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Report

The cyclin-dependent kinase inhibitor roscovitine and the nucleoside analog sangivamycin induce apoptosis in caspase-3 deficient breast cancer cells independent of caspase mediated P-glycoprotein cleavage

Implications for therapy of drug resistant breast cancers

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Abbreviations: CDK, cyclin-dependent kinase; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MDR, multidrug resistance; PE, phycoerythrin; P-gp, 170-kDa P-glycoprotein; PI, propidium iodide; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis

Key words: P-glycoprotein, drug resistance, breast cancer, cleavage, apoptosis, caspase-3, roscovitine

Resistance to multiple chemotherapeutic agents is a common clinical problem which can arise during cancer treatment. Drug resistance often involves overexpression of the multidrug resistance *MDR1* gene, encoding P-glycoprotein (P-gp), a 170-kDa glycoprotein belonging to the ATP-binding cassette superfamily of membrane transporters. We have recently demonstrated apoptosis-induced, caspase-3-dependent P-gp cleavage in human T-lymphoblastoid CEM-R VBL100 cells. However, P-gp contain many aspartate residues which could be targeted by caspases other than caspase-3. To test whether other caspases could cleave P-gp in vivo, we investigated the fate of P-gp during roscovitine- and sangivamycin- induced apoptosis in MCF7 human breast cancer cells, as they lack functional caspase-3. MCF7 cells were stably transfected with human cDNA encoding P-gp. P-gp was cleaved in vitro by purified recombinant caspase-3, -6 and -7. However, P-gp cleavage was not detected in vivo in MCF7 cells induced to undergoing apoptosis by either roscovitine or sangivamycin, despite activation of both caspase-6 and -7. Interestingly, P-gp overexpressing MCF7 cells were more sensitive to either roscovitine or sangivamycin than wild-type cells, suggesting a novel potential therapeutic strategy against P-gp overexpressing cells. Taken together, our results support the concept that caspase-3 is the only caspase responsible for in vivo cleavage of P-gp and also highlight small molecules which could be effective in treating P-gp overexpressing cancers.

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Introduction

Cancer patients who undergo chemotherapy often experience intrinsic or acquired resistance to a wide spectrum of chemotherapeutic agents. One type of drug resistance to anticancer compounds is mediated by the overexpression of the 170-kDa P-glycoprotein (P-gp), the product of multidrug resistance (*MDR1*) gene.[1] P-gp is a transmembrane glycoprotein and a member of the ATP-binding cassette (ABC) superfamily of membrane transport proteins. P-gp works like an energy-dependent efflux pump which can extrude various structurally unrelated chemicals at the expense of ATP depletion, resulting in decrease of the intracellular cytotoxic drug accumulation in cancer cells.[2] Remarkably, expression of P-gp has been documented in putative cancer stem cells.[3-5]

It has been reported that resistance to different drugs due to the expression and function of P-gp, leads to a parallel resistance to a number of apoptotic stimuli.[6,7] It has been suggested that overexpression of P-gp impairs the activation of caspases,[8] a family of proteases which play fundamental roles in apoptosis.[9-13] We recently demonstrated that in P-gp overexpressing CEM-R T-lymphoblastoid cells, P-gp was cleaved in vivo by caspase-3 during apoptosis induced by a number of unrelated stimuli.[14] Subsequently, it has been suggested that P-gp cleavage primes a caspase-3-mediated amplification of apoptotic signaling pathways and could be considered as a step toward a no-return pathway for apoptosis in tumor cells overexpressing P-gp.[15] Caspases are specific cysteine proteases recognizing four amino acids, named S4, S3, S2, S1. The preferred S3 position is an invariant glutamine for all mammalian caspases, while S1 is an aspartate residue. Thus,

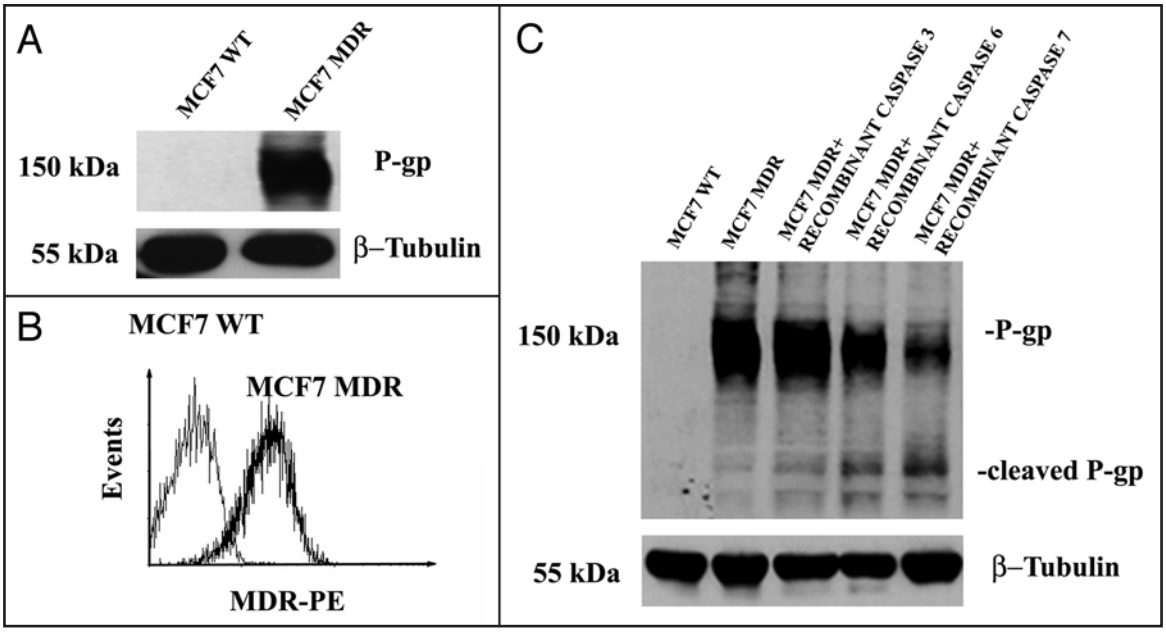

Figure 1. Expression and in vitro cleavage of P-gp in MCF7 MDR cells. (A) western blot analysis documenting expression of P-gp in stably transfected MCF7 MDR cells. MCF7 WT: parental cells. Forty µg of proteins were loaded per lane. Antibody to β-tubulin demonstrated equal loading. (B) flow cytometric analysis. Cells were stained for surface P-gp using a monoclonal antibody conjugated to PE. At least 10,000 events were analyzed. (C) western blot analysis demonstrating in vitro cleavage of P-gp by purified recombinant human capsase-3, -6 and -7. Antibody to β-tubulin demonstrated equal loading. In (A and C), molecular weights are indicated at left.

specificity for caspase cleavage can be better described as X-Glu-X-Asp.[16] The cleavage site of P-gp during apoptosis has not been identified so far.

However, P-gp protein sequence contains over 30 aspartate residues which could act as cleavage site(s).[17] It is also unclear whether only caspase-3 cleaves P-gp during apoptosis, or other executioner caspases, including caspase-6 and -7, could be involved. To address the issue of whether caspases other than caspase-3 could cleave P-gp, we took advantage of the MCF7 human breast cancer cell line, as these cells are devoid of functional caspase-3.[18] However, they express other functional executioner caspases, including caspase-6 and -7.[19] Here, we demonstrate that recombinant caspase-3, -6 and -7, all cleaved P-gp in vitro in lysates prepared from P-gp overexpressing MCF7 cells. However, when MCF7 cells were induced to undergo apoptosis by either roscovitine or sangivamycin, no in vivo P-gp cleavage was detected, despite activation of both caspase-6 and -7. Therefore, our results suggest that caspase-3 is critical for in vivo cleavage of P-gp.

Results

In vitro caspase-dependent P-gp cleavage. Initially, we evaluated P-gp expression levels in human MCF7 cells stably transfected with *MDR1* cDNA. Western blot analysis documented P-gp expression (Fig. 1A). This phenotypic profile was also confirmed by flow cytometry (Fig. 1B). Since we previously documented both in vitro and in vivo caspase-3-dependent P-gp cleavage in CEM-R cells, we assessed the in vitro cleavage action of recombinant effector caspase-3, -6 and -7, using MCF7 MDR cell extracts. All three caspases were able to cleave P-gp, as indicated by the appearance of the 85-kDa cleavage product in western blots (Fig. 1C). However, caspase-6 and -7 were more effective than caspase-3.

MCF7 MDR cells are susceptible to roscovitine- and sangivamycin-induced apoptosis. Since human MCF7 breast cancer cells are relatively resistant to conventional chemotherapy, due to the lack of caspase-3 activity, we exposed MCF7 cells to roscovitine and sangivamycin, two inhibitors which exert proapoptotic activity in MCF7 cells.[19,25] Apoptotic cell death of MCF7 WT and MCF7 MDR cells was documented by AnnexinV-FITC/PI staining and also confirmed by flow cytometric analysis of PI-stained samples (Fig. 2A and B). Both techniques gave similar results and highlighted that MCF7 MDR cells were significantly ($p < 0.05$) more sensitive to the inhibitors than MCF7 WT cells.

Lack of caspase-dependent in vivo P-gp cleavage in response to roscovitive and sangivamycin. Western blot analysis documented that in MCF7 MDR cells, there was no in vivo P-gp cleavage in response to either roscovitine or sangivamycin (Fig. 2C), in spite of the activation of both caspase-6 and -7 (Fig. 2D). Caspase-7 activation was demonstrated by the appearance of its 20-kDa cleaved product, while caspase-6 activation was observed by the decrease of its native form. Interestingly, caspase inhibitors decreased caspase activation, suggesting that caspase-6 and -7 could cleave each other. Surprisingly, both roscovitine and sangivamycin, caused an increase in full length P-gp, which was strongly opposed by the caspase 6-inhibitor and, to a lesser extent, by the caspase 7-inhibitor (Fig. 2C). Both caspase inhibitors prevented apoptotic cell death (not shown).

P-gp gene expression analysis. To investigate if the increase in full length P-gp occurred as a result of increased gene expression, we performed Real Time RT-PCR analysis (Fig. 3). P-gp mRNA

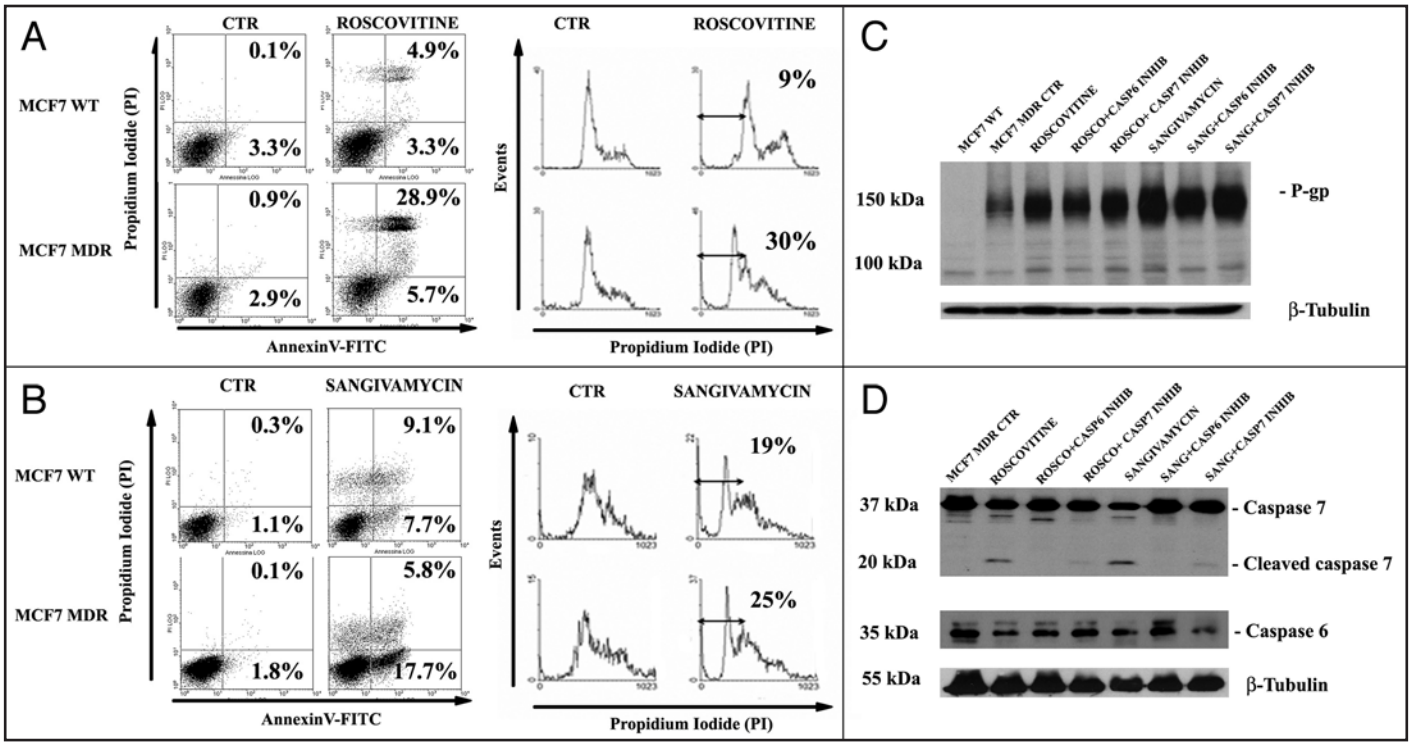

Figure 2. Roscovitine and sangivamycin are proapoptotic in MCF7 cells. (A and B) Annexin V-FITC/PI staining and cell cycle analysis of MCF7 cells treated with either roscovitine or sangivamycin. CTR, Control (untreated) cells. At least 10,000 events were analyzed. In both (A and B), one representative of three separate experiments is shown. In (A), the percentage of early (lower right quadrant) and late (upper right quadrant) apoptotic cells are indicated. In (B), the percentage of sub-G_0/G_1 cells is highlighted. The differences between MCF7 WT and MCF7 MDR documented in (A and B) were found significant ($p < 0.05$), when statistically analyzed. (C) western blot analysis for P-gp expression in samples treated with either roscovitine or sangivamycin. (D) western blot analysis for caspase-6 and -7 in samples treated with either roscovitine or sangivamycin. In both (C and D), 40 μg of protein were loaded per lane, and antibody to β-tubulin demonstrated equal loading. The blots in (C and D) are representative of three separate experiments. Molecular weights are indicated at left.

level increased as a result of sangivamycin treatment and decreased in samples pre-incubated with either caspase 6- or caspase-7 inhibitor. In contrast, at 24 hr of roscovitine treatment a marked decrease in P-gp mRNA was observed, which was not prevented by either caspase 6- or caspase 7-inhibitor (Fig. 3).

Discussion

In tumor cells, apoptotic pathways are frequently deregulated due to inactivation or downregulation of pro-apoptotic genes, including caspase-3.[26] Indeed, a significant majority of human breast tumors lack caspase-3 expression, suggesting that loss of caspase-3 may represent an important cell survival mechanism in breast cancer patients.[27] Moreover, it is established that in roscovitine-treated MCF7 breast carcinoma cells, different apoptotic pathways are initialized depending on the status of caspase-3.[25] Recently, it has been proposed that a proapoptotic self-enhancing mechanism, depending on caspase-3 cleavage of P-gp, regulated apoptosis in a mithocondrial-dependent manner.[15]

Caspase-3 deficient MCF7 cells were chosen as an optimal model to test the hypothesis that caspase-3 activation in vivo is pivotal to apoptotic P-gp cleavage in P-pg overexpressing cells. Because pharmacological selection of cell lines expressing P-gp could cause genetic modifications, thus affecting cell sensitivity to death stimuli, MCF7 cells were transduced with *MDR1* cDNA. Resistance to apoptotic stimuli has been reported frequently in MCF7 cells,[27] thus apoptosis was induced with two non-P-gp substrates, proapoptotic drugs, roscovitine and sangivamycin. Roscovitine, an olomoucine-related purine flavopiridol, acts as a potent inhibitor for the activity of cyclin-dependent kinase (CDK), CDK1, CDK2, CDK5 and CDK7. Roscovitine arrested human MCF7 cancer cells in the G_2/M phase of the cell cycle and concomitantly induced apoptosis.[25] Sangivamycin, a protein kinase C inhibitor, displayed a cytotoxic effect in multidrug-resistant MCF7 cells, causing massive apoptotic cell death, probably via activation of caspase-6, -7 and -9.[19]

In MCF7 MDR cells, both roscovitine and sangivamycin induced apoptosis, through the activation of caspase-6 and -7, suggesting loss of caspase-3 is compensated by the activation of other executioner caspases.[19] In our hands the activation of these executioner caspases was sufficient to induce MCF7 MDR to undergo apoptosis, but did not lead to the characteristic in vivo P-gp cleavage we previously observed in apoptotic lymphoblastoid CEM-R cells (i.e., the 85-kDa fragment formation). Therefore, the roscovitine- and sangivamycin-activated apoptotic pathways, were not able to affect P-gp integrity in MCF7 MDR cells. In contrast, when MCF7 MDR cell extracts were incubated in vitro with recombinant caspase-3, -6 or -7, P-gp was cleaved, and we detected the typical 85 kDa P-gp-cleavage product. Although it is clear that

roscovitine and sangivamycin were proapoptotic in MCF7 MDR cells, they also increased expression of full length P-gp, presumably in a caspase-dependent manner. In fact, pre-treatment with caspase-6 or -7 inhibitors, resulted in a decreased expression of full length P-gp.

It has been demonstrated that in MCF7 cells, roscovitine induced a site-specific phosphorylation of p53 protein on Ser46 and its accumulation in the nucleus where it could regulate P-gp expression[25] In addition, sangivamicyn, through c-Jun N-terminal kinase (JNK) pathway activation[19] may modulate p53 and P-gp expression. Accordingly, we would have expected a reduction in P-gp expression in samples treated with sangivamycin, as JNK has been reported to negatively affect P-gp gene expression through c-Jun.[23,28] Only in sangivamicyn-treated MCF7 MDR cells were the changes in P-gp mRNA expression correlated to changes in P-gp protein levels. The promoter of the *MDR1* gene harbors stress-responsive elements, which could be induced by cytostatic drugs.[29] In MCF7 MDR cells, sangivamycin inhibited cell cycle progression by inducing an arrest in the G_1 phase (data not shown). Therefore, we speculate that sangivamycin could lead to *MDR1* gene transcription through a mechanism similar to that of cytostatic drugs. In contrast, in roscovitine-treated MCF7 MDR cells this trend was not observed. Therefore, in this case, increased P-gp protein expression could be due to other mechanisms, such as protein stabilization and increased half-life.

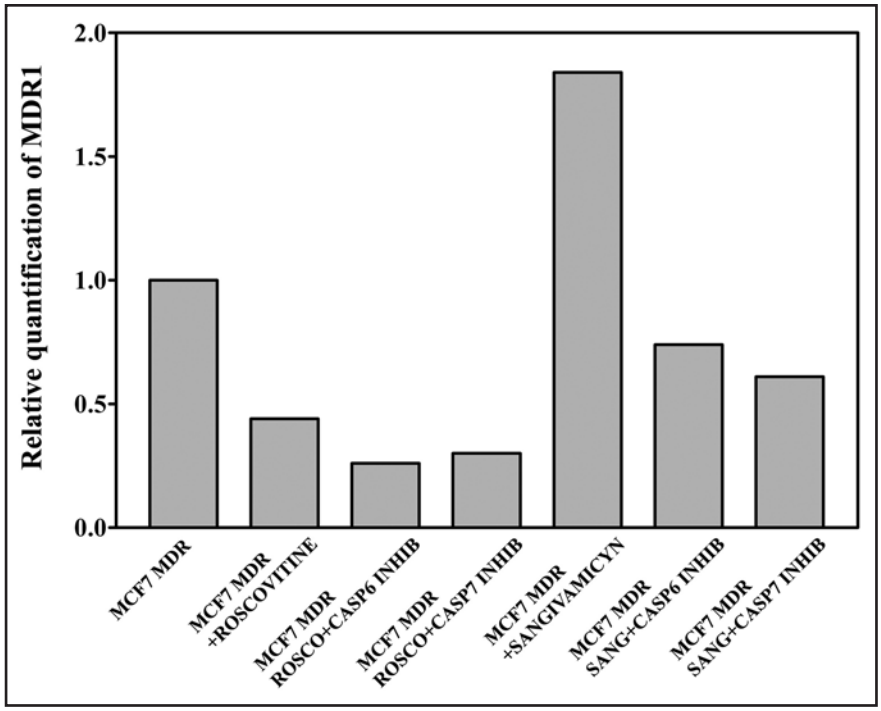

Figure 3. Effects of roscovitine and sangivamycin on P-gp mRNA expression levels. Real-Time RT-PCR was performed as described in Materials and Methods. GAPDH was used as the housekeeping gene. One representative of three different experiments which gave similar results is presented.

An interesting finding is that MCF7 MDR cells were more sensitive to both roscovitine and sangivamycin than parental cells. This observation could be clinically relevant, as roscovitine is now undergoing clinical trials for cancer patients.[30-32]

In conclusion, our findings demonstrate that caspase-3 plays a fundamental role in vivo in apoptotic P-gp cleavage and that, in cases where caspase-3 is lacking, P-gp cleavage could not occur, in spite of caspase-6 and -7 activation, whereas in vitro both caspase-6 and -7 could substitute for caspase-3. The reasons for this phenomenon are at present unknown, but they may be related to a different subcellular localization of capase-6 and -7 relative to P-gp, which would preclude them from cleaving P-gp in vivo. Future investigations should clarify this issue. However, in P-gp overexpressing cells, apoptosis did take place even if P-gp was not cleaved by caspases, indicating that cleavage itself is not a prerequisite for cell death to occur.

Materials and Methods

Cell culture and induction of apoptosis. The parental human mammary adenocarcinoma cell line MCF7 (MCF7 WT) was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown as a monolayer up to 60–70% confluence in Minimum Essential Medium supplemented with 10% fetal bovine serum, and 5 mM L-glutamine at 37°C. For apoptosis induction, cells were treated with either roscovitine (30 μM, Sigma-Aldrich Co., St. Louis, MO, USA) for 24 hr, or with sangivamycin (5 μM, Calbiochem, La Jolla, CA, USA) for 16 hr. In some experiments, cells, prior to apoptosis induction were preincubated for two hr with 40 μM of either caspase-3/7 inhibitor I or Z-VEID-FMK (a caspase-6 inhibitor), both from Calbiochem.

Cell transfection. The full length 3.843 Kb cDNA for human *MDR1*,[20] was amplified by PCR using Accuprime Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and cloned into BamHI/XbAI sites of the pcDNA 3.0 (Invitrogen) expression vector into using the following primers: 5'-CGC GGA TCC GCG ATG GAT CTT GAA GGG GAC CG-3' and 5'-GCT CTA GAG CTC ACT GGC GCT TTG TTC CAG-3'. MCF7 cells were electroporated using the Cell Line Nucleofector Kit V (Amaxa Instrumentation Laboratories, Koln, Germany) following the manufacturer's instructions. Selection of the stable MCF7 *MDR1*-overexpressing clone was performed in the presence of G418 (2 mg/ml). After three weeks of selection, cell surface expression of P-gp was detected by flow cytometry using phycoerythrin (PE)-conjugated anti-P-gp monoclonal antibody (BD Biosciences Pharmingen, Milan, Italy)[14] After selection, positive cultures (thereafter named MCF7 MDR) were routinely maintained in 800 μg/ml G418.

In vitro P-gp cleavage. Cells were lysed in caspase lysis buffer (12.5 mM Hepes-NaOH, pH 7.4, 10% sucrose, 0.1% CHAPS, 2 mM EDTA, 5 mM DTT). Samples were incubated on ice for 1 hr, passed five times trough a 26 gauge needle, and then centri-

fuged for 15 min at 4°C at 17,000 xg. Protein concentration in the supernatants was determined by standard techniques. Samples (300 μg of protein) were incubated for 4 hr at 37°C in the presence of recombinant human caspase-3, -6 or -7 (50 Units/each, all from from Calbiochem), as reported elsewhere.[14] Electrophoresis sample buffer was then added to stop the reaction.

Preparation of whole cell lysates and western blot analysis. Cells were lysed as described elsewhere,[21] and proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes, as previously reported.[22] Membranes were incubated overnight at 4°C with the following antibodies: mouse monoclonal antibody to P-gp (clone F4, Sigma-Aldrich), rabbit antibody to either caspase-6 or -7 (Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibody to β-tubulin (Sigma-Aldrich). Nitrocellulose membranes were then processed as described elsewhere.[23]

Flow cytometric analysis of cell cycle and apoptosis. Cell cycle distribution was analyzed by flow cytometric analysis of samples stained with propidium iodide (PI, 20 μg/ml) for 0.5 hr. At least 10,000 events/sample were recorded. Apoptosis was detected using an Annexin V-FITC/PI kit (Bender MedSystems, Vienna, Austria) following the manufacturer's instructions. Samples were then analyzed by flow cytometry using an XL flow cytometer (Beckman Coulter, Miami, FL, USA).[24]

Real-time RT-PCR. To analyze and quantify the levels of *MDR1* gene expression, we used a TaqMan isoform-specific probe (Assay n° Hs00184491_m1, Applied Biosystems, Foster City, CA). Reactions were run on the ABI Prism 7300 sequence detection system (Applied Biosystems) with the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Quantification analysis was performed using the ΔΔCt method and statistically analyzed by GraphPad Prism 4.0 software.

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