Identification and validation of compounds selectively killing resistant cancer: delineating cell line specific effects from P-glycoportein-induced toxicity

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Abstract

Despite significant progress, resistance to chemotherapy is still the main reason why cancer remains a deadly disease. An attractive strategy is to target the collateral sensitivity of otherwise multidrug resistant (MDR) cancer. In this study our aim was to catalogue various compounds that were reported to elicit increased toxicity in P-glycoprotein (Pgp) overexpressing MDR cells. We show that the activity of most of the serendipitously identified compounds reported to target MDR cells is in fact cell-line specific, and is not influenced significantly by the function of Pgp. In contrast, novel 8-hydroxyquinoline derivatives that we identify in the NCI DTP drug repository possess a robust Pgp-dependent toxic activity across diverse cell lines. Pgp expression associated with the resistance of the doxorubicin resistant $Brca1^{-/-}$;$p53^{-/-}$ spontaneous mouse mammary carcinoma cells could be eliminated by a single treatment with NSC57969, suggesting that MDR-selective compounds can effectively revert the MDR phenotype of cells expressing Pgp at clinically relevant levels. The discovery of new MDR-selective compounds shows the potential of this emerging technology and highlights the 8-hydroxyquinoline scaffold as a promising starting point for the development of compounds targeting the Achilles heel of drug resistant cancer.

Introduction

Targeted therapies, designed to counter cancer-specific pathways, have a substantial impact on patient survival in several cancers including breast cancer, chronic myelogenous leukaemia, non-small-cell lung cancer or metastatic melanoma. However, even among those patients that show initial response to targeted therapy, drug-resistant clones frequently evolve by a variety of mechanisms, especially when single drugs are used. Ultimately, resistance to chemotherapy results in treatment failure, which is the main reason why cancer remains a deadly disease. Common resistance mechanisms rely on mutations that alter the target so that the drug is no longer able to interact with it. In addition, resistant cells often upregulate alternative tumor promoting pathways that reignite tumor growth. Similarly to the strategies deployed against drug-resistant infections, patient-specific combination of targeted drugs may represent a powerful approach for treating resistant cancer (1). In principle, by targeting several pathways, combination therapies increase the complexity of resistance mechanisms that are required to combat treatment. Unfortunately, side effects usually limit the scope of combinations. In addition, cancer cells respond with mechanisms that are able to confer simultaneous resistance against several drugs. By modifying pathways controlling apoptosis, epithelial–mesenchymal transition (EMT) or DNA repair, multidrug resistant (MDR) cancer cells are able to cope with multiple cytotoxic agents with distinct targets (2). One of the best characterized mechanisms of multidrug resistance is the increased drug efflux mediated by ATP-binding cassette (ABC) transporters (3)(4). ABC proteins, present in all living organisms from prokaryotes to mammals, are transmembrane proteins that control the passage of their substrates across membrane barriers. ABC transporters make up a complex cellular defense system responsible for the recognition and the energy-dependent removal of environmental toxic agents entering the cells or organisms (5). In cancer, MDR transporters act as a primary

shield that keeps intracellular chemotherapy drug levels below a cell-killing threshold. Cancer cells overexpressing pumps become multidrug resistant, as the promiscuity of the transporters allow the efflux of most clinically used anticancer agents. Both clinically and experimentally, the most important MDR transporter proved to be P-glycoprotein (Pgp), showing the widest substrate specificity encompassing anticancer drugs, HIV-protease inhibitors, immunosuppressive agents, antiepileptics and several targeted agents. The contribution of Pgp to poor chemotherapy response was convincingly demonstrated in hematological malignancies, sarcomas, breast cancer, and other solid cancers (4). Recently, acquired doxorubicin resistance was associated with increased expression of the mouse Mdr1 genes in a genetically engineered mouse model for BRCA1-related breast cancer. Significantly, even moderate increases of Mdr1 expression were found to be sufficient to cause doxorubicin resistance, which could be reversed by the third-generation Pgp inhibitor tariquidar (6). These results confirm that Pgp indeed plays a pivotal role in causing drug resistance in a realistic model of cancer (7).

There is a constant need to develop drugs that retain activity in resistant cancer with minimal side-effects. Unfortunately, clinical studies conducted with several generations of Pgp inhibitions have failed, and the pharmaceutical industry seems to have abandoned the concept of Pgp inhibition. Thus, new ideas, leading to novel therapeutic solutions are needed to circumvent efflux-based resistance mechanisms. An alternative strategy to overcome MDR may rely on the concept of collateral sensitivity (CS). Since the first description of CS in 1952 (8), several investigators have attempted to exploit increased susceptibility of otherwise resistant bacteria. In cancer, CS may be considered as a form of synthetic lethality ensued by the resistance phenotype (such as Pgp) and the toxicity of a drug (9). Historically, one of the first compounds shown to elicit selective toxicity against Chinese Hamster Ovary (CHO) cells

selected to be resistant against vincristine or colchicine (10) (11) was verapamil, which was also one of the first Pgp-inhibitors tested in clinical trials (12). The paradoxical hypersensitivity of Pgp-expressing multidrug-resistant cells was initially perceived as a curious anomaly (13). As more and more compounds were shown to specifically target resistant cells, it became clear that the CS conveyed by Pgp represents a promising strategy for targeting MDR cancer (14). However, a major limitation of these findings is that the causative role of P-glycoprotein in CS was not assessed systematically, and thus the contribution of further cellular alterations associated with the particular model could not be excluded. In this study our aim was to catalogue serendipitously identified compounds that were reported in the literature to elicit increased toxicity in Pgp overexpressing MDR cells. In particular, we wanted to assess the influence of Pgp on the toxicity by using a panel of parental and MDR cell lines. We show that the activity of most of the compounds reported to target Pgp-expressing MDR cells is in fact cell-line specific, and is not significantly influenced by the function of P-glycoprotein. In contrast, novel MDR-selective compounds that we identify in the NCI DTP drug repository retain a P-glycoprotein–dependent toxic activity across diverse cell lines and primary cells established from a realistic mouse model of MDR breast cancer (15).

Materials and methods

Drug database. ABCB1 mRNA levels of the NCI-60 cell panel were correlated to the toxicity profiles of approximately 50,000 molecules (December 2010 release), of which more than 15% were not accessible at the time of our previous study (16). Candidate MDR-selective compounds were identified based on correlation of ABCB1 expression to the cytotoxicity patterns of drugs that were measured inside the range of cytotoxicity with no >50% missing values (16,17).

Chemicals. Unless otherwise stated, NSC-compounds were acquired from DTP's drug repository; chemicals were purchased from Sigma Aldrich. NSC733435 was purchased from ChemBridge (San Diego, C., USA), Dp44mT was obtained from Merck. P85 was kind gift from Dr. R. Mészáros (ELTE University, Hungary). Tariquidar was a kind gift from Dr. S. Bates (NCI, NIH). Desmosdumotin B analogues were synthesized by Szintekon (Debrecen, Hungary). KP772 was synthesized as described by Hart and Laming with slight modifications (18).

Cell lines and culture conditions MES-SA, the doxorubicin selected MES-SA/Dx5, MDCKII and A431 cells were obtained from ATCC where they were characterized by DNA fingerprinting; KB-3-1 and the vinblastine selected KB-V1 were a kind gift from Dr. Michael M. Gottesman (NIH); HCT-15 cells were obtained from DTP (DCTD Tumor Repository, National Cancer Institute at Frederick, Maryland), where the identity of cell lines is extensively characterized. MDCKII-ABCB1 and A431-ABCB1 cell lines were established as described (19). KB-V1, and MES-SA/Dx5 cells were treated with 300 nmol/L vinblastine, and 500 nmol/L doxorubicin, respectively, to ensure Pgp expression. Multiple frozen aliquots were established upon the acquisition of cells. Experiments were performed using cells passaged fewer than 20 times after reviving from liquid N_2. Cells were periodically tested and resulted negative for mycoplasma contamination with the MycoAlert™ mycoplasma detection kit (Lonza, Hungary). Cells were cultured in DMEM (SigmaAldrich, Hungary) or RPMI (HCT-15), supplemented with 10% fetal bovine serum, 5 mmol/L glutamine, and 50 units/mL penicillin and streptomycin (Life Technologies). All cell lines were cultivated at 37 °C, 5% CO2.

Cell viability assay. Viability was assessed by the MTT as described previously (16); or the PrestoBlue® assay (Life Technologies, USA), according to the manufacturer's instructions. Curves were fitted by Graph Pad Prism 5 software using the sigmoidal dose–

response model. Curve fit statistics were used to determine IC_{50} values. Differences between the IC_{50} values were analyzed by two-sided unpaired Student's t test and results were considered statistically significant at a P value of <0.05 (*) or 0.01 (**).

Immunocytochemistry. 5000 or 20000 cells (MES-SA, MES-SA/Dx5 and KB-3-1, KB-V1, respectively) were plated in 8-well chambers (Thermo Scientific). 24 hours later the cells were fixed with 4% paraformaldehyde in Dulbecco's modified phosphate-buffered saline (DPBS) for 15 min at room temperature. After three washing steps with DPBS, cells were treated with DPBS containing 2 mg/mL BSA, 1% fish gelatin, 5% goat serum, and 0.1% Triton-X 100 for 1 h at room temperature. The samples were then incubated for 1 h at room temperature with anti-P-glycoprotein antibody (1:500 MRK16, Kamiya Biomedical) or LAMP1 (1:200 L1418 polyclonal, Sigma Aldrich). After washing with DPBS, the cells were incubated for 1 h at room temperature with phycoerythrin- or Alexa488-conjugated (Pgp) goat anti-mouse IgG antibody (Life Technologies), and AlexaFluor546 (LAMP1) goat anti-rabbit IgG antibody diluted in blocking solution (1:250). Nuclei were stained with DAPI (Dojindo Molecular Technologies). The stained samples were examined by an Axioscope 2 fluorescent microscope (Zeiss, Thornwood, NY) and a Zeiss LSM 710 confocal laser scanning microscope.

Flow cytometry. Annexin V/Propidium iodide (PI) based apoptosis quantification was performed by using the Annexin V, FITC Apoptosis Detection kit (Dojindo Molecular Technologies) according to the protocol provided by the manufacturer. Briefly, cells were seeded in T25 flasks with 10^6 cells/flask density, treated with the given drugs for 72h, then the supernatant containing apoptotic cells were removed, attached cells were washed with PBS, detached with trypsin and added to the earlier removed supernatant. 300.000 cells were stained for 15 min at RT in dark with 5 µl Annexin V and 5 ul PI solution in a 10-fold diluted Annexin V Binding Solution. Samples were analyzed with a FACSCANTO II flow cytometer

(BD Biosciences). Calcein assay was performed as described by Homolya et al. (32). After treatment with the indicated compounds, cells were washed with PBS, detached with trypsin, collected in FBS-free DMEM, and 250.000 cells were incubated with 0.25 μM calcein AM (Dojindo Molecular Technologies) with or without 10 μM verapamil for 10 min at 37°C. Cells were washed with ice-cold PBS and calcein accumulation was measured with a FACSCANTO II flow cytometer. Dead cells were excluded based on TO-PRO3 (Life Technologies) positivity.

Video microscopy. Cells were seeded in a 96-well plate at 5000 cells/well density. After 24h 1.5 μM NSC57969 was added to every well, and annexin positivity was monitored using the Annexin V, FITC Apoptosis Detection kit (Dojindo Molecular Technologies). Briefly, cells were washed with PBS and stained for 15 min at RT in dark with 5 μl Annexin V and 5 ul PI solution in a 10-fold diluted Annexin V Binding Solution. Images were captured with a JuLI Stage Real-Time Cell History Recorder (NanoEnTek) inside an incubator using bright, GFP (ex.466/40, em.525/50) and RFP (ex.525/50, em.580LP) channels.

Animal experiments. All animal protocols were approved by the Hungarian Animal Health and Animal Welfare Directorate according to the EU's directives. All surgical procedures were performed according to the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Institute of Enzymology, RCNS in Budapest, Hungary (22.1/2291/3/2010). Drug resistance of tumor implants was induced as described by Rottenberg et al. (20). Briefly, tissue pieces (1–2 mm in diameter) obtained from $Brca1^{-/-};p53^{-/-}$ FVB mouse mammary tumors (a kind gift from Sven Rottenberg, NKI) mouse were transplanted orthotopically into the mammary fat pad of wild type FVB mice (Harlan, Europe) under anesthesia (20 mg/kg zolazepam, 12.5 mg/kg xylazine, 3 mg/kg butorphanol,

20 mg/kg tiletamine). The tumor size was monitored at least 3 times per week by caliper measurements after the tumors became palpable. Tumor volume was calculated using the V=length×(width^2/2) formula. When the volume of the tumors reached ~200 mm^3, doxorubicin (Doxorubicin-TEVA) treatment using the maximum tolerable dose (MTD, 5 mg/kg iv) was started. Doxorubicin treatment using the MTD was repeated every 10 days unless the size of the tumors decreased to 50% of its original volume. In that case treatment was repeated when the tumor relapsed to its original size. Animals were sacrificed when the tumor volume reached ~2000 mm^3.

Isolation of primary breast tumor cells. Tumor-bearing mice were sacrificed by cervical dislocation. The removed tumor was washed once in 70% ethanol and twice in ice cold PBS. Peritumoral tissues were removed with fine scissors and the inner tumor mass was cut into ~1mm^3 pieces. The pieces were transferred to a 50 ml conical tube containing 20 ml of 200 U/ml type IV collagenase and 0,6 U/ml dispase (Life Technologies) in completed DMEM, and digested for 2 hours at 37°C with vortexing every 15 min for 1 min. The cell suspension was filtered through a 70 micron nylon filter insert (BD Biosciences), centrifuged for 5 min at 300×g. The supernatant was removed, and the pellet was dissolved in completed DMEM/F-12.

Results

Cytotoxicity assays in a diverse panel of MDR cells indicate lack of Pgp-potentiated toxicity of serendipitously identified MDR-targeting compounds

In order to systematically evaluate compounds reported to target MDR cells, we compiled a cell line panel consisting of parental and drug resistant cells. By testing a range of cell lines expressing functional Pgp, we wanted to determine if there were any exceptions to the MDR-

selective toxicity of the investigated compounds. The panel contains two drug selected lines expressing high levels of Pgp: KB-V1 cells overexpress Pgp as a result of a long term selection of parental KB-3-1 (HeLa) epidermoid carcinoma cells in vinblastine (21); MES-SA/Dx5 is a drug-resistant cell line derived from a human uterine sarcoma line (MES-SA) by doxorubicin selection (22). To determine if heterologous expression of the human P-glycoprotein is sufficient to sensitize cells against the tested compounds, we used the Sleeping Beauty transposon-based gene delivery system to establish MDCKII cell lines stably expressing wild-type human Pgp. Finally, we used high affinity Pgp-inhibitors Tariquidar or PSC833 to establish the requirement for functional Pgp to mediate sensitization of the cells.

For each cell line pair the MDR-selective ratio (SR) was determined using viability assays (23). Except for the electron transport chain inhibitors rotenone and Pluronic P85 that were not selective to any of the model cells, our results confirm the increased toxicity of the tested compounds against one or the other MDR line (Table 1). Verapamil and R121, suggested to exhaust MDR cells by engaging Pgp molecules in permanent activation, were indeed more toxic to MES-SA/Dx5 cells than to the Pgp negative parental MES-SA cell line. However, these high affinity substrates were equally toxic to the parental and resistant sublines within the KB and MDCK cell pairs. Similarly, MES-SA/Dx5 showed modest collateral sensitivity to TritonX-100, but this increased sensitivity was not eliminated in the presence of the Pgp inhibitor. Strikingly, desmosdumotin derivatives, reported to be 3-400 times more toxic to KB-Vin cells (as compared to the parental cell KB line), showed but a slight preferential toxicity in KB-V1 cells, and were barely toxic in the other MDR models. Dp44mT, reported to preferentially target MDR cells by an increased Pgp-mediated lysosomal accumulation (24), demonstrated elevated toxicity only in MES-SA/Dx5 cells. Regrettably, collateral sensitivity of MES-SA/Dx5 cells was not abrogated by inhibition of Pgp with Tariquidar, nor could be reproduced in the KB-3-1/KB-V1 or the MDCK cell pairs. Additional MDR models,

including the retrovirally transduced A431/A431-B1 cells or a cell line expressing high levels of endogenous Pgp (HCT-15) also failed to confirm the Pgp-dependent MDR-selective toxicity of Dp44mT (Figure 1A). Selective toxicity of Dp44mT was shown to occur via Pgp mediated lysosomal accumulation of a redox-active copper complex, leading to lysosomal-membrane permeabilization and apoptosis (24). In agreement with studies showing that Pgp primarily localizes to the plasma membrane (25), we were unable to detect Pgp in the lysosomes of KB-V-1 cells, explaining our inability to reproduce reported results (Figure 1B). Overexpression of membrane proteins are known to interfere with intracellular targeting (25–27). The relevance of the particular in vitro conditions leading to the reported lysosomal expression of Pgp remains to be verified in clinical studies.

The compounds listed in Table 1 were selected based on published evidence linking their toxicity to the collateral sensitivity of MDR cells expressing Pgp. However, the results obtained using the cell line panel containing 3 MDR cell line pairs indicate that even if the activity of Pgp may increase the potency of these compounds in certain cells, it cannot exclusively account for the collateral sensitivity of MDR cells.

Pgp is both necessary and sufficient to induce collateral sensitivity against new MDR-selective compounds identified in the DTP database

We and others have shown that by correlating Pgp mRNA levels and the publicly available DTP drug screening toxicity profiles across the NCI-60 panel it is possible to identify Pgp substrates (28) and MDR-selective compounds such as NSC73306, whose toxicity is increased by P-glycoprotein (16)(17). Cells become hypersensitive to NSC73306 in proportion to their Pgp function, and this selectivity is abrogated by functional inhibition or downregulation of Pgp (29). Irrespective of variations in cell line background, NSC73306 consistently

demonstrated a Pgp-potentiated MDR-selective toxicity (23,30). To identify further MDR-selective compounds, we computed the correlation of the cytotoxicity pattern of 49169 molecules released in 2010 by DTP to the expression of Pgp across the NCI60 cells. Our analysis yielded 82 putative MDR-selective compounds (Pearson's correlation coefficient>0.4), of which 61 were already identified in our earlier work (16). The 21 novel putative MDR-selective compounds are shown in Table 2, the structures of the most relevant compounds are shown in Figure 2; the complete list is shown in Supplementary Table S1. To put the 21 novel putative MDR-selective compounds to the same test we used to characterize the serendipitously identified MDR targeting compounds shown in Table 1, we measured toxicity across the panel containing three MDR cell line pairs. Of the 21 candidate compounds only 14 were made available by DTP for in vitro testing; one additional compound (NSC733435) was obtained from commercial sources. Two compounds (NSC627452, NSC733435) showed no toxicity in any of the cells, while NSC672035 and NSC15372 were comparably toxic in all cell lines. The remaining 11 compounds proved to be more toxic to at least one of the MDR cells. In agreement with our earlier observations, the newly identified compounds are enriched in structures enabling metal chelation (16). The isatin-β-thiosemicarbazone backbone of NSC716771 has been previously linked to Pgp-potentiated cytotoxicity (shown next to NSC73306, the first MDR-selective compound identified in the DTP database (14)(16)). The compound set is particularly abundant in 8-hydroxyquinoline derivatives, suggesting a strong association of the 8OHQ backbone with MDR-selective toxicity. The 8OHQ cluster contains six derivatives substituted at the 7-, 5- or 2-positions. While the unsubstituted 8OHQ core structure does not display MDR-selective activity, derivatives substituted at the 5-position (NSC79544 and NSC48892) or the 2-position (NSC67090) show moderate SR values between 1.5 and 3. In contrast, substituents in R7 (NSC57969, NSC693871) have a strong preference for targeting MDR cell lines (SR=5).

While chelation of metal ions might be possible for the distant 8OHQ analog NSC72881, it is unlikely for the 1,4-benzopyrone derivative NSC609800, the 1-(3,5-trimethoxyphenyl)-imidazole NSC733435, or the naphthyl derivative NSC15372. Accordingly, these compounds demonstrate comparably low toxicity throughout the cell line panel, regardless of the resistance status of the cells. Conversely, 1,10-phenanthroline has significant MDR-selective toxicity. This effect is Pgp-dependent, and is detected in the entire panel. Notably, KP722, the only serendipitously identified MDR-targeting compound that was found active across the panel, is a lanthanoid tris-phenathroline complex, shown also in Table 2 as a reference compound (31), next to two new 1,10-phenanthroline complexes formed with tin (NSC608465) and palladium (NSC676735). Interestingly, complexation of phenanthroline with either La, Sn or Pd does not modify selective toxicity (SR 1.4-7). Additional compounds identified in the screen show some degree of diversity. The urea derivative NSC672035 shows remote similarity to the TSC structures, yet the compound was not selectively toxic. Of the two compounds containing a quinoidic system NSC13977 showed weak but significant MDR-selective toxicity, and NSC740469 was unavailable from DTP. The remaining molecules are quite dissimilar. NSC748494, NSC726708, NSC689209 were not available, NSC627452 was not toxic, but NSC17551 showed MDR-selective toxicity.

To elucidate the mechanism of cell death, annexin staining was monitored by video microscopy and FACS. As shown in Figure 3A, treatment with 1.5 uM NSC57969 resulted in a marked induction of apoptosis in MES-SA/Dx5 cells. Selective induction of apoptosis in Pgp-expressing cells was confirmed using FACS analysis (Figure 3B). Taken together, these results confirm that the correlative approach identifies several *bona fide* MDR-selective compounds against which Pgp expressing MDR cells show robust collateral sensitivity. Collateral sensitivity against these MDR-selective compounds is linked to Pgp-function, independently of the context provided by specific alterations present in a given cell line. In

contrast to serendipitously identified compounds, MDR-selective compounds identified in the pharmacogenomic approach such as NSC57969, NSC693871 or NSC297366 are selectively toxic throughout the entire MDR panel, irrespectively of the mechanism driving Pgp expression or the tissue of origin of the cell (see Supplementary Figure 1 for results obtained in additional cell lines). Tariquidar, a high affinity Pgp-inhibitor, was able to eliminate collateral sensitivity, demonstrating the requirement for functional Pgp to mediate sensitization of MDR cells.

A single, high-dose treatment with MDR-selective compounds results in the loss of Pgp expression and re-sensitization to chemotherapy

Continuous exposure of cells to MDR selective compounds such as NSC73306 was described to result in a gradual loss of Pgp expression, supporting the causal link between toxicity and Pgp function (29). To characterize the ability of the compounds analyzed in this study to modulate the expression of Pgp, we incubated MES-SA/Dx5 cells with IC_{20} concentrations of the compounds (killing 80% of the cells in the cytotoxicity assays) and followed the expression of Pgp in the surviving cell population. The activity of Pgp was characterized by a FACS-based assay, measuring the accumulation of calcein in the cells (32). NSC57969, which showed MDR selective toxicity across the 3 different MDR cells, completely eradicated Pgp-expressing cells (Figure 4D). Treatment with NSC293766 gave identical results (Supplementary Figure 2), while MES-SA/Dx5 cells treated with 8-hydroxyquinoline did not lose Pgp, in line with the lack of MDR selective toxicity of the unsubstituted quinolinole scaffold (Figure 4C and 4G). Loss of Pgp-expression occurred gradually, as indicated by the progressive loss of calcein-dim cells from the population. Loss of Pgp-mediated drug efflux immediately re-sensitized cells to Pgp substrate drugs such as doxorubicin (Figure 4E). In parallel, the cells acquired cross-resistance to MDR-selective compounds, providing yet another evidence supporting the causal role of Pgp in the initial

collateral sensitivity of MES-SA-Dx5 cells. Lack of Pgp function may be attributed to chemical inhibition. However, confocal images of the cells demonstrated that the MDR-selective treatment resulted in a complete loss of Pgp expression (Figure 4G). Next, we followed the effect of several putative MDR selective compounds on the functional expression of Pgp in MES-SA/Dx5 cells for up to 3 months. Strikingly, four compounds eradicated Pgp expression after but a single treatment (Figure 5). NSC57969 and NSC297366 exhibited submicromolar toxicity and significant selectivity in the cytotoxicity assays, while NSC608468 and 1,10-phenanthroline were less toxic and less selective (Table 1), suggesting that the ability of the compounds to rapidly eliminate Pgp expression is not strictly related to the degree of selective toxicity. Another group of compounds, consisting of NSC73306, NSC17551, NSC67090 and KP772 was also able to eliminate Pgp-expressing cells, though only after 3-5 treatment cycles, while verapamil and NSC79644 (showing marginally selective toxicity in the screens) reduced the fraction of Pgp-expressing MES-SA/Dx5 cells by 50%. 8-OHQ and NSC13977 had no effect on the expression of Pgp.

NSC57969 eradicates P-glycoprotein expression in a primary breast tumor culture obtained from doxorubicin resistant brca1$^{-/-}$/p53$^{-/-}$ spontaneous mouse mammary carcinoma

To test if the MDR-selective toxicity of the compounds identified in this study is maintained in a more realistic model of clinical drug resistance, we used a genetically engineered model of resistant murine mammary cancer (33). Since a pharmacologically active formulation was not achievable, we tested the *in vitro* effect of the MDR-selective compounds on primary cultures established from doxorubicin resistant spontaneous mouse mammary carcinoma (Figure 6 and Supplementary Figure 3). Functional expression of Pgp was evaluated by the calcein assay, which indicated that a fraction of the primary cells established from the drug resistant tumor possess a low but significant efflux capacity (6). In vitro treatment of the

primary culture with doxorubicin efficiently enriched the population in Pgp-expressing cells. Conversely, Pgp expression associated with the resistance of the doxorubicin resistant Brca1$^{-/-}$;p53$^{-/-}$ spontaneous mouse mammary carcinoma cells could be eliminated by a single treatment with NSC57969, suggesting that that MDR-selective compounds can effectively revert the MDR phenotype of cells that express Pgp at clinically relevant levels (Figure 6).

Discussion

Although there is some controversy regarding the contribution of multidrug transporters to the development of clinical MDR, it is widely accepted that ABC transporters are overexpressed in at least 50% of human cancers some time during treatment (34). Despite the established importance of tumor drug resistance to cancer mortality, therapeutic solutions remain incomplete (35). Attempts to overcome Pgp-mediated drug resistance by chemically inhibiting the transporter were considered failures due to unwanted toxicity and unconvincing clinical trials (36–38). Thus, continued research with a renewed effort to combat multidrug resistance is warranted. Collateral sensitivity of MDR cells indicates that resistance can be interpreted as a trait that may be targeted by new drugs. This concept deviates from the failed strategy of transporter inhibition, which requires the coadministration of cytotoxic drugs with small-molecule inhibitors devoid of intrinsic toxicity. In contrast, the activity of MDR-selective compounds is increased, rather than diminished in MDR cells. The notion that the function of Pgp may be causally related to collateral sensitivity is based on early reports showing the increased sensitivity of MDR cell lines. In a recent review we compiled the list of serendipitously discovered MDR-selective compounds reported in the literature (14)– here we use an in vitro test system to systematically establish the contribution of Pgp to the toxicity of the most relevant molecules.

Selective toxicity of verapamil (10)(11) and other high affinity Pgp substrates such as the dipeptide reversin121 (39) was explained by the increased energy depletion of MDR cells, induced by the ATP consumption associated with the futile effort of Pgp to reduce intracellular drug levels (40). Though not through interaction with Pgp, the D-glucose analogue 2-deoxy-D-glucose (2-DG) (41), the mitochondrial electron chain transport inhibitor rotenone (40) and the polymeric surfactant Pluronic (42) were also suggested to target MDR cells by the selective depletion of cellular ATP levels. In particular, selective toxicity of pluronic (P85) seemed to depend on functional P-glycoprotein in several cell lines (43,43,44). The exact mechanism has not been elucidated - it was hypothesized that selective toxicity of rotenone and P85 is mediated by a preferential inhibition of mitochondrial enzymes, resulting in selective ATP depletion and oxidative stress in MDR cells (42). Unfortunately, our results did not confirm the selective toxicity of rotenone or P85, suggesting that Pgp is not sufficient to convey collateral sensitivity to these compounds. Interpretation of studies suggesting changes associated with Pgp expression is difficult, because of the alterations linked to the presence of cytotoxic drugs and the long term selection of experimental cells. Thus, the confirmed selective toxicity of TritonX in MES-SA/DX5 cells is likely due to host adaptation rather than the expression of P-glycoprotein. Similarly, the reported MDR-selective toxicity of desmosdumotin analogs is likely tied to specific cell lines. The flavonoid desmosdumotin B was identified from the root bark of *Desmos dumosus*. Following the demonstration of its selective toxicity, further analogs were explicitly developed to target MDR cells (45). The most potent 6,8,8-triethyldesmosdumotin B analogs showed up to 460 fold (!) selectivity in P-glycoprotein expressing KB-VIN cells (46) and significantly lower selectivity in Hep3B-VIN cells (47). Remarkably, KB-VIN cells were derived from parental KB nasopharyngeal carcinoma cells by selection in vincristine (46) and thus do not belong to the extensively characterized series of resistant HeLa derivatives (21). Since desmosdumotins were not

available commercially or through academic material transfer, the three most active analogs were synthesized. While we were able to confirm marginal selective toxicity in KB-V1 cells, MES-SA/Dx5 cells and MDCK-B1 cells were not hypersensitive suggesting that the MDR-selective toxicity of desmosdumotin analogs is tied to specific characteristics of the KB-VIN and Hep3B-VIN cells.

In some cases, we were able to reproduce the preferential toxicity of reported MDR-selective compounds, at least in a subset of the MDR cells. Dp44mT was described to preferentially suppress the proliferation of etoposide resistant breast cancer (MCF-7/VP) and vinblastine resistant epidermoid carcinoma (KB-V1) cells (48). Notably, selectivity was reversible by inhibiting or silencing Pgp, showing that in these cell lines Pgp contributes to the cytotoxity of Dp44mT. While we were unable to reproduce the result obtained with KB-V1 cells, our results confirm the elevated toxicity of Dp44mT in MES-SA/Dx5 cells. Unfortunately, additional MDR models also failed to confirm the Pgp-dependent MDR-selective toxicity of Dp44mT. Thus, we conclude that, with the exception of the KP772, the serendipitously identified MDR-selective compounds did not show consistent Pgp-dependent toxicity. In contrast, the toxicity of several novel compounds identified by the correlative approach was found to be consistently increased by Pgp in several MDR cell lines selected with cytotoxins, or genetically engineered to overexpress Pgp. Our results reveal three scaffolds associated with Pgp-potentiated toxicity. In addition to the previously reported isatin-β-thiosemicarbazone (TSC) and 1-10 phenanthroline backbones (14), we find that 8-hydroxyquinoline derivatives possess MDR-selective toxicity. We identify NSC297366 as a particularly active compound with submicromolar activity in MDR cells and a selectivity ratio of 10-19. Our results establish the 8-hydroxyquinoline (8OHQ) backbone as a promising scaffold for designing MDR-targeting compounds. Using NSC297366 as a starting point, we

are currently exploring further 8OHQ derivatives substituted at the 7-position to establish structure-activity relationships governing selective toxicity.

A potential approach to harvest the cost of Pgp expression would be to reverse drug resistance by treating resistant cells with MDR-selective compounds. To avoid complex changes associated with prolonged drug exposure, we treated MES-SA/Dx5 cells with a single high concentration of MDR-selective compounds. We find that three newly identified MDR-selective compounds (NSC57969, NSC297366, NSC608465) and 1,10-phenantroline completely eradicate the Pgp-positive MES-SA/Dx5 cells following only one, high-dose treatment. Following this single treatment MES-SA/Dx5 cells exhibit a stable phenotype characterized by the loss of Pgp and a renewed sensitivity to doxorubicin. This newly acquired Pgp-negative, chemotherapy-sensitive phenotype was stable for up to 30 passages (~4 months), suggesting that treatment stoppage does not lead to the re-expression of Pgp (Supplementary Figure 4). A similar switch was observed following multiple cycles of treatment with further confirmed MDR-selective compounds including NSC73306, NSC17551, NSC67090 and KP772. This group of compounds lacked consistent MDR-selective toxicity in the short term cytotoxicity assay, but nevertheless were able to deplete MES-SA/Dx5 of Pgp-positive cells.

The mechanism of Pgp-potentiated toxicity is unknown. The most promising compounds are all metal chelators, suggesting that metal-ion interaction is key to preferential cytotoxicity. In a recent review, we have suggested several possible mechanisms to explain how efflux transporters may convey collateral sensitivity to anticancer chelators (14). Pgp-mediated efflux may deprive cells of essential metals, specifically sensitizing Pgp-expressing MDR cells. Since the anticancer activity of metal complexes often relies on reduction−oxidation (redox) cycling, an elevated sensitivity to reactive oxygen species (ROS), linked to activity of transporters may also explain the collateral sensitivity of multidrug-resistant cells to MDR-

selective compounds. A caveat in these explanations is that chelation is not sufficient to convey MDR-selective toxicity, as evidenced by the lack of Pgp-potentiated activity of the unsubstituted 8OHQ core structure. Taken together, these results confirm that chelation is not sufficient to convey MDR-selective activity. Analysis of the structure activity relationship and the regulatory mechanisms responsible for the phenotype-switch of MDR cells is the subject of current research in our laboratory.

The MDR-selective compounds identified by the correlative approach efficiently exploit the fitness cost and the ensuing paradoxical hypersensitivity that is invariably associated with the expression of Pgp. Collateral sensitivity of MDR cell lines may suggest that drug resistant cancer expressing P-glycoprotein can be selectively targeted. However, if artefacts linked to the in vitro propagation of cell lines, the continuous selection for growth, or the extreme Pgp levels contribute to the elevated toxicity of MDR-selective compounds, it is unlikely that the concept of MDR-targeted therapy will be transferred from the bench to the bedside. In fact, our inability to confirm literature data may be explained if a certain threshold level of Pgp expression was required for the MDR-selective toxicity of the serendipitously identified compounds. For example, selective toxicity of Dp44mT may be restricted to cells in which massive overexpression of Pgp results in lysosomal localization. This is relevant since clinical studies suggest that the expression of P-glycoprotein in tumor cells is far inferior to that observed in MDR cell lines (49,50). We have shown that the toxicity of NSC73306 is proportional to Pgp expression in a series of KB cells encompassing a wide range of Pgp expression levels (29). Here our aim was to characterize the newly identified MDR-selective compounds in a genetically engineered mouse model of drug resistant cancer. In full accordance with published results, we confirm that doxorubicin resistance of brca1$^{-/-}$/;p53$^{-/-}$ spontaneous mouse mammary tumors is indeed conveyed by a moderate increase in the expression of the Abcb1a and b genes (6) (51). We isolated primary cells and evaluated the

effect of in vitro treatment at a low (<10) passage number. We show that treatment with NSC57969 results in a significant reduction of Pgp-mediated calcein efflux capacity in primary mammary carcinoma cells, suggesting that MDR-selective compounds can effectively revert the MDR phenotype of cells that express Pgp at clinically relevant levels.

Because MDR-selective compounds do not inhibit efflux, a profound effect on drug absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) is unlikely. However, there are many normal cell types that express P-glycoprotein at significant levels, and these might be subject to toxicity from the agents that induce collateral sensitivity. Considering the failure of ABC transporter inhibitors in cancer trials, the potential effect of MDR-selective compounds on physiological Pgp may well limit the therapeutic potential of our strategy. To address this concern, we analyzed the effect of NSC57969 on hCMEC/D3 cells, which are routinely used as an *in vitro* blood brain barrier (BBB) model (52,53). As shown in Figure 7, hCMEC/D3 cells express significant levels of functional Pgp- consistently with the high Pgp expression of the human brain microvascular endothelium. Despite high expression levels, treatment of hCMEC/D3 cells with NSC57969 does not induce loss of Pgp (Figure 7). These results suggest that normal tissues expressing Pgp are not differentially sensitive to MDR-selective agents, raising the possibility that it is the combination of the malignantly transformed state with the expression of Pgp that leads to toxicity. In future studies we will test if the collateral sensitivity of MDR cancer cells can be exploited using a strategy in which treatment with doxorubicin is preceded or followed by MDR-selective therapy to prevent or revert MDR cancer.

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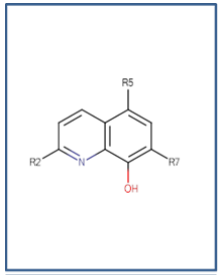
Table 1

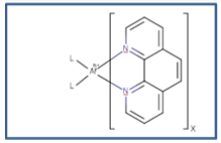
	KB-3-1	KBV-1	KB-3-1/ KBV-1	MDCK II	MDCK II-B1	MDCK II/ MDCK II-B1	Mes-Sa	Dx5	Mes-Sa/ Dx5	Mes-Sa (i)	Dx5 (i)	Mes-Sa/ Dx5(i)		Reported as MDR selective compound
verapamil	55,2	69,5	0,8	52,6	43,6	1,2	49,4	33,2	1,5**	30,2	17,7	1,7**	a	(10)
reversin121	NT	NT	-	NT	NT	-	131,0	13,0	10,8**	95,4	12,3	7,6*	a	(39)
TritonX-100	55,4	47,8	1,2	73,7	97,6	0,8	29,9	9,3	3,4**	24,2	9,9	2,6*	b	(54)
TEDB	198	100	2,0**	NT	NT	-	91,7	67,2	1,4	-	-	-		(47)
4'-Me-TEDB	26,4	14,6	2,1*	97,4	96,6	1,0	36,3	29,1	1,3	-	-	-		(47)
4'-Et-TEDB	13,0	7,7	1,7*	NT	NT	-	41,5	19,9	1,9	-	-	-		(47)
Pluronic P85	126	129	1,0	80,7	93,4	0,9	45,9	49,1	1,0	-	-	-		(43)
Dp44mT	0,071	0,055	1,4	0,0035	0,0026	1,3	0,045	0,019	2,7*	0,046	0,010	4,8**	b	(48)
Rotenone	0,136	0,136	1,1	0,150	0,115	1,4	0,090	0,078	1,2	-	-	-		(40)

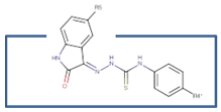
Table 1. Cytotoxicity (IC_{50}) of compounds (μM) reported to elicit increased toxicity against Pgp expressing MDR cells. A selectivity ratio (SR) >1 indicates that the compound kills Pgp-expressing cells more effectively than parental cells, demonstrating MDR-selective activity. NT: Not toxic up to 150 μM (reversin121) or 200 μM (desmosdumotins). (i): Pgp inhibitor tariquidar (a) or PSC-833 (b). Values represent the average of at least three independent experiments. TEDB: 6,8,8-triethyldesmosdumotin; Dp44mT: Di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone. *p<0.05; **p<0.01

Table 2

NSC code	KB-3-1	KBV-1	KB-3-1/ KBV-1	MDCK II	MDCK II-B1	MDCK II/ MDCK II-B1	Mes-Sa	Dx5	Mes-Sa/ Dx5	Mes-Sa (TQ)	Dx5 (TQ)	Mes-Sa/ Dx5 (TQ)
NSC733435	NT	NT		NT	NT		NT	NT				
NSC15372	13,4	13,6	1,0	40,7	38,0	1,1	28,3	32,8	0,8			
NSC72881	16,7	10,8	1,7	18,5	13,6	1,4	34,9	12,7	2,8**	32,9	33,6	1,1
NSC609800	NA	NA		NA	NA		NA	NA		NA	NA	
NSC297366	2,1	0,1	17,6**	2,9	0,3	10,5**	2,1	0,1	19,0*	1,7	1,6	1,1
NSC1014	5,8	4,4	1,4	10,9	5,6	1,9**	18,0	10,8	1,7	17,6	11,8	1,5
NSC57969	8,6	0,6	14,6*	4,5	0,8	5,7**	4,9	0,6	8,2**	4,8	3,6	1,4
NSC693871	14,7	2,5	7,4**	15,0	3,2	5,1**	5,0	1,3	4,0**	4,9	3,5	1,4
NSC48892	3,2	1,9	1,7**	3,6	2,3	1,6*	3,7	2,0	1,9*	3,8	2,8	1,3
NSC79544	2,2	0,7	3,0**	1,3	1,1	1,2	2,6	1,8	1,5	2,2	1,9	1,1
NSC67090	4,8	2,8	1,6	3,9	1,8	2,3**	3,6	1,3	2,8**	2,4	1,4	1,7
8-OH-Q	20,1	10,0	2,2	3,3	2,8	1,2	4,8	2,8	1,7**	4,1	2,1	1,9**
1,10-phen	32,9	11,0	3,4*	5,1	2,6	2,0**	4,7	1,7	3,1**	4,6	3,7	1,3
NSC676735	7,2	3,5	2,7*	12,9	9,2	1,4**	21,8	7,1	3,2*	21,3	17,6	1,3
KP772	64,0	12,2	7,0**	6,6	4,0	1,6*	4,7	1,5	3,0**	4,9	4,3	1,2
NSC608465	2,6	1,1	2,6*	2,6	1,4	2,3*	3,6	1,2	3,0*	4,9	3,8	1,6
NSC748494	NA	NA		NA	NA		NA	NA		NA	NA	
NSC726708	NA	NA		NA	NA		NA	NA		NA	NA	
NSC740469	NA	NA		NA	NA		NA	NA		NA	NA	
NSC13977	11,3	4,6	2,6*	9,5	4,4	2,1*	8,2	3,1	2,6*	8,0	5,5	1,5
NSC672035	75,1	57,7	1,2	31,4	54,0	0,6	51,9	53,6	1,0			
NSC73306	7,5	3,7	2,2**	6,8	2,8	2,4*	6,4	3,1	2,0**	5,3	4,0	1,3*
NSC716771	13,1	5,8	2,3	NA	NA		NA	NA		NA	NA	
NSC689209	NA	NA		NA	NA		NA	NA		NA	NA	
NSC17551	8,7	4,6	2,0**	14,6	4,5	3,3*	23,7	3,7	6,3**	23,6	10,9	2,2**
NSC627452	NT	NT		NT	NT		NT	NT				







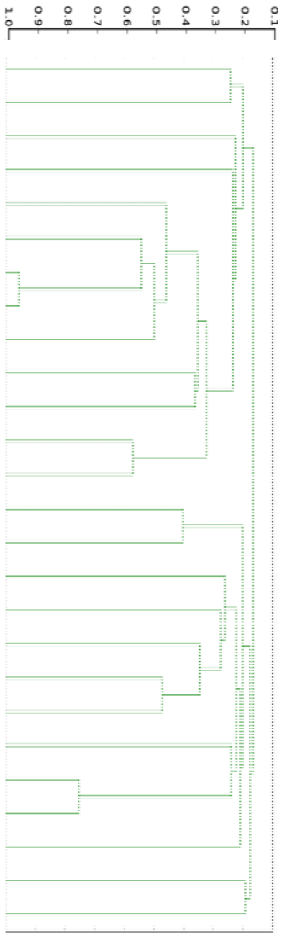


Table 2. Novel compounds predicted to show Pgp-potentiated MDR-selective toxicity based on the correlation between Pgp expression and DTP determined efficacy patterns across the NCI-60 cell line panel.

The dendrogram shows the average linkage hierarchical clustering of 21 new putative MDR-selective compounds (NSC codes) and reference molecules (NSC693871; 8OHQ; KP772; 1,10 Phenanthroline; NSC73306) based on Tanimoto similarity indices (Chemaxon (55)). NA, compound not available for testing; in bold, confirmed MDR-selective activity; underlined: cell line specific MDR-selective activity. Clusters of structurally related compounds where one or more analogues are active are highlighted by their core common structure (structures for all compounds are shown in Supplementary Table S1). Values indicate cytotoxicity (IC_{50}) of compounds (μM) in parental and Pgp expressing MDR cells. The IC_{50} values of KP772 are given in ligand equivalents. A selectivity ratio >1 indicates that the compound kills Pgp-expressing cells more effectively than parental cells, demonstrating MDR-selective activity. NT: not toxic up to 100 uM; TQ: tariquidar. *Significance level, $P < 0.05$;**$P<0.01$. Data for NSC716771 were taken from (30).

Figure Legends

Figure 1. Dp44mT does not show robust MDR-selective toxicity A. Whereas MES-SA/Dx5 cells are hypersensitive to Dp44mT, Pgp-expressing A431 B1 and HCT-15 cells do not show collateral sensitivity as compared to control cells transfected with a control plasmid (A431) or treated with the Pgp inhibitor Tariquidar (HCT-15+TQ). B. Determination of the subcellular localization of P-glycoprotein in KB-V-1 epidermoid carcinoma cells. Pgp was visualized using the monoclonal antibody MRK16 (green); lysosomes (red) and nuclei (blue) were labeled with LAMP1 and DAPI, respectively. Scale bar: 10 μm.

Figure 2. Structures of the most relevant novel MDR-selective compounds reported in this study

Figure 3. NSC57969 selectively induces apoptosis in Pgp-expressing cells. MES-SA (MES) and MES-SA/Dx5 (Dx5) cells were cultured in a 96-well plate at 5000 cells/well density in the presence of NSC57969 (1.5 μM). Scale bar: 250 μm. (A). The proportion of cells undergoing apoptosis at 72 hours of treatment was determined by FACS analysis (B).

Figure 4. Treatment with NSC57969 results in the loss of Pgp expression and re-sensitization to chemotherapy. Following incubation with Calcein AM, MES-SA cells (A) show high fluorescence, whereas MES-SA/Dx5 cells (B) remain dim because calcein is effluxed from the cells by Pgp. Calcein accumulation was measured in MES-SA/Dx5 cells following a 14 day-long treatment with 8-hidroxyquinoline (MES-SA/Dx5-8OHQ) (C) or 15 days after treatment with NSC57969 (MES-SA/Dx5-57969) (D). Grey histograms show calcein accumulation measured in the presence of the Pgp-inhibitor verapamil. (E) MES-SA/Dx5-57969 cells loose resistance to doxorubicin and (F) also loose collateral sensitivity to NSC297366. (G) P-glycoprotein expression (red) of MES-SA, MES-SA/Dx5 and MES-

SA/Dx5-57969 cells detected with the MRK16 antibody. Nuclei were stained with DAPI (blue). Magnification ×40, Scale bar: 50 μm..

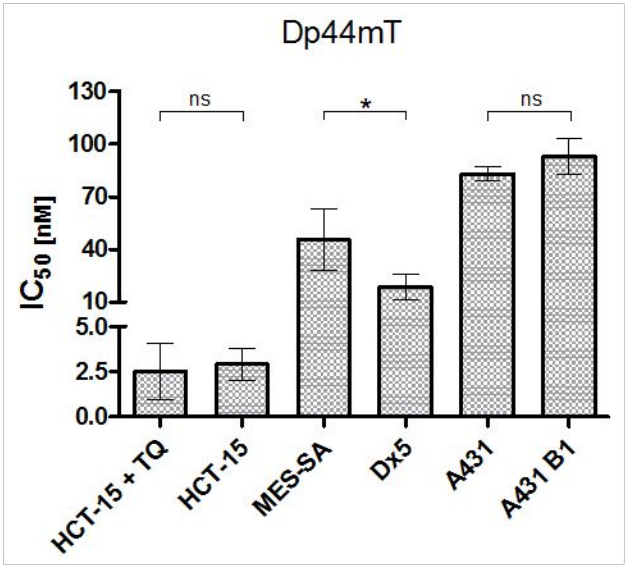
Figure 5. Time dependent loss of Pgp-positive cells following treatment of MES-SA/Dx5 cells with putative MDR-selective compounds. Data points represent the fraction of Pgp-negative cells as determined by the Calcein assay.

Figure 6. Primary cells isolated from drug resistant Brca1$^{-/-}$;p53$^{-/-}$ spontaneous mouse mammary carcinoma contain a P-glycoprotein expressing subpopulation that can be eradicated in vitro by NSC57969. Tumor pieces derived from Brca1$^{-/-}$;p53$^{-/-}$ mouse mammary tumors were transplanted orthotopically into the mammary fat pad of wild type FVB mice as described in (9). Primary cell cultures were established from drug resistant tumors. Pgp activity was quantified with the calcein assay before and after treatment with either 5 μM NSC57969 or 0.5 μM doxorubicine. Drug resistant tumors contain a significant fraction of Pgp positive cells (31%) that can be eliminated by a single treatment with NSC 57969 (1.9%), whereas treatment with doxorubicine further increases the fraction of Pgp positive cells (47.6%). Vertical lines represent gates set according to the verapamil treated samples corresponding to complete Pgp inhibition.

Figure 7. NSC57969 does not influence the expression of functional P-glycoprotein in hCMEC/D3 cells. Calcein accumulation in untreated (A) and treated (B) hCMEC/D3 cells following a 14 day-long treatment with NSC57969. Grey histograms show calcein accumulation measured in the presence of the Pgp-inhibitor verapamil.

Figure 1

A


Dp44mT
ns
*
ns
130
100
70
40
10
5.0
2.5
0.0
IC50 [nM]
HCT-15 + TQ
HCT-15
MES-SA
Dx5
A431
A431 B1

B

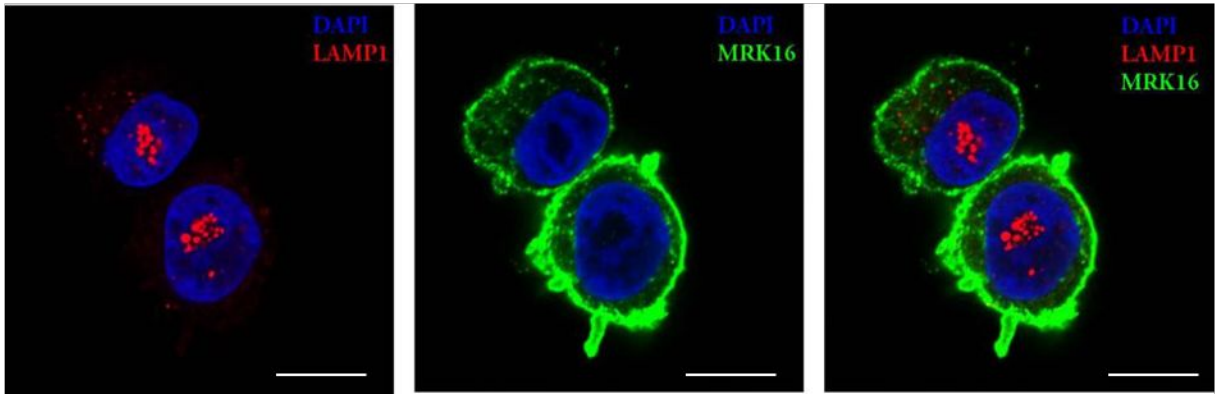
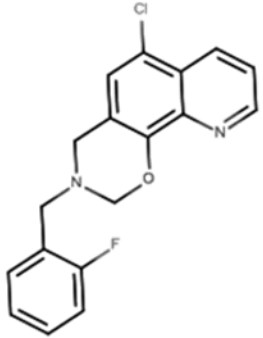
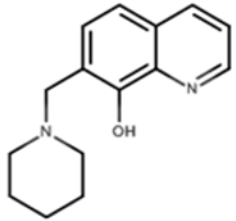

DAPI
LAMP1
DAPI
MRK16
DAPI
LAMP1
MRK16

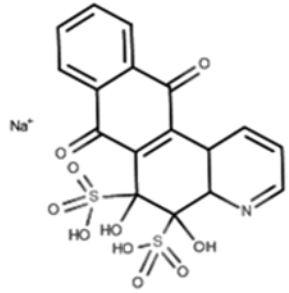
Figure 2



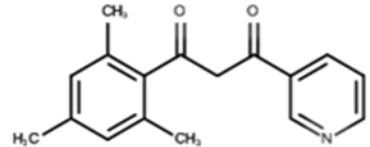
NSC297366



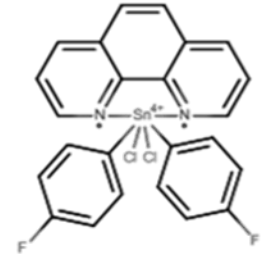
NSC57969



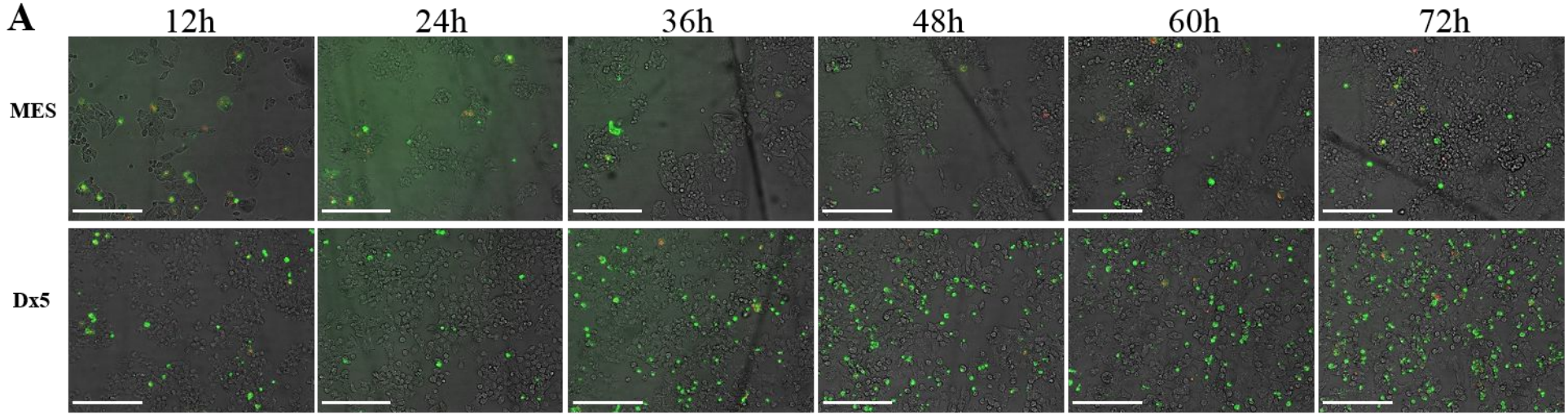
NSC608465



NSC17551



NSC13977


A
12h
24h
36h
48h
60h
72h
MES
Dx5

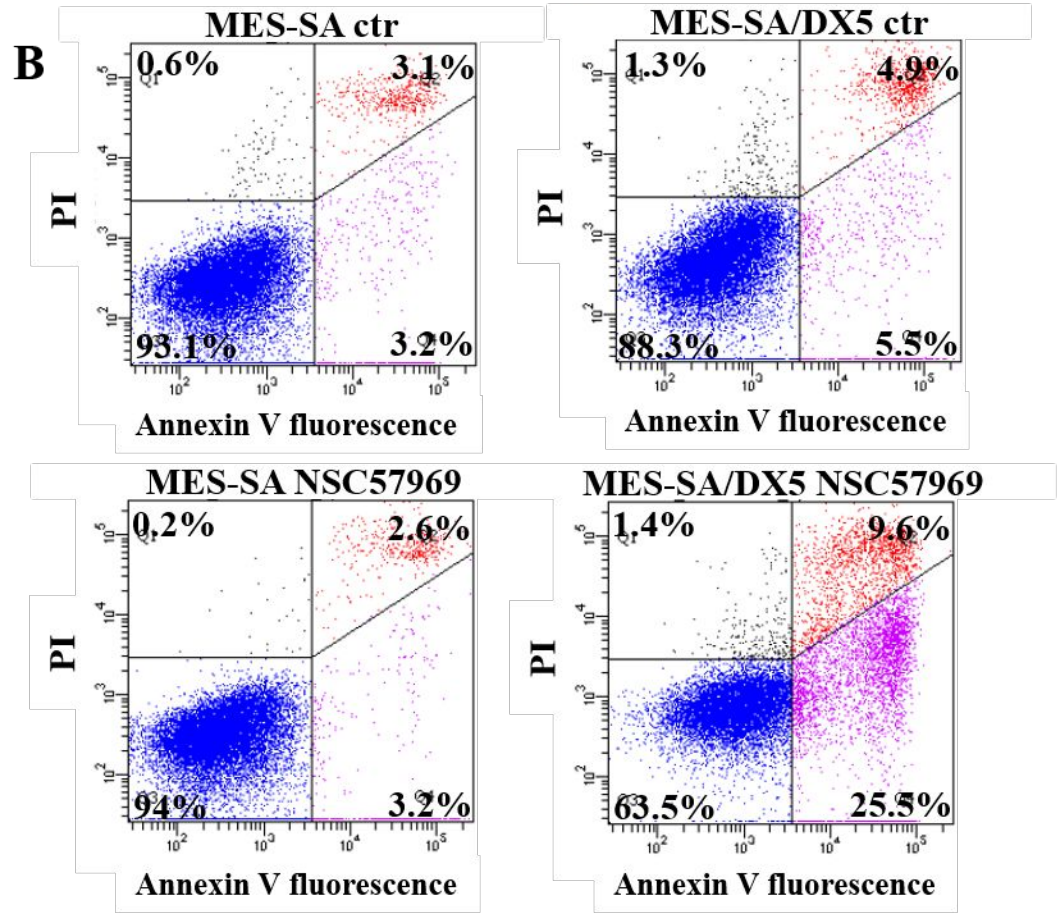

B
MES-SA ctr
0.6%
3.1%
93.1%
3.2%
PI
Annexin V fluorescence
MES-SA/DX5 ctr
1.3%
4.9%
88.3%
5.5%
PI
Annexin V fluorescence
MES-SA NSC57969
0.2%
2.6%
94%
3.2%
PI
Annexin V fluorescence
MES-SA/DX5 NSC57969
1.4%
9.6%
63.5%
25.5%
PI
Annexin V fluorescence

Figure 4

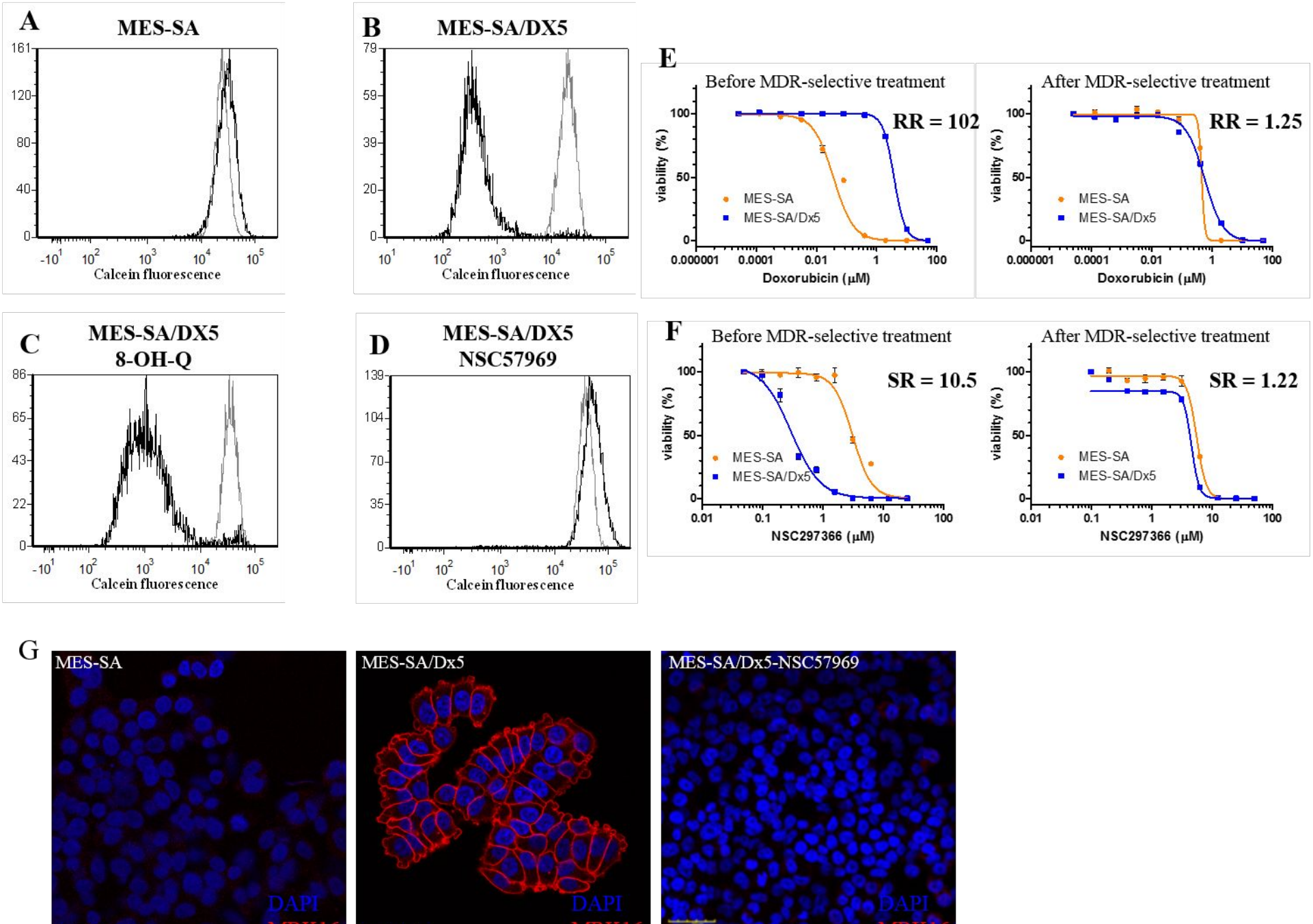

A
MES-SA
Calcein fluorescence
B
MES-SA/DX5
Calcein fluorescence
C
MES-SA/DX5
8-OH-Q
Calcein fluorescence
D
MES-SA/DX5
NSC57969
Calcein fluorescence
E
Before MDR-selective treatment
RR = 102
viability (%)
MES-SA
MES-SA/Dx5
Doxorubicin (μM)
After MDR-selective treatment
RR = 1.25
viability (%)
MES-SA
MES-SA/Dx5
Doxorubicin (μM)
F
Before MDR-selective treatment
SR = 10.5
viability (%)
MES-SA
MES-SA/Dx5
NSC297366 (μM)
After MDR-selective treatment
SR = 1.22
viability (%)
MES-SA
MES-SA/Dx5
NSC297366 (μM)
G
MES-SA
DAPI
MRK16
MES-SA/Dx5
DAPI
MRK16
MES-SA/Dx5-NSC57969
DAPI
MRK16

Figure 5

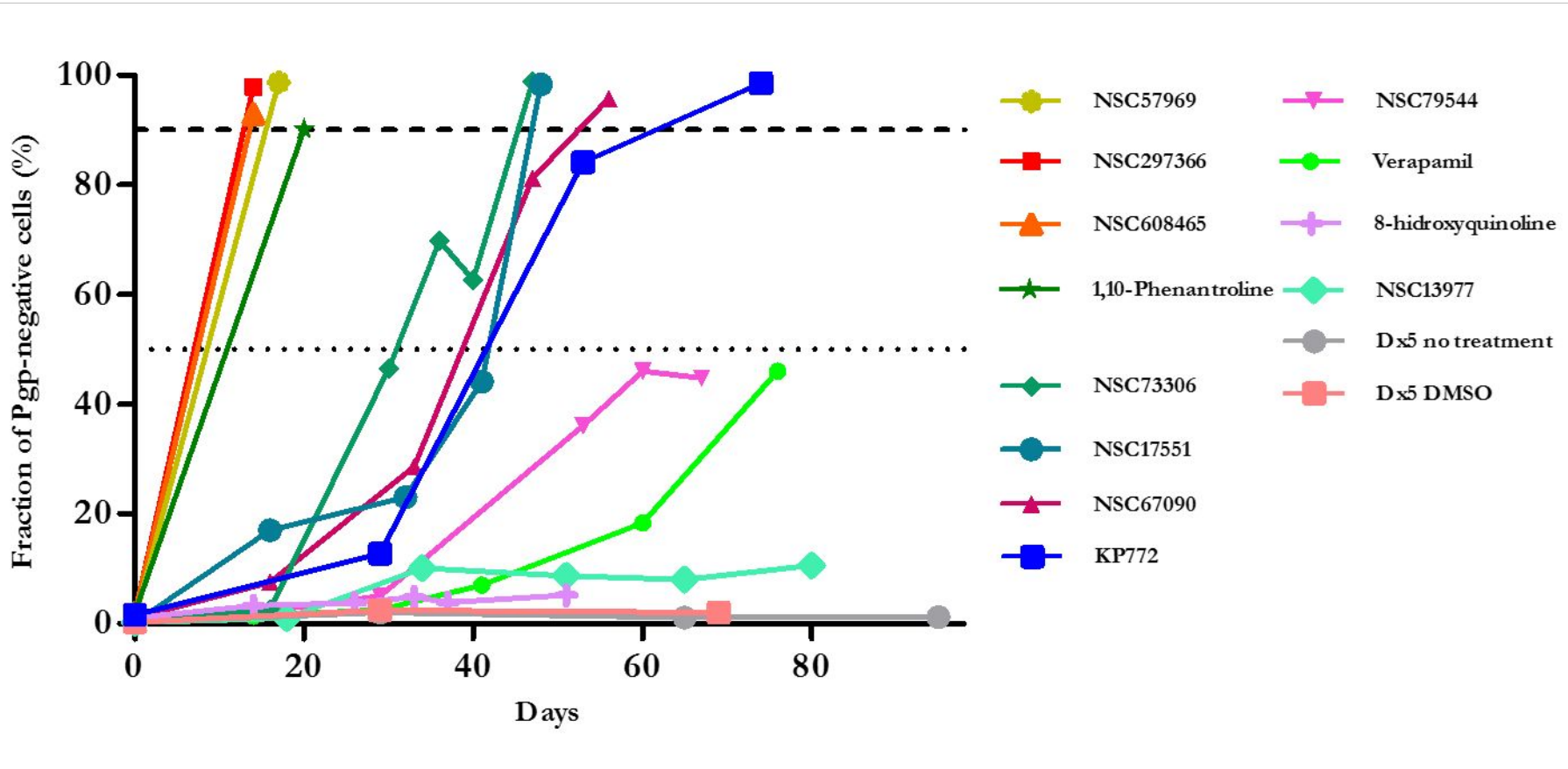
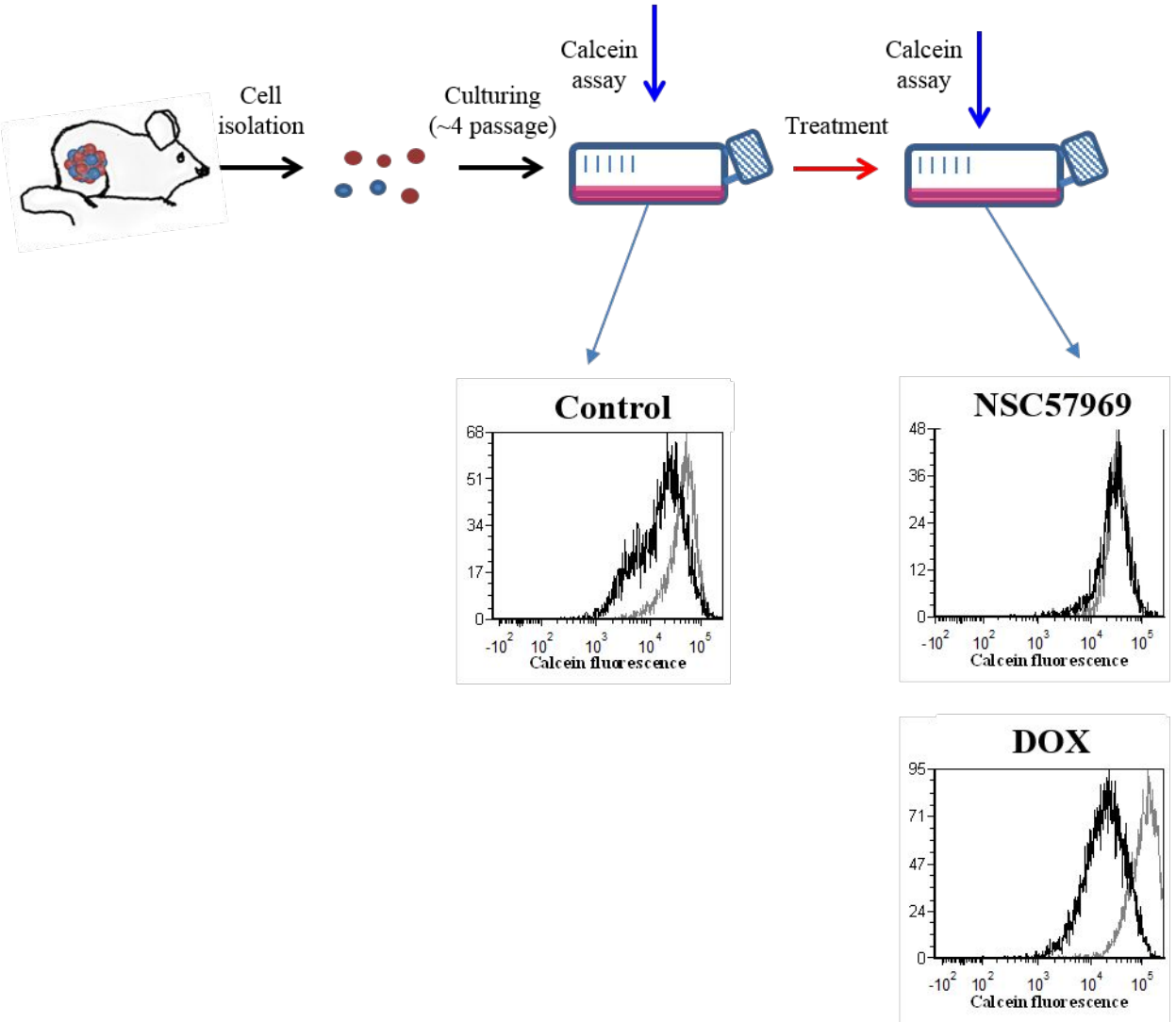
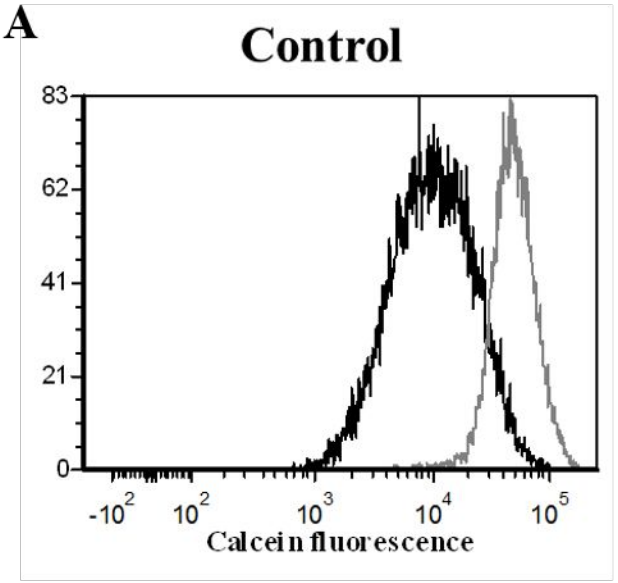
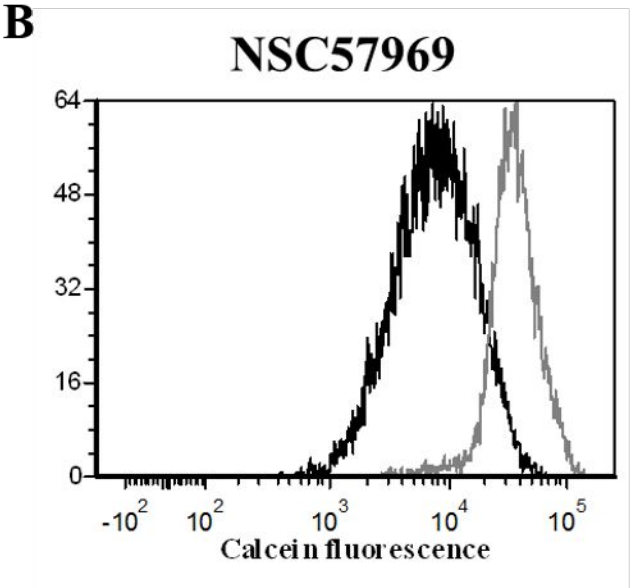

Fraction of Pgp-negative cells (%)
100
80
60
40
20
0
0
20
40
60
80
Days
NSC57969
NSC297366
NSC608465
1,10-Phenantroline
NSC73306
NSC17551
NSC67090
KP772
NSC79544
Verapamil
8-hidroxyquinoline
NSC13977
Dx5 no treatment
Dx5 DMSO

Figure 6


Cell isolation
Culturing (~4 passage)
Calcein assay
Treatment
Calcein assay
Control
NSC57969
DOX
Calcein fluorescence
Calcein fluorescence
Calcein fluorescence


A
Control
83
62
41
21
0
-10^2
10^2
10^3
10^4
10^5
Calcein fluorescence


B
NSC57969
64
48
32
16
0
-10^2
10^2
10^3
10^4
10^5
Calcein fluorescence