differential expression of all other genes between the parental MCF7 and the drug resistant MCF7/AdVp3000 cells was confirmed although the fold of difference observed by real-time PCR for some genes varies from the AmpArray analysis. The variation is largely due to the semi-quantitative nature of the AmpArray method (see Discussion below). As summarized in Table 4A, 8 genes (ABCA5, ABCA7, ABCA12, ABCB4, ABCB10, ABCC2, ABCC4, and ABCC11) maintained their altered expression in the revertant cell line. Thus, they may not have significant contribution to the drug-resistant phenotype of MCF7/AdVp3000 cells although they may still contribute to the residual resistance in the revertant cell line (see Discussion below). As summarized in Table 4B, 5 genes (ABCA4, ABCB3, ABCC3, ABCC8, and ABCG2) fully reversed their altered expression in the revertant cell line and three other genes (ABCA3, ABCA10, and ABCC5) partially reversed their expression in the revertant cell line (Table 4B). Of these genes, ABCA4, ABCC3, and ABCG2 have the most dramatic up-regulation in the drug resistant MCF7/AdVp3000 cells with complete reversion in the revertant cell line and they are most likely responsible for the drug resistance observed in the MCF7/AdVp3000 cells. To further validate the expression of these three genes, we performed western blot analysis. As shown in Fig. 3, both ABCC3 and ABCG2 proteins were not detected in MCF7 cells but were detected at a much higher level in MCF7/AdVp3000 cells followed by a decrease in the revertant cell line. However, we were unable to detect ABCA4 protein in both plasma membranes and whole cell lysates using two different antibodies (data not shown), suggesting that ABCA4 protein may not be expressed in MCF7/AdVp3000 cells despite that its mRNA transcripts were expressed at high levels. It is possible that the expression of ABCA4 is under post-transcriptional regulation.

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To assess whether the increased ABCC3 expression in MCF7/AdVp3000 cells contributes to the selected Adriamycin resistance, we performed an experiment to silence ABCC3 expression in MCF7/AdVp3000 cells followed by determining the possible changes in Adriamycin resistance. For this purpose, two siRNAs were designed (see Materials and Methods) and only one (ABCC3_121) was able to silence ABCC3 expression even after 9 days of transfection (Fig. 4A). We next performed SRB assay to determine the effect of silenced ABCC3 expression on Adriamycin resistance in the presence of FTC or GF120918 (ABCG2 inhibitors) to eliminate ABCG2-mediated resistance. As shown in Fig. 4B and 4C, silencing ABCC3 expression significantly reduced the resistance level of MCF7/AdVp3000 cells to Adriamycin.

Discussion

In this study, we used AmpArray in combination with real-time RT-PCR analysis to profile the expression of 47 ABC transporters in the drug resistant MCF7/AdVp3000 cell compared with its parental sensitive MCF7 cell. In addition to ABCG2 which has been thought previously to be responsible for the drug selected resistance in MCF7/AdVp3000 cells, we found 15 other ABC transporters that have significant changes in their expressions. By comparing with the revertant MCF7/AdVpRev cells, we found that only 5 ABC transporters (ABCA4, ABCB3, ABCC3, ABCC8, and ABCG2) of these 16 genes had complete reversion in their expression and they are likely contributing to decreased cellular drug accumulation and increased drug resistance in MCF7/AdVp3000 cells.

Recently, a micro array of 38 ABC transporters has been developed and validated with several multidrug resistant cells lines (Gillet et al., 2004). However, because only a limited number of ABC transporter genes are included, its application is likely also limited. On the other hand, Gottesman's laboratory has developed an ABC-ToxChip for microarray analysis of

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toxicological response genes (Annereau et al., 2004). By comparing the KB-3-1 and DU-145 cells to their corresponding resistant derivative cell lines selected using colchicine (KB-8-5) and 9-nitro-camptothecin (RCO.1), respectively, they showed that ABCB1 and ABCC2 had dramatic over-expression in the two drug resistant cell lines, respectively. Later, the same group developed a more focused real-time RT-PCR analysis on the 48 ABC transporters and used this approach to profile the expression of ABC transporters in 60 diverse cancer cell lines (the NCI-60) (Szakacs et al., 2004). While the real-time PCR approach is a focused assay on ABC transporters in the later study, performing this assay routinely on all 48 genes is time consuming and limited for routine applications. However, the AmpArray approach described in this study is a quick semi quantitative assay to profile expressions of ABC transporters. The use of 96-well plates makes it convenient and economical to assay 47 transporters plus a GAPDH control at the same time on a regular thermal cycler that houses a 96-well plate format.

The disadvantage of AmpArray, however, is that the assay is semi quantitative and there is a potential problem in accuracy for genes that have small changes as demonstrated by the study on ABCB3 and ABCA7. Both ABCA7 and ABCB3, in fact, changed little as determined by both AmpArray and real-time PCR despite the fact that the direction of changes is different by the two methods of detection. Thus, this disadvantage of AmpArray assay is likely associated with the genes with minimal changes. The second limit of AmpArray is that it is insensitive to extremely high levels of mRNAs. For instance, ABCG2 was found to be up-regulated ~3000 times as determined by real-time PCR. However, only a ~6-fold difference was observed between MCF7 and MCF7/AdVp3000 cells when the AmpArray was used.

Because the limitations of the AmpArray as described above, validation using real-time quantitative RT-PCR is recommended to rule out false positive findings. Because AmpArray, similar to other available gene array technologies, measures only at the RNA level, the changes

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at the protein level need to be confirmed by western blot if antibody is available. In this study, we found that although ABCA4 is expressed at the RNA level with increased expression in MCF7/AdVp3000 cells. However, we failed to detect ABCA4 protein by western blot analysis using two different antibodies, suggesting that its mRNA may not be translated into proteins or the protein has very low stability. Furthermore, it should be noted that some genes with no or <1.5-fold alterations as determined by AmpArray may be false negatives. Although we did not perform real-time PCR analysis to validate all these ABC transporter genes, we did analyze ABCA2 gene using real-time PCR and found that it indeed had <1.5-fold decrease in the MCF7/AdVp3000 cells similar to that determined by AmpArray (data not shown).

Of the 47 ABC transporters tested using AmpArray, only 16 (including ABCG2) were found to have altered expression in MCF7/AdVp3000 cells. The remaining genes were either not detected or had less than 1.5-fold difference between MCF7 and MCF7/AdVp3000 cells. Some of these genes, such as ABCB1 and ABCC1 which are known to cause drug resistance when over-expressed, were not elevated in MCF7/AdVp3000 cells. These observations are consistent with previous finding that the use of verapamil, an inhibitor of ABCB1 and ABCC1, suppressed the selected over-expression of these two genes.

Of the 16 ABC transporters (Table 3) that had ≥1.5-fold changes in expression between the drug resistant MCF7/AdVp3000 and the parental MCF7 cells as detected by AmpArray, two genes (ABCA7 and ABCB3) were false positives as demonstrated by real-time RT-PCR (Fig. 2). The expression of both ABCA7 and ABCB3 was found to be decreased slightly in MCF7/AdVp3000 cells compared with the parental MCF7 cells using real-time RT-PCR instead of an increase as shown using AmpArray. The differential expression of the remaining 14 ABC genes were confirmed although the level of differences of some genes is different from that of the AmpArray analysis due to the semi quantitative nature of the AmpArray assay.

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Of these 16 genes, the expression of 8 genes (ABCA5, ABCA7, ABCA12, ABCB4, ABCB10, ABCC2, ABCC4, and ABCC11) did not reverse in MCF7/AdVpRev cells (Table 4A). However, the expression of ABCB10 and ABCC2 was increased significantly in the drug resistant MCF7/AdVp3000 cells as determined by real-time RT-PCR analysis (Fig. 2 and Table 4A). Because MCF7/AdVpRev is a partial revertant, ABCB10 and ABCC2 may still contribute to the reduced drug accumulation and increased resistance in MCF7/AdVp3000 cells despite that their altered expression was not reversed in the MCF7/AdVpRev cells. ABCC2 has previously been shown to cause drug resistance when over-expressed (Dean et al., 2001; Konig et al., 2003) whereas ABCB10 is a half transporter located in mitochondria without any known functions (Allikmets et al., 1995; Zhang et al., 2000).

The other 8 genes reversed their altered expression either completely or partially in the revertant MCF7/AdVpRev cells (Table 4B). Of these 8 genes, ABCG2, which was increased drastically in MCF7/AdVp3000 cells and experienced a complete reversion in MCF7/AdVpRev cells, has been shown previously to be one of the causes of drug resistance in MCF7/AdVp3000 cells (Doyle et al., 1998; Miyake et al., 1999).

Three transporters (ABCA3, ABCB3 and ABCA10) had partial or full reversion in the revertant MCF7/AdVpRev cell line and were down regulated in MCF7/AdVp3000 cells. None of these three transporters has been found previously to associate with transport of anticancer drugs. Because their expressions are down-regulated in the drug resistant MCF7/AdVp3000 cells, they may not be involved in increasing the efflux of anticancer drugs and, thereby, to effectively decrease cellular accumulation of these drugs in MCF7/AdVp3000 cells. However, ABCA3 has been suggested to transport lipid within cells (Yamano et al., 2001) while the substrate of ABCA10 is currently unknown and ABCB3 was thought to involve transporting peptide into ER lumen for antigen presentation (de la Salle et al., 1994). It is, thus, speculative that the altered

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lipid composition in MCF7/AdVp3000 cells due to decreased ABCA3 expression could contribute to the decreased drug accumulation and increased drug resistance in MCF7/AdVp3000 cells.

Four remaining genes (ABCA4, ABCC3, ABCC5, and ABCC8) were all found up-regulated in the resistant MCF7/AdVp3000 cells and their expressions were either completely or partially reversed in the revertant MCF7/AdVpRev cells. ABCA4 was previously thought to be exclusively expressed in retina and its mutation has been thought to be the cause of Stargardt's diseases (Cremers et al., 1998; Martinez-Mir et al., 1998). Currently, there is no report which suggests that ABCA4 could be involved in effluxing anticancer drugs. Its up-regulated expression by 120 fold in MCF7/AdVp3000 cells as determined by real-time RT-PCR (Fig. 2) and complete reversion in the revertant MCF7/AdVpRev cells suggests that it may also be able to transport anticancer drugs such as Adriamycin and mitoxantrone. However, we were unable to detect any ABCA4 at protein level in the plasma membranes or total cell lysates of MCF7/AdVp3000 cells by western blot analysis, casting doubt on if ABCA4 really contributes to the selected resistance in MCF7/AdVp3000 cells.

Both ABCC3 and ABCC5 have been associated with resistance to certain anticancer drugs (Borst and Elferink, 2002; Dean et al., 2001). We found that ABCC5 is up-regulated in MCF7/AdVp3000 cells by 2-3 fold and its reversion in MCF7/AdVpRev is limited. Furthermore, the substrates for ABCC5 are likely nucleoside analogue drugs (Wijnholds et al., 2000). Thus, ABCC5 may contribute little to the selected drug resistance in MCF7/AdVp3000 cells although its involvement in resistance to Adriamycin cannot be excluded, especially considering that its elevated expression was not reversed which could also contribute to the residual resistance in MCF7/AdVpRev cells. However, the expression of ABCC3 is up-regulated by 459 fold in MCF7/AdVp3000 cells and its expression is completely reversed in the MCF7/AdVpRev cells as

shown by real-time RT-PCR (Fig. 2) and confirmed by western blot (Fig. 3). Thus, ABCC3 likely contributes significantly to the decreased drug accumulation and increased resistance in MCF7/AdVp3000 cells. Indeed, we clearly demonstrated that silencing ABCC3 expression in the selected MCF7/AdVp3000 cells using siRNA reduced the Adriamycin resistance level, demonstrating that ABCC3 directly contributes to Adriamycin resistance. ABCC3 has been shown to transport several anticancer drugs including epipodophyllotoxins etoposide and teniposide, methotrexate, and vincristine although its direct role in Adriamycin resistance has not been demonstrated by introducing ABCC3 cDNA into HEK293 or murine fibroblast-like cells in the past (Kool et al., 1999; Zelcer et al., 2001; Zeng et al., 1999; Zeng et al., 2001; Zeng et al., 2000). The reason for the discrepancy between the current and these previous studies is not clear. However, it is possibly that the ABCC3 gene in MCF7/AdVp3000 cells selected by Adriamycin contains a spontaneous mutation which increases its affinity to Adriamycin. Such mutation ($G^{185}\rightarrow V^{185}$) which increases resistance to colchicine has been associated with ABCB1 in a cell line selected by colchicine (Choi, 1988). A spontaneous mutation of R^{482} in ABCG2 in drug-selected cells has also been observed to alter substrate specificity (Honjo et al., 2001). We are currently testing this possibility by cloning and sequencing the ABCC3 gene from MCF7/AdVp3000 cells.

ABCC8, also known as sulfonylurea receptor 1 (SUR1), functions as a modulator of ATP-sensitive potassium channels and insulin release (Matsuo et al., 2003). Previously, no studies have been reported on the potential role of ABCC8 in effluxing anticancer drugs and, thus, drug resistance. The finding that its expression is increased by 4 fold (Fig. 2) in MCF7/AdVp3000 cells and that this increase is completely reversed in the revertant MCF7/AdVpRev suggests that ABCC8 may contribute to some degree to the drug resistance in MCF7/AdVp3000 cells.

ABCA4, ABCC3, and ABCG2 are the only three ABC transporters that were increased drastically at mRNA level (~130-3000 fold) in the MCF7/AdVp3000 cells and then completely reversed in the revertant MCF7/AdVpRev cells. Previously, it has been shown that the ABCG2 gene was amplified in the MCF7/AdVp3000 cells (Miyake et al., 1999). While the ABCG2 gene maps to 4q22, the ABCA4 and ABCC3 genes map to 1p21.3 and 17q21.3, respectively (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowTOC&rid=mono_001.TOC&depth=2). Thus, it is unlikely that both ABCA4 and ABCC3 were amplified in the same amplicon together with ABCG2. It is, however, possible that these genes were selected independently during the stepwise selection of drug resistant MCF7 sublines which awaits further investigation.

Interestingly, in the Szakacs's study correlations have been found between drug sensitivity and expression of some ABC transporters by profiling 60 cancer cell lines in comparison with sensitivity to 1429 compounds (Szakacs et al., 2004). For example, ABCA4 expression was found to inversely correlate with cell sensitivity to 4 compounds. ABCC3 expression was inversely correlated with cell sensitivity to 11 compounds. Similarly, ABCC8 expression was also found to inversely correlate with sensitivity to one compound. These findings, together with our results, strongly suggest that the elevated expression of ABCC3 and ABCC8 are likely contributing to the drug resistance observed in MCF7/AdVp3000 cells.

In summary, we found in this study that the expression of several ABC transporters in addition to ABCG2 are changed in the drug-selected MCF7/AdVp3000 cells and that some of these transporters may contribute to the decreased accumulation of anticancer drugs and, thus, resistance to these drugs by the MCF7/AdVp3000 cells. This phenomenon of elevated expression of multiple ABC transporters may be wide spread in many cancer cell lines that were selected by anticancer drugs for resistant phenotypes and in cancers that have poor clinical

prognosis. Future studies on many drug-selected cell lines and clinical samples using AmpArray may likely help address this issue.

Acknowledgement

The authors wish to thank Dr. Susan Bates for providing the MCF7/AdVp3000, MCF7/AdVpRev cell lines and FTC, Dr. Robert S. Molday for providing ABCA4 monoclonal antibody, and Youmin Shu at GeneCopoeia for technical service on AmpArray analysis.

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Molecular Pharmacology Fast Forward. Published on May 18, 2005 as DOI: 10.1124/mol.105.011015
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Footnote

This work was supported in part by National Institutes of Health grants CA64539 and CA94961.

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Legends for Figures

Figure 1. AmpArray profiling of ABC transporters. AmpArray was performed as described in Materials and Methods using a cycler with a 96 well format. The PCR products were then separated by agarose gel electrophoresis (A). Panel B shows the template of ABC gene positions. S=sensitive (MCF7); R=resistant (MCF7/AdVp3000).

Figure 2. Validation using real-time RT-PCR analysis. Real-time RT-PCR was performed as described in Materials and Methods. Relative mRNA levels were measured using SYBR Green and calculated in the fold change ($2^{\Delta Ct}$) relative to MCF7 cells after normalization by the internal control, GAPDH.

Figure 3. Validation using western blot analysis. Western blots were performed using 10 μg of plasma membranes separated by SDS-PAGE followed by transferring to PVDF membranes probed using antibodies specific to ABCC3 (upper) and ABCG2 (middle). The equal loading of proteins was verified by a gel stained with commassie blue (lower panel).

Figure 4. Silencing and role of ABCC3 in Adriamycin resistance. MCF7/AdVp3000 cells were transfected with siRNAs targeting ABCC3 (si) or negative control siRNAs (scr) followed by western blot analysis to determine ABCC3 expression (A) or by SRB assay to determine the effect of decreased ABCC3 expression on resistance to Adriamycin in the presence of ABCG2 inhibitors FTC (B) and GF120918 (C).

Tables

Table 1. Primers for real-time RT-PCR

ABC Genes	Forward Primer	Reverse Primer
ABCA3	5’-CAGCTGGGCGAAGGTTTTC	5’-GAGATCTGGCTCACGGAGTAGTC
ABCA4	5’-GCTAAACAGCAGACTGAAAGTCATG	5’-AAAGATCAGTCCTGGGCTTGTC
ABCA5	5’-GGCTTATAAAATTCCTAAGGAAGATGTT	5’-CAAAAGCATGTTTAGCTTCTTCCA
ABCA7	5’-CTTCCTTTGGAACAGCCTTTTG	5’-GCTATGGGAGGTGAGCATCAC
ABCA10	5’-TCCTCTTTAATGGCGTATAAGTTACCT	5’-TCTGTTTCATCGCCTCTAACTTGA
ABCA12	5’-ACTCTGGAAGAGGTTTTCATCAACTT	5’-GGAACCTTGGCTGCTGGTATC
ABCB3	5’-AGCTGCGAAGATGATAAGGTGAT	5’-CCCTTCTCCCCTACATCTGTGT
ABCB4	5’-AACCTCAAATCCTCCTGTTGGAT	5’-AGGGCTTCTTGGACAACCTTTT
ABCB10	5’-CCAGTGTGGCTGAGATCCAA	5’-CAATCGCAATCCGCTGTTT
ABCC2	5’-TGTGGCCAGCCTGCAACT	5’-CCTCTGGCCTATGCTCAGGTT
ABCC3	5’-ACCCAGTTTGATACCTGCACTGT	5’-GGACCCTGGTGTAGTCCATGA
ABCC4	5’-TTGGACACGGTAACTGTTGCA	5’-GGAATGTCGGTTAGAGGTTTGG
ABCC5	5’-ATTTGGACCCCTTCAACCAGTAC	5’-GGTAGCTGAGCAATACATTCTTTCAT
ABCC8	5’-CACCATCGCGCATCGA	5’-GAGCAGCTTCTCTGGCTTATCG
ABCC11	5’-CACCGTGCTCGTCATTGC	5’-AATTCTACCACCTTCCCATTGC
ABCG2	5’-GCTTTCTACCTGCACGAAAACCAGTTGAG	5’-ATGGCGTTGAGACCAG

Table 2A. ABC genes that were not detected in either drug resistant MCF7/AdVp3000 and parental MCF7 cells.

Genes	Accession #
ABCA1	NM_005502
ABCA6	NM_080284
ABCA8	NM_007168
ABCA9	NM_080283
ABCA13	NM_152701
ABCB1	NM_000927
ABCB5	XM_291215
ABCB8	NM_007188
ABCB11	NM_003742
ABCC6	NM_001171
ABCC7	NM_000492
ABCC9	NM_005691
ABCC12	NM_033226
ABCC13	NM_138726
ABCD2	NM_005164
ABCG4	NM_022169
ABCG5	NM_022436
ABCG8	NM_022437

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Table 2B. ABC transporters with expression altered by <1.5 fold in drug resistant MCF7/AdVp3000 cells compared to parental MCF7 cells

Genes	Accession #
ABCA2	NM_001606
ABCB2	NM_000593
ABCB6	NM_005689
ABCB7	NM_004299
ABCB9	NM_019624
ABCC1	NM_004996
ABCC10	NM_033450
ABCD1	NM_000033
ABCD3	NM_002858
ABCD4	NM_005050
ABCF2	NM_005692
ABCF3	NM_018358
ABCG1	NM_004915

Table 3. ABC transporters with expression altered by ≥1.5 fold in drug resistant MCF7/AdVp3000 cells compared to parental MCF7 cells

Genes	Accession #	Fold ↑	Fold ↓
ABCA3*	NM_001089		1.5[b]
ABCA4*	NM_000350	12.8	
ABCA5*	NM_018672	2.0	
ABCA7[a]	NM_019112	2.0	
ABCA10*	NM_080282		2.7
ABCA12*	NM_015657		4.3
ABCB3[a]	NM_000544	2.1	
ABCB4*	NM_000443		2.0
ABCB10*	NM_012089	2.0	
ABCC2*	NM_000392	8.3	
ABCC3*	NM_003786	41.5	
ABCC4*	NM_005845		1.7
ABCC5*	NM_005688	1.6	
ABCC8*	NM_000352	11.0	
ABCC11*	NM_032583		6.5
ABCG2*	NM_004827	5.6	

*Confirmed by real-time PCR

[a] Different from real-time PCR

[b] The numbers are averages from two experiments

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Table 4A. ABC transporters without reversed expression in the revertant MCF7/AdVpRev cells

Genes	Fold Changes in Expression in MCF7/AdVp3000[a]	Reversed Expression in MCF7/AdVpRev
ABCA5	↑ 1.5 ± 0.4	No
ABCA7	↓ 1.7 ± 0.2	No
ABCA12	↓ 20 ± 4	No
ABCB4	↓ 4.2 ± 0.3	No
ABCB10	↑ 12 ± 8	No
ABCC2	↑ 4.3 ± 1.0	No
ABCC4	↓ 1.6 ± 0.3	No
ABCC11	↓ 3.1 ± 0.6	No

[a] The numbers were from a representative real-time RT-PCR experiment.

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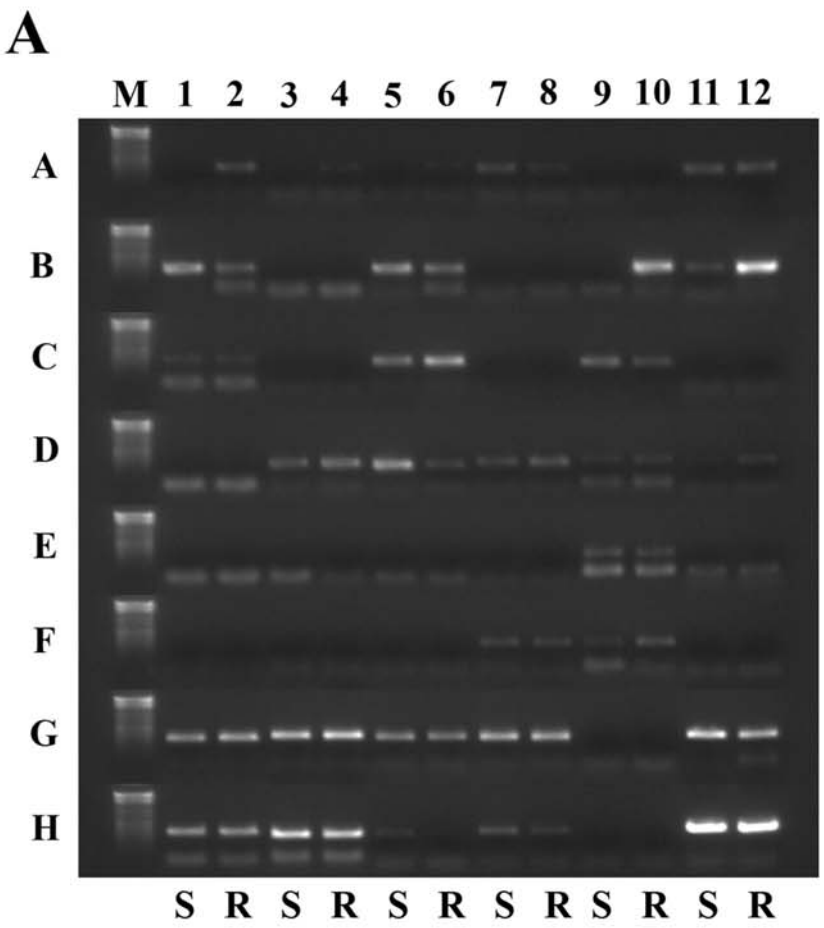
Table 4B. ABC transporters with reversed expression in the revertant MCF7/AdVpRev cells

Genes	Fold Changes in Expression in MCF7/AdVp3000 [a]	Reversed Expression in MCF7/AdVpRev
ABCA3	↓ 2 ± 0.2	Partial
ABCA4	↑ 132 ± 26	Full
ABCA10	↓ 14 ± 3	Partial
ABCB3	↓ 1.4 ± 0.01	Full
ABCC3	↑ 459 ± 79	Full
ABCC5	↑ 3 ± 0.2	Partial
ABCC8	↑ 4 ± 1	Full
ABCG2	↑ 2858 ± 620	Full

[a] The numbers were from a representative real-time RT-PCR experiment



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABCA4		ABCC8		ABCC2		ABCB4		ABCC7		ABCB2	
B	ABCA3		ABCC6		ABCA2		ABCB11		ABCC3		ABCG2	
C	ABCG1		ABCA1		ABCC5		ABCC9		ABCC4		ABCA8	
D	ABCB8		ABCB10		ABCA12		ABCA5		ABCA7		ABCB9	
E	ABCG4		ABCG5		ABCG8		ABCC12		ABCC10		ABCA9	
F	ABCA6		ABCA13		ABCB5		ABCD1		ABCB3		ABCB1	
G	ABCD3		ABCB7		ABCC1		ABCD4		ABCD2		ABCB6	
H	ABCF2		ABCF3		ABCC11		ABCA10		ABCC13		GAPDH	
	S	R	S	R	S	R	S	R	S	R	S	R

Figure 1

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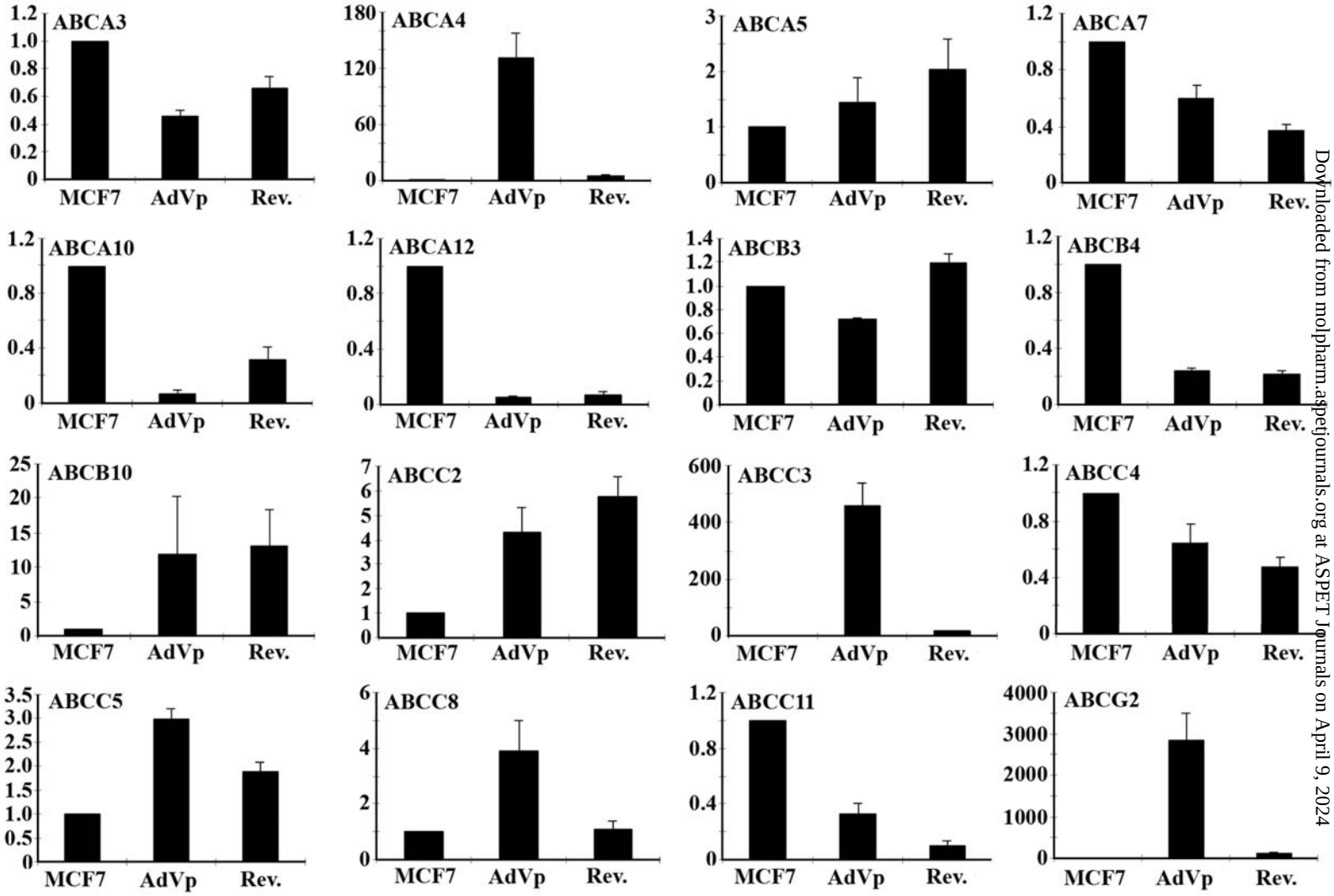

Figure 2

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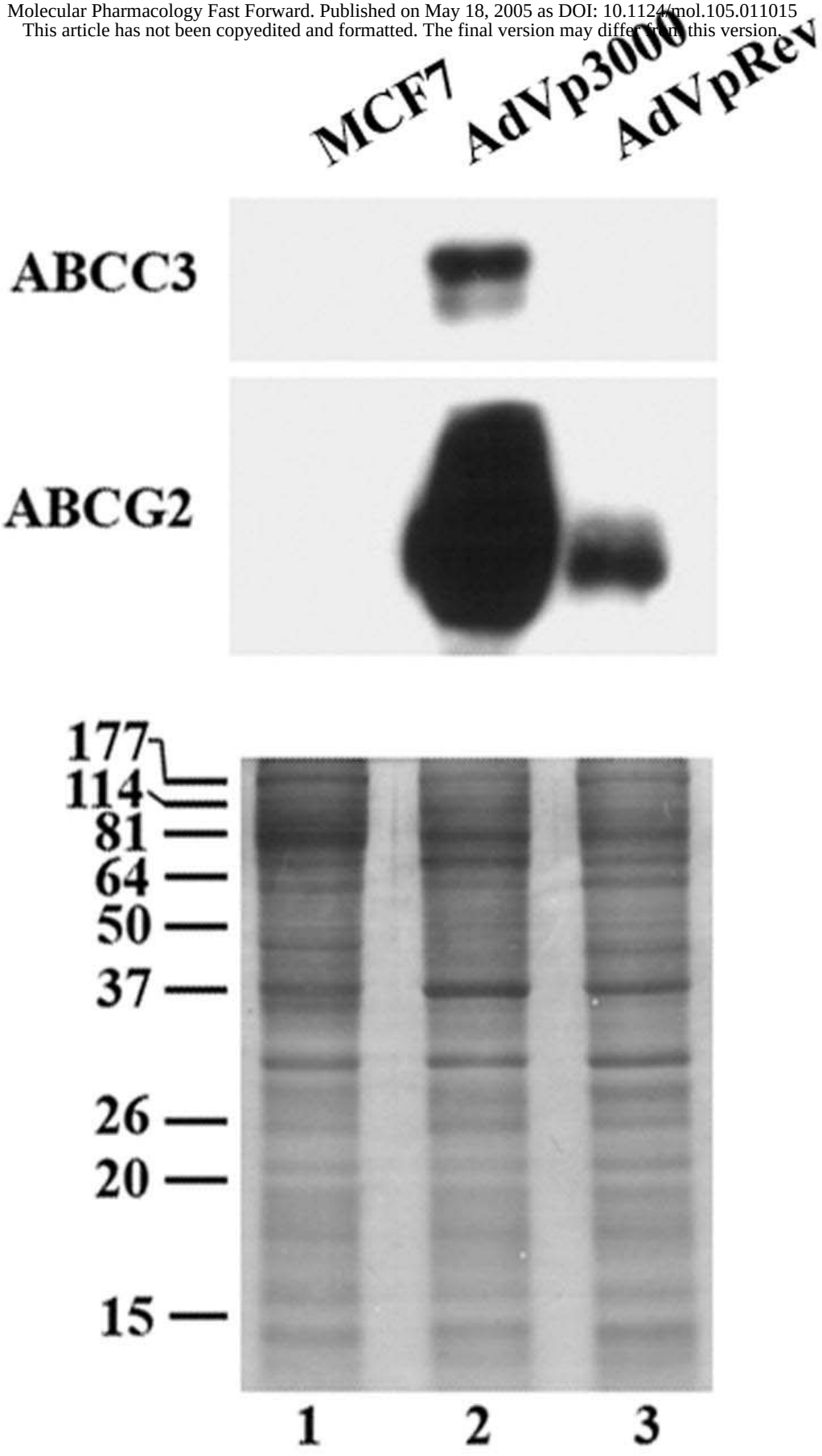

Figure 3

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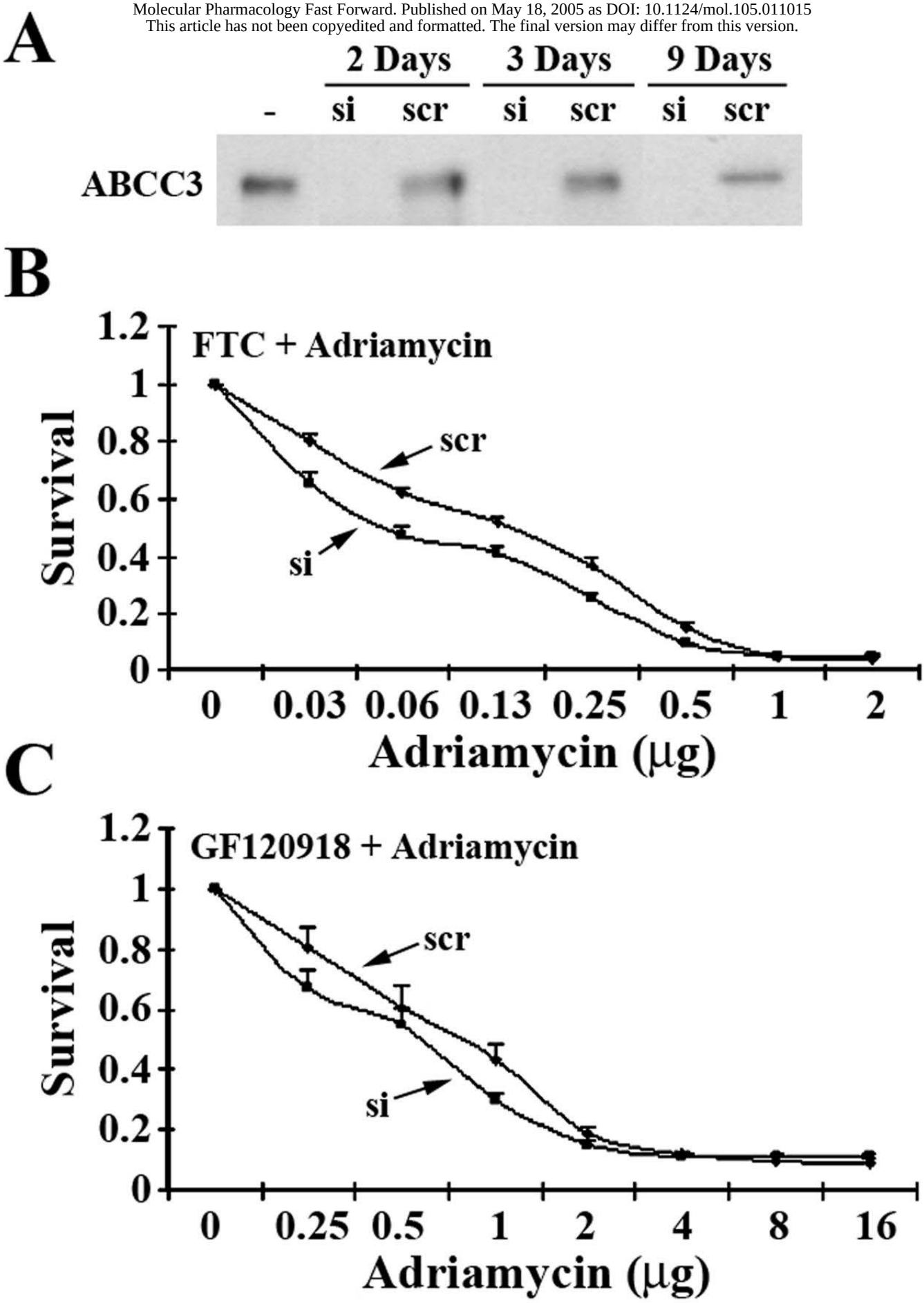

Figure 4