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# The molecular mechanisms of drug resistance in single-step and multi-step drug-selected cancer cells

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## Abstract

Multidrug resistance (MDR) remains one of the key determinants in chemotherapeutic success of cancer patients. Often, acquired resistance is mediated by the overexpression of ATP-binding cassette (ABC) drug transporters. To study the mechanisms involved in the MDR phenotype, investigators have generated a variety of *in vitro* cell culture models using both multi-step and single-step drug selections. Sublines produced from multi-step selections have led to the discovery of several crucial drug transporters including ABCB1, ABCC1 and ABCG2. Additionally, a number of mechanisms causing gene overexpression have been elucidated. To more closely mimic *in vivo* conditions, investigators have also established MDR sublines with single-step drug selections. Here we examine some of the multi-step and single-step selected cell lines generated to elucidate the mechanisms involved in the development of MDR in cancer cells.

### Keywords

Multidrug resistance; multi-step selection; single-step selection; ABC transporter; gene amplification; epigenetic changes

## Introduction

Cancer is one of the top ten leading causes of death in the world. In the United States alone, one of every four deaths will be due to cancer in 2008 (1). Advancements in early detection and cancer treatments have yielded significant progress, yet cancer deaths still outnumber deaths due to heart disease in people less than 85 years of age in the United States. A major factor in therapeutic failure for cancer involves the development of drug resistance to a variety of structurally unrelated anti-cancer drugs, also known as multidrug resistance (MDR) (2).

MDR can develop in several different ways, with the predominant mechanism being the overexpression of ATP-binding Cassette (ABC) drug transporters on the plasma membrane of tumor cells. These transporters act as energy driven pumps (3), and as such, maintain intracellular drug concentrations below toxic levels. Thus, the tumor survives, rendering the treatment ineffective. Tumors can demonstrate intrinsic drug resistance in which the tumor is innately resistant to treatment; this occurs in tumors originating from epithelial cells such as renal or adrenal tumors which naturally express ABC drug transporters (4). Acquired resistance, on the other hand, arises following therapy, and tumors normally present with the MDR phenotype subsequent to various genetic changes.

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ABC drug transporters belong to the largest superfamily of transporter proteins (5). Members of this family are recognized by a consensus ATP-binding site from 90 to 110 amino acids in length which also includes a linker region between two Walker motifs. In addition to two ATP-binding sites, ABC transporters normally possess two transmembrane domains. The 50 human ABC transporter genes are further subdivided into seven subfamilies (A-G) based on similar gene structure, order of the domains, and on sequence homology in their consensus domains (6). ABC drug transporters such as ABCB1, ABCC1 and ABCG2 are expressed in normal and tumor cells and are localized to different plasma membrane surfaces; the normal function of a number of these transporters is to efflux endogenous and xenobiotic metabolites from the cell (7). The substrate specificity for ABCB1, ABCG2, and the various ABCC family members overlaps extensively although the primary sequences of these transporters vary significantly (7). This phenomenon makes treatment of multidrug-resistant cancer unsuccessful in spite of the multitude of drugs available. Reports show that patients with ABCB1-positive tumors are three times more likely to fail a course of therapy than those who have tumors which are ABCB1-negative (8). Even more taxing for patients are tumors which express multiple ABC transporters, since overexpression is not mutually exclusive and a tumor can overexpress several MDR-linked ABC transporters in tandem.

Although over 12 of these transporters have been linked to MDR (9), little is known about the regulation of these transporters. Often multi-step drug selections have been employed to study the MDR phenotype. Several drawbacks are associated with this technique. The multi-step selections utilize higher concentrations of drug than those found in patients as well as extended periods of exposure. These factors produce pleiotropic effects. To avoid such issues, we recently employed a single-step selection to evaluate ABC transporter regulation. We reported that ABC transporter mRNA expression patterns vary with single vs. multi-step selection with doxorubicin in MCF-7 breast cancer cells (10). In multi-step selections with doxorubicin, ABCB1 is often the dominant ABC transporter causing MDR; we have shown that following a single-step selection using low concentrations of doxorubicin other transporters including ABCC2, ABCC4 and ABCG2 are overexpressed. In this chapter, we will review a number of the multi-step and single-step selected cancer cell lines which have been established and used extensively to investigate MDR (Table 1). In addition, we will discuss the mechanisms that have been ascertained in the development of these drug-resistant cell lines.

## Multi-Step Selected Cell Lines

To study MDR *in vitro*, investigators have utilized drug selections to generate resistant cell lines for over 30 years. Selections can be performed on individual clones or on mass populations of cells (11). To establish individual clones, the cells must be cloned so that an individual cell is the source for the entire population which will then be selected with the drug of choice. This technique boosts one major advantage in that a single gene will be responsible for the MDR. Alternatively, multifactorial MDR will result when an entire cell population is selected (11). The selection of a cell population, on the other hand, more closely mimics the clinical situation and can stem from a spectrum of mechanisms.

One of the original multi-step selected cell lines was established by the selection of an individual clone of KB epidermoid carcinoma cells with colchicine (12). The subsequent resistant sublines were generated with increasing single-step selections beginning with KB-3-1, an individual clone from a population of the KB cells. The *MDR*1 (*ABCB*1) gene was first identified from these cells. Using this same methodology, four additional KB sublines were created with selections in high concentrations of colchicine, vinblastine or doxorubicin (13). These sublines were selected with a step-wise selection, and all express

high levels of ABCB1. Since their establishment, the various resistant sublines of KB cells have been widely used to investigate MDR mediated by ABCB1.

In contrast to the KB cells, MCF-7 breast cancer cells were selected with doxorubicin using the mass population method, yet also expressed ABCB1 (14). This original selection was performed with increasing concentrations of doxorubicin beginning with 10 nM. The final resistant subline, MCF-7 AdrR, was capable of surviving in 10 μM doxorubicin. Later these original doxorubicin-resistant MCF-7 cells were determined to actually be OVCAR-8 ovarian cancer cells, which were resistant to doxorubicin (15). Their nomenclature has changed accordingly to NCI/ADR-Res or OVCAR-8/ADR (16). Other laboratories independently generated doxorubicin-resistant MCF-7 cells, and one such subline was established by culturing MCF-7 cells in 0.025 μg/ml doxorubicin and increasing the selection pressure by 2-fold until the cells grew in the presence of 2 μg/ml doxorubicin (17). Interestingly, these resistant cells also overexpressed ABCB1 and were karyotyped to match MCF-7 cells from ATCC (18). Doxorubicin-resistant sarcoma cells (MES-SA/Dx5) were also one of the early MDR models expressing ABCB1; these cells were selected with increasing concentrations of doxorubicin (19). Lastly, MCF-7 and MDA-MB-231 breast cancer cells exposed to increasing concentrations of docetaxel (up to 30 μM), known as MCF-7 TAX30 and MDA-MB-231 TAX30, were also found to overexpress ABCB1 (20). Moreover, ABCB1 was involved in MDR in highly resistant cell lines derived from both the individual clone and population selection techniques.

Yet in other more recent studies with MCF-7 cells using multi-step selections with lower concentrations of doxorubicin, both ABCG2 and ABCB1 were expressed (21; 22). In these studies concentrations ranging from 9 nM to 300 nM doxorubicin were employed. Similarly, MCF-7 cells selected with a multi-step selection with paclitaxel, 0.56 nM to a final concentration of 6.6 nM, also express both transporters, but at a lower level than the doxorubicin-selected MCF-7 cells (21; 22). Remarkably, paclitaxel is not a substrate of ABCG2, yet its selection pressure caused the overexpression of ABCG2. Furthermore, the investigators demonstrated that the clones were isogenic and that MDR was a consequence of adaptation and not a selection of a clone within the population. Involvement of other ABC transporters at lower selection concentrations suggests that ABCB1 may be dominant only at the later stages of MDR in particular cell types.

ABCC1 was first reported in a resistant cell line produced with a stepwise doxorubicin selection in a small cell lung cancer cell line, NCI-H69; this was called H69AR and did not express ABCB1 (23). Large-cell (COR-L23), small-cell (H69) and adenocarcinoma (MOR) lung tumor lines continuously selected with increasing concentrations of doxorubicin were also found to express ABCC1 (24; 25). Another small cell lung carcinoma cell line, $GLC_4$, was utilized in resistance studies, and ABCC1 was overexpressed when selected with doxorubicin concentrations augmented from 18 nM to 1,152 nM (26). Surprisingly, etoposide-selected MCF-7 cells also showed overexpression of ABCC1 instead of ABCB1. These cells were generated with a stepwise selection in increasing concentrations of etoposide starting with 200 nM up to 10 μM, and revertants were prevented by occasional reselection in 4 μM etoposide (27). Investigators reported that etoposide-selected H69 small cell lung cancer cells expressed both ABCB1 and ABCC1 at the mRNA and protein levels (28). During this selection process, ABCC1 expression preceded that of ABCB1. At a moderate level of ABCC1 expression, rather than continue increasing the expression of ABCC1, the cells turned on the *ABCB*1 gene (28). It also appears that cell type dictates the particular ABC transporter which is induced and that cells can activate more than one ABC transporter.

Investigators have also prepared a variety of resistant cell lines overexpressing ABCG2. For instance, MCF-7 cells selected with increasing concentrations of flavopiridol, MCF-7/FLV1000, expressed wild-type ABCG2 (29). MCF-7 cells selected in the presence of both doxorubicin and verapamil also overexpressed ABCG2; these cells are known as MCF-7 AdVp3000 (30). Unlike the MCF-7/FLV1000, the MCF-7 AdVp3000 expressed the mutant ABCG2 R482T. MCF-7 cells were also exposed to mitoxantrone and were found to overexpress wild-type ABCG2 (31). When S1 human colon carcinoma cells were selected with mitoxantrone, ABCG2 was also overexpressed (29). Additional sublines were generated when these original S1-M1-3.2 (32) were exposed to higher concentrations of drug, up to a final concentration of 80 μM. These S1 sublines also expressed a mutant R482G ABCG2 protein. Another example of MDR mediated by ABCG2 overexpression occurred in IGROV-1 human ovarian carcinoma cells selected with either topotecan or mitoxantrone (33). Similarly, SF295 human glioblastoma cells showed ABCG2 overexpression when selected in increasing concentrations of mitoxantrone ( 50 to 500 nM) (34). Unexpectedly, when a mitoxantrone-resistant small cell lung cancer cell line, $GLC_4$-MITO, was established, ABCA2 upregulation was found (35). In the case of ABCG2-overexpressing cell lines *in vitro*, two gain of function mutations have been identified (R482T and R482G). To our knowledge, no such mutations have been reported in clinical samples positive for ABCG2 to date.

## Mechanisms of Overexpression in Multi-Step Selected Cell Lines

Gene amplification is the most common method for drug-resistant cells to overexpress a particular ABC transporter. In a comprehensive analysis of 23 drug-resistant cancer cell lines derived from 10 different human cancers, it was revealed that changes in gene copy number were implicated in acquired drug resistance (36). Comparative genomic hybridization (CGH) was executed on drug sensitive and their corresponding drug-resistant sublines, and the regions of gain within the drug-resistant cell lines were consistent with regions encoding ABC transporters in 19 of the 23 cell lines. These changes were further confirmed by fluorescence *in situ* hybridization (FISH) analysis in these cells. Of particular interest were ABCA3, ABCB1 and ABCC9 which had a greater than 2-fold increase in copy numbers. Furthermore, gene amplification was in line with gene expression changes present in these resistant cells.

Amplified genes are either present in homogeneously staining regions or on extrachromosomal elements such as double-minutes. Investigations of resistant KB cells also showed gene amplification of the *ABCB*1 gene (37; 38). Double-minute chromosomes were identified in these KB resistant cell lines. Investigators also determined that KB cells could easily lose their resistance when no selection pressure was present because the gene amplification was only found in the form of double-minutes. Furthermore, in this KB resistant series, it was uncovered that in the less resistant sublines the *ABCB*1 was activated while in the more highly resistant sublines gene amplification occurred (39). For instance, later studies showed that with the higher colchicine selection pressure, double-minutes were stably maintained even after several months of continuous passaging in culture. The exact formation of the double-minutes in the sequential series of resistant sublines was closely examined using gel electrophoresis. Submicroscopic extrachromosomal circular DNA was revealed in the less resistant sublines. Consequently, the double-minutes uncovered in the more resistant sublines were formed by multimerization of these submicroscopic circular DNAs (39).

Gene amplification has also been reported for some of the other multi-step selected cell lines previously described. MES-SA/Dx5, doxorubicin-resistant sarcoma cells, displayed 7q21 alterations and gene amplification (40). However, these cells, unlike the KB resistant cells,

did not possess double-minutes as seen by FISH analysis. In the two breast cancer cell lines selected with docetaxel, MCF-7 TAX30 and MDA-MB-231 TAX30, gene amplification of chromosome 7q in the region which encodes for ABCB1 was discovered using CGH (20).

*ABCB*1 overexpression can also be caused at the transcription level. Investigators have found that in drug-sensitive cells only one transcription start site is used for *ABCB*1; nonetheless, drug-resistant cells which do not exhibit gene amplification often exploited more than one downstream transcription start site for *ABCB*1 (41). This substitution of other downstream transcription start sites for *ABCB*1 within the same cell line was a distinct mechanism which led to the identification of the MED-1 (Multiple start site Element Downstream) in many of the genes with a TATA-less promoter which have multiple start sites such as ABCB1 (42; 43). This MED-1, GCTCCC/G, was crucial for *ABCB*1 expression in drug-resistant cells in the cell lines examined. Likewise MEF1, *MDR*1 promoter-enhancing factor 1, also activated transcription but through an upstream promoter element, −118 to −111 (44).

Often drug selections can also cause alterations in genes that appear as gene rearrangements through nonhomologous recombinations. For example, investigators first reported a hybrid *ABCB*1 mRNA resulting from such a chromosomal rearrangement in a doxorubicin-selected S48-3s human colon adenocarcinoma cell line (45). In these cells, there was a 4;7 translocation resulting with the 3′ end of *ABCB*1 adjacent to a transcriptionally active chromosome 4 gene, thus triggering the activation of *ABCB*1 by the promoter sequences on the adjacent chromosome 4. For this particular gene rearrangement, the subsequent ABCB1 protein structure remained unaltered due to the rearrangement occurring within the 5′ region of *ABCB*1. Follow-up studies illustrated that eight other selected cells and two clinical samples had gene rearrangements (45; 46). Unexpectedly, these gene hybrids differed, suggesting that *ABCB*1 merely required a sufficiently active promoter to activate it and that the specific promoter was irrelevant. Other *ABCB*1 mRNA hybrids have also been reported (47). These hybrids were regulated by nearby genomic sequences with similarity to a retroviral element. Nevertheless, no chromosomal rearrangements were discovered in these hybrids.

Epigenetic changes have also been reported to activate *ABCB*1. In the repressed state, the *ABCB*1 promoter is methylated and enriched in methyl-CpG binding protein 2 (MeCP2). In MCF-7 cells exposed to a stepwise selection with doxorubicin, ABCB1 is overexpressed and in the resistant cells the *ABCB*1 promoter is completely unmethylated (48). The promoter methylation status of *ABCB*1 is inversely correlated to the expression of the *ABCB*1 gene. The loss of methylation at the promoter facilitates the activation of *ABCB*1 in these resistant cells.

In the resistant cell lines which displayed ABCC1 overexpression, gene amplification was also the most common mechanism. The original ABCC1-overexpressing cell line H69AR demonstrated gene amplification with Southern blot analysis (23). Many double-minutes of chromosome 16 were discovered in the COR-L23/R cells, while the MOR cells exhibited an enlarged copy of chromosome 16 with homogeneously staining regions (24). As with the cells overexpressing *ABCB*1, highly resistant cell lines such as $GLC_4$/ADR (26) , COR-L23/R and MOR/R predominantly displayed gene amplification of *ABCC*1 as the mode of gene overexpression. On the contrary, transcriptional activation of *ABCC*1 was solely responsible for gene overexpression in the weakly resistant SW-1573 30.3M subline, which had been selected with low concentrations of doxorubicin (49) . Of the highly resistant cell lines, only MOR/R presented a combination of gene amplification and gene activation, whereas gene amplification was the main mechanism for gene overexpression in the $GLC_4$/ADR and COR-L23/R selected cell lines.

For resistant cell lines overexpressing ABCG2, Southern analysis of MCF-7 AdVp3000 and S1-M1-80 sublines uncovered that only MCF-7 AdVp3000 had gene amplification (50). In later studies, these cell lines as well as MCF-7/MX were further characterized by CGH, FISH, spectral karyotyping and Southern blotting (51). No amplification was confirmed in the S1-M1-80 subline, while the other two cell lines showed amplification with multiple translocations of chromosome 4. Other investigators evaluated the MCF-7/MX subline and also showed gene amplification by Southern blot analysis (52). In the SF295 MX selected cells, ABCG2 was found amplified by Southern analysis. The sublines selected with the lower concentrations displayed double-minutes containing the *ABCG*2 gene when examined with FISH and spectral karyotyping. At 500 nM mitoxantrone selection pressure, a homogeneously staining region was found integrated into the chromosome causing *ABCG*2 overexpression (34). Furthermore, Boonstra and colleagues also found gene amplification with CGH in the $GLC_4$-MITO cells for ABCA2 on chromosome 9 (35).

Analogous to results from *ABCB*1 promoter studies in drug-resistant cells overexpressing ABCB1, *ABCG*2 has also been shown to have multiple transcription start sites in drug-selected cells (53). Investigators have also reported the expression of novel 5′UTR variants of transcripts that possess different translation efficiencies. Thus, the ABCG2 protein expression is directed at the posttranscriptional level as a consequence of these 5′UTR variants. However, no gene rearrangements in the 5′UTR region were seen.

Various epigenetic changes have also been found in multi-step selected cell lines that overexpress ABCG2. Chromatin immunoprecipitation (ChIP) has been used to identify histone modifications in these multi-step selected cells. In the S1-M1-80 cells, which show no gene amplification, the *ABCG*2 proximal promoter displayed histone H3 acetylation (54). Further epigenetic changes were present in this subline as HDAC1 and HDAC3 bound less to the proximal promoter of *ABCG*2. More importantly, Pol II binding, an indicator of *ABCG*2 promoter activity, was enhanced in these resistant cells.

MicroRNAs (miRNA) have also been implicated in the regulation of genes. Recent reports investigated the effects of miRNA on *ABCG*2 expression. S1 colon cells possessed a longer 3′UTR in the *ABCG*2 mRNA where a putative hsa-miR-519c binding site exists (55). This miRNA binds to this site, causing translational repression and mRNA degradation in the sensitive parental cells. Conversely, the S1-M1-80 cells utilize a noncanonical AUUAAA poly (A) signal to yield a much shorter 3′UTR lacking this miRNA binding site, thus eluding degradation mediated by hsa-miR-519c. It appears that a combination of epigenetic and miRNA mediated changes are responsible for the overexpression of ABCG2 in the highly resistant S1-M1-80 cells.

## Single-Step Selected Cell Lines

These and many other multidrug resistant cancer cell lines have been established *in vitro* through continual drug selection. Although they offer a sufficient means for investigating the regulation of ABC transporter expression and function, rarely do these continual drug selections emulate what is found in the clinical setting. Thus, insight into the development of MDR by ABC transporters at clinically relevant concentrations and ascertaining which factors induce upregulation of ABC transporters during the initial steps of MDR will afford more advantageous measures to circumvent MDR. Our goal was to evaluate the expression of ABC transporters following a short low dose selection which would simulate the conditions encountered *in vivo* and to compare the gene expression levels of MDR-linked ABC transporters in these sublines selected by a low-dose single step to an established high-dose doxorubicin selected subline (17) and to determine if overexpression of the same ABC transporters occurs.

We have recently found that ABC transporter mRNA expression patterns vary with single- vs. multi-step treatment with doxorubicin in MCF-7 breast cancer cells. We established single-step doxorubicin-selected MCF-7 sublines using very low concentrations (14 or 21 nM) (10). Individual clones were selected from a population of 10,000 cells in a 100 × 20mm tissue culture dish exposed to drug for 10 days. Clones were then maintained in drug-free medium following the initial drug selection. We compared these single-step sublines to a previously established multi-step doxorubicin-selected MCF-7 subline (17) known to overexpress only ABCB1 at the mRNA and protein levels (Figure 1) due to gene amplification. We evaluated a number of ABC transporters and found that *ABCC*2, *ABCC*4 and *ABCG*2 were overexpressed at the mRNA level in these single-step selected sublines (Figure 2). Yet, only ABCC4 and ABCG2 were overexpressed at the protein level. Both 14 and 21 nM single-step doxorubicin-selected sublines exhibited nearly 5-fold resistance to doxorubicin compared to parental MCF-7 cells. However, ABCC4 did not confer resistance to this drug, suggesting that ABCG2 was the major transporter responsible for the development of doxorubicin resistance. Sequencing of ABCG2 in the single-step selected sublines revealed that our *in vitro* selection resulted in the overexpression of the wild-type ABCG2 and not the gain of function mutations either G or T at amino acid 482. SiRNA studies further confirmed that mainly ABCG2 confered drug resistance in these clones. We also observed that the upregulation of ABCG2 was facilitated by histone hyperacetylation of H3 at the proximal promoter of *ABCG*2. Similar to what was found in the S1-M1-80 cells, Pol II binding was increased while HDAC1 binding was decreased in the single-step selected sublines. This was the first report of ABCG2 overexpression in MCF-7 cells following a short term low dose selection with doxorubicin.

To further evaluate if this ABCG2 overexpression was drug and cell line independent, we generated additional sublines of MCF-7 cells with a single-step selection using 300 nM etoposide and two different cancer cell lines, IGROV-1 ovarian cancer cells and S-1 colon tumor cells, with 14 and 21 nM doxorubicin, respectively. To ensure that we were not selecting for a resistant clone, several lower etoposide concentrations, 50, 100 and 200 nM, were first evaluated. These lower selections indicated that all MCF-7 parental cells were able to survive. For IGROV-1 cells, only the 14 nM doxorubicin selection yielded resistant cells. Five sublines derived from IGROV-1 cells obtained using 14nm doxorubicin and S-1-resistant clones, obtained at a 21 nM doxorubicin concentration were further characterized. Furthermore, we were also able to replicate the upregulation of the same ABC transporters in the MCF-7 cells using this single-step selection with 300 nM etoposide. ABCG2 was also the dominant overexpressed ABC transporter for these additional sublines. This suggests that even a low dose selection can bring about MDR and that ABCG2 overexpression mediates the early stages of MDR development in certain cell lines. ABCG2 may be protecting against the cytotoxic effects of drugs in our single-step-selected clones, as it does in stem cells (56). Analysis of other mammary stem cell markers in our single-step sublines demonstrated that we did not enrich for cancer stem cells during the single-step selections of these clones. Taken together with the epigenetic alterations that were discovered in these resistant sublines, adaptation as opposed to selection appears to be the mitigating factor for this selection process.

Single-step selections have also been performed with the MES-SA, human sarcoma cell line. This protocol used the mass population selection technique, where cells were first plated in a 96-well plate, treated for two weeks with 40 nM doxorubicin, grown drug free for two additional weeks, and then individually harvested from each well (57). As with the MES-SA/Dx5, all clones examined expressed ABCB1. The authors used fluctuation analysis to determine that the doxorubicin-resistant clones were derived due to spontaneous mutations. Additionally, no chromosomal alteration or gene amplification was discovered in these single-step mutants (40). When either etoposide or paclitaxel was used in single-step

selections with MES-SA cells, authors found that either no ABCB1 overexpression occurred (58) or that only 44% of the clones expressed ABCB1 (59), respectively. Etoposide-selected MES-SA cells showed a reduction in topoisomerase II but no ABC transporter increases. This suggests that ABCB1 substrates have different effects when selecting for ABCB1-expressing clones. Furthermore, a single-step selection with 40 nM doxorubicin in the presence of an ABCB1 inhibitor, PSC833, also produced no detectable levels of ABCB1 but rather decreased levels of topoisomerase II α (60).

In recent follow-up studies, the authors found that an increase in acetylated H3 modified the chromatin structure of *ABCB*1 far upstream, 968-bp proximal to the upstream promoter, and initiated upstream transcripts for these single-step selections (61). Equally important, the authors confirmed that these upstream *ABCB*1 transcripts were spontaneous in nature given that a clonal variant expanded to several million cells without any drug selection also produced these *ABCB*1 upstream variants.

Other single-step selections have generated sublines which overexpress ABCC1. One such example was the H82, a variant of small cell lung cancer, which was selected for 18 hours with 69 nM epirubicin. This initial selection yielded a drug-resistant cell line which was then subsequently selected for 18 hours with 14 nM epirubicin, producing an even more resistant line known as H82/E8 (62). These sublines displayed two- to nine-fold resistance. Remarkably, the H82/E8 subline remained stably resistant for over two years without further drug treatment. Investigators also selected H69 cells with eight treatments of 14 nM epirubicin followed by maintenance in drug-free medium. This subline is referred to as H69/E8. Only the H82/E8 increased *ABCC*1 expression (62) while neither cell line expressed *ABCB*1. Other investigators using a 50 nM single-step doxorubicin-selection with $GLC_4$ small cell lung cancer cells attributed an increase in ABCC1 expression to the activation of the JNK pathway (63).

## Conclusion

Drug selections with both clonal and cell populations have aided in the study of MDR mediated by ABC transporters. For instance, these *in vitro* techniques have led to the identification of at least three of the most influential ABC drug transporters for MDR. The overexpression of a particular ABC transporter during drug selection appears to depend on a multitude of factors which include but are not limited to the cell type, the selection regimen, the drug used for selection pressure as well as the concentrations utilized. These factors suggest that a number of ABC transporters should be evaluated following drug selection in addition to ABCB1. The single-step selection is capable of generating sublines with the MDR phenotype at clinically relevant concentrations while eliminating pleiotropic effects due to long-term drug exposure. With advancements in techniques for analysis at the molecular level and better understanding of gene regulation in the presence of drug, future studies should focus on translational research to improve the success rate of cancer therapies.

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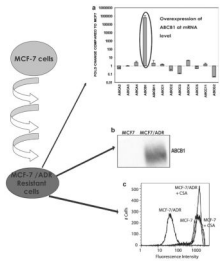


**Figure 1. Analysis of ABC transporter expression and function in the multi-step doxorubicin-selected MCF7/ADR cell line**

**A)** The delta-delta CT method was used to determine the fold change of ABC transporter gene expression in the multi-step doxorubicin-selected cells, MCF7/ADR (17), compared to their parental line, MCF-7. The values represent the mean and standard deviation (n=2). The overexpression of ABCB1 is highlighted by the black circle. **B)** Using C219, the ABCB1-specific monoclonal antibody, the relative quantities of ABCB1 were determined for MCF7/ADR and MCF-7 in whole-cell lysates. Lane 1, MCF-7 control and lane 2, MCF7/ADR, (100,000 cells for all samples). **C)** Calcein-AM efflux assays were performed using flow cytometry. Assays compared MCF-7 and MCF7/ADR. Charcoal grey histogram, MCF7/ADR; dark grey histogram, MCF7/ADR cells in the presence of 10 μM cyclosporine A (CSA); black histogram, MCF-7, and light grey histogram, MCF-7 in the presence of cyclosporine A. The schematic on the far left side depicts the multi-step selection with doxorubicin in 0.025 μg/ml doxorubicin and increasing the selection pressure by 2-fold until the cells grew in the presence of 2 μg/ml doxorubicin.

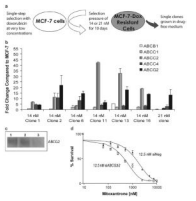

**Figure 2. Single-step doxorubicin-selected clones overexpress ABCG2**

**A)** The schematic of the single-step selection for the MCF-7 cells with doxorubicin. **B)** Characterization of selected ABC transporter gene expression levels in several single-step clones. Doxorubicin-resistant MCF-7 clones were established employing a single step selection with either 14 or 21 nM treatment for 10 days followed by culturing continuously in drug-free medium. The average fold change compared to parental MCF-7 cells ± SD (n=4) was calculated using delta delta Ct method from real-time RT-PCR data. Reference gene is *PMCA*4 (64). The key for selected five ABC transporters is given in the figure. **C)** Western blotting analysis of ABCG2 protein using BXP-21 antibody following no treatment (lane 1), 50 nM negative siRNA treatment (lane 2) and 50 nM G2-2 siRNA treatment (lane 3). **D)** Cytotoxicity assays with mitoxantrone evaluating the effect of silencing ABCG2 in the 21 nM single-step clone. Dose response curves were derived from three independent experiments using the CCK-8 assay. White box, 21 nM cells with 12.5 nM G2-2 siRNA and black triangle, 21 nM cells with 12.5 nM negative siRNA. Error bars indicate standard deviation (n=3).

**Table 1**

List of Select Multi-step and Single-Step Selected Cell Lines Overexpressing ABC Transporters

| Selection Regimen | Cell Line | Drug | ABC Transporter Overexpressed |
|---|---|---|---|
| Multi-step | KB-3-1 | Colchicine | ABCB1 (*13*) |
| | | Doxorubicin | ABCB1 (*13*) |
| | | Vinblastine | ABCB1 (*13*) |
| Multi-step | OVCAR-8 | Doxorubicin | ABCB1 (*14; 15*) |
| Multi-step | MCF-7 | Doxorubicin | ABCB1 (*17*) |
| Multi-step | MES-SA | Doxorubicin | ABCB1 (*19*) |
| Multi-step | MCF-7 | Docetaxel | ABCB1 (*20*) |
| Multi-step | MDA-MB-231 | Docetaxel | ABCB1 (*20*) |
| Multi-step | MCF-7 | Doxorubicin | ABCB1 and ABCG2 (*21; 22*) |
| Multi-step | MCF-7 | Paclitaxel | ABCB1 and ABCG2 (*21; 22*) |
| Multi-step | NCI-H69 | Doxorubicin | ABCC1 (*23*) |
| Multi-step | COR-L23 | Doxorubicin | ABCC1 (*24; 25*) |
| Multi-step | MOR | Doxorubicin | ABCC1 (*24; 25*) |
| Multi-step | $GLC_4$ | Doxorubicin | ABCC1 (*26*) |
| Multi-step | MCF-7 | Etoposide | ABCC1 (*27*) |
| Multi-step | NCI-H69 | Etoposide | ABCB1 and ABCC1 (*28*) |
| Multi-step | MCF-7 | Flavopiridol | ABCG2 (*29*) |
| Multi-step | MCF-7 | Doxorubicin and verapamil | ABCG2 (*30*) |
| Multi-step | MCF-7 | Mitoxantrone | ABCG2 (*31*) |
| Multi-step | S1 | Mitoxantrone | ABCG2 (*32*) |
| Multi-step | IGROV-1 | Topotecan | ABCG2 (*33*) |
| Multi-step | IGROV-1 | Mitoxantrone | ABCG2 (*33*) |
| Multi-step | SF295 | Mitoxantrone | ABCG2 (*34*) |
| Multi-step | $GLC_4$ | Mitoxantrone | ABCA2 (*35*) |
| Single-step | MCF-7 | Doxorubicin | ABCC4 and ABCG2* (*10*) |
| Single-step | MCF-7 | Etoposide | ABCG2 (*10*) |
| Single-step | IGROV-1 | Doxorubicin | ABCG2 (*10*) |
| Single-step | S1 | Doxorubicin | ABCG2 (*10*) |
| Single-step | MES-SA | Doxorubicin | ABCB1 (*57*) |
| Single-step | MES-SA | Paclitaxel | ABCB1 (*59*) |
| Single-step | NCI-H82 | Epirubicin | ABCC1 (*62*) |
| Single-step | $GLC_4$ | Doxorubicin | ABCC1 (*63*) |

In all cases the overexpression of an ABC transporter was demonstrated at the functional level and the references are given in the parenthesis.

* ABCG2 is the ABC transporter responsible for MDR and ABCC4 does not confer resistance to doxorubicin.