J Med Biochem 2013; 32 (4) DOI: 10.2478/jomb-2013-0050

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 32: 406–416, 2013

Original paper
Originalni naučni rad

NO-MODIFIED SAQUINAVIR IS EQUALLY EFFICIENT AGAINST DOXORUBICIN SENSITIVE AND RESISTANT NON-SMALL CELL LUNG CARCINOMA CELLS

MODIFIKOVANA FORMA SAKVINAVIRA EFIKASNO SUPRIMIRA RAST ĆELIJA NESITNOĆELIJSKOG KARCINOMA PLUĆA RAZLIČITE OSETLJIVOSTI NA DOKSORUBICIN

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Summary

Background: The NO-modified form of the HIV inhibitor saquinavir (Saq-NO) inhibited the growth of a variety of cancer cell lines *in vitro* and *in vivo* more potently than the original compound in a nontoxic fashion. In addition, chemo- and immunosensitizing properties were observed. The aim of the present study was to evaluate its anticancer action against non-small cell lung carcinoma cells in their doxorubicin (DOXO) sensitive and resistant phenotype (NCI-H460 and NCI-H460/R).
Methods: The viability of cells was analyzed by MTT and crystal violet assays. DR5 expression was estimated by real time RT-PCR and flow cytometry. Activity of P-glycoprotein (P-gp) pumps was evaluated by the Rho123 accumulation assay.
Results: Saq-NO diminished the viability of lung cancer cells through induction of cell cycle arrest in the G_0/G_1 phase independently of the overexpression of the P-gp pumps. In addition, Saq-NO elevated or completely reconstituted the doxorubicin efficacy in NCI-H460 and NCI-H460/R, respectively. The chemosensitizing effect in DOXO resistant cells was a consequence of P-gp inhibition which was found to be

Kratak sadržaj

Uvod: Inhibitor HIV proteaze – sakvinavir nakon modifikacije kovalentnim vezivanjem NO (Saq-NO) gubi toksična svojstva dok potentnije od originalnog jedinjenja inhibira *in vitro* i *in vivo* rast brojnih ćelijskih linija kancera. Pored direktnog antitumorskog delovanja, Saq-NO povećava osetljivost ćelija kancera na antitumorski imunski odgovor i konvencionalnu hemioterapiju. Ova studija je imala za cilj ispitivanje antitumorskog potencijala Saq-NO na ćelijskim linijama nesitnoćelijskog karcinoma pluća, senzitivnim (NCI-H460), odnosno rezistentnim (NCI-H460/R) na doksorubicin.
Metode: Vijabilitet ćelija je evaluiran testovima MTT i »kristal violet«. Ekspresija receptora DR5 je procenjivana metodom RT-PCR u realnom vremenu i protočnom citofluorimetrijom. Aktivnost P-gp pumpi određivana je akumulacionim testom Rho123.
Rezultati: Saq-NO inhibira rast ćelija kancera pluća zaustavljanjem ćelija u fazi G_0/G_1 ćelijskog ciklusa a zapaženi efekat nije oslabljen povećanjem ekspresije P-gp pumpi. Pored toga, Saq-NO povećava osetljivost NCI-H460 ćelija, dok u slučaju rezistentne forme, NCI-H460/R, potpuno rekonsti-

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more potent than that observed with dex-verapamil, a conventional inhibitor of P-gp. Sensitization to DOXO upon Saq-NO was accompanied by elevated DR5 expression, but the resistance to TRAIL was not abrogated.

Conclusions: The NO-modified HIV inhibitor saquinavir displayed equal antiproliferative and chemosensitizing properties in DOXO sensitive and resistant non-small cell lung carcinoma cells, suggesting the importance of the evaluation of this drug as an antineoplastic agent.

Keywords: chemosensitization, non-small cell lung carcinoma cells, saquinavir, saquinavir-NO, tumor

tuiše njihovu osetljivost na doksorubicin. Efekat hemosenzitizacije je posledica inhibicije P-gp pumpi, što Saq-NO čini potentnijim od deksverapamila, uobičajenog inhibitora P-gp. Opisani fenomen je praćen povećanjem ekspresije receptora DR5 na genskom i membranskom nivou, ali time rezistencija na molekul TRAIL nije ukinuta.

Zaključak: Saq-NO pokazuje značajan antiproliferativan i hemosenzitizujući potencijal na ćelijama nesitnoćelijskog kancera pluća nezavisno od njihove osetljivosti odnosno rezistencije na doksorubicin, ukazujući na potrebu daljeg ispitivanja ovog jedinjenja u svojstvu potencijalnog antineoplastičnog agensa.

Ključne reči: tumor, sakvinavir, sakvinavir-NO, nesitnoćelijski karcinom pluća, hemosenzitizacija

Introduction

Lung cancer is a complex malignant disease with a variety of histological types. Non-small cell lung carcinoma (NSCLC) is one of the most frequent types of lung cancer and it accounts for approximately 75% of all lung carcinomas. Currently, chemotherapy remains the mainstay of treatment; unfortunately, the 5-year survival of patients is still poor, fixed at 15%, without significant improvement over the last two decades (1).

Loss of sensitivity to death receptor-mediated killing by immune system products, as well as the rapid development of the chemoresistant phenotype, are recognized as the leading causes of the low rate of curability of NSCLC. Both phenomena are frequently connected to the establishment of intracellular resistance to induction of apoptosis (2). Therefore, new drugs with the ability to overcome this barrier and remove the death pathway blockage are extremely desirable. Saquinavir-NO (Saq-NO) represents a modality of the HIV protease inhibitor saquinavir. Saquinavir (Saq) was created to inhibit the formation of infective viral particles through blockage of the HIV enzyme active site (3). However, it was found that this drug effectively suppressed the development of HIV-related malignancies or reduced their presence (4). This property was subsequently investigated, and it was shown that inhibition of the PI3K-Akt signaling pathway and proteosome activity were connected with its antitumor feature (4–12). Unfortunately, the presence of side effects seriously limited Saq usage and presented the opportunity for designing different forms of this drug with an advanced pharmacological profile (13). Attachment of the nitric oxide (NO) moiety to saquinavir generated a new drug with significantly improved antitumor characteristics (14). Saq-NO was completely nontoxic *in vitro* and *in vivo*, while its activity against a wide range of cell lines was qualitatively and quantitatively increased in comparison to the parental drug (14). The novel agent was efficient in numerous syngeneic and xenograft tumor models without exhibiting any signs of biochemical or visible toxicity (14–20). The unique mechanism of its tumoricidal action includes nonaggressive inhibition of proliferation due to suppression of S6 protein which is important in the translation of 5′ terminal oligopyrimidine (TOP) containing mRNA and induction of differentiation towards the normal nonmalignant phenotype (18). Apart from these important features, Saq-NO was able to sensitize the tumor cells to chemo- and immune response and therefore to prevent the growth of tumors with an intrinsic resistance to apoptosis (15, 18). Saq-NO was found to be efficient in prostate cancer cells which overexpressed MDR (20), but there are no published data concerning the efficacy of Saq-NO against one of the most aggressive forms of cancer, NSCLC, which is either sensitive or resistant to chemotherapy. Results presented in this study clearly indicate that Saq-NO nonselectively affected both NSLC phenotypes either susceptible or not to the conventionally used chemotherapeutic drug doxorubicin. Besides its direct antiproliferative potential, Saq-NO was effective against both doxorubicin sensitive and resistant NSCLC cell lines. Finally, Saq-NO amplified the expression of TRAIL specific death receptor without restoration of its functionality.

Material and Methods

Reagents and cells

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). The NCI-H460 human non-small lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VI). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 0.01% sodium pyruvate, and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. After trypsinization, cells were seeded at 4×10^3/well in 96-well plates for viability determination and 1.5 ×

10^5/well in 6-well plates for flow cytometric analysis. Resistant NCI-H460/R cells were selected originally from NCI-H460 cells by exposure to gradually increasing concentrations of doxorubicin (21).

Determination of cell viability by MTT and crystal violet assay

For the determination of cell viability, the crystal violet (CV) and mitochondrial dehydrogenase tests (MTT) were performed. Cells (4×10^3/well) were cultivated in the presence of a wide range of drug doses for 48 h and viability was determined as previously described (22). The results are presented as percentage of the control (untreated cells), that was arbitrarily set to 100%.

Cell cycle analysis

Cells were treated with 18.8 μmol/L of Saq and Saq-NO, trypsinized and fixed in 70% ethanol at 4 °C overnight. Cells were stained with a solution containing PI (20 μg/mL) and RNase (0.1 mg/mL) for 30 min at 37 °C in the dark. After each step, cells were washed with PBS, and red fluorescence was analyzed on a FACS Calibur flow cytometer (BD, Heidelberg, Germany). The distribution of cells in different cell cycle phases was determined with Cell Quest Pro software (BD) (23).

Isobologram analysis

Isobologram analysis was used to determine the type of interaction between Saq-NO and recombinant human TRAIL. Isobolograms were created after treatment with a wide range of Saq-NO concentrations (4.7–18.8 μmol/L) with different concentrations of TRAIL (6.2–50 ng/mL). Combinations reaching 30–70% of cytotoxicity were expressed as concentration of single agent alone leading to the observed amount of toxicity. Analysis was done using dose-response curves after exposure to Saq-NO alone, TRAIL alone, or their combination for 24 h (15, 24).

RNA isolation and relative quantification of DR5 mRNA by real time RT-PCR

Total RNA was isolated from NCI-H460 and NCI-H460/R cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two μg of RNA were converted to cDNA by reverse transcription (RT) reactions in a 100 μL volume with random hexamer primers using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. The reactions were performed under RNase-free conditions at 25 °C for 10 min and 37 °C for 2 h. Each RT reaction was accompanied by a no-RT control in which the reverse transcriptase was replaced by DEPC treated water.

The expression of DR5 mRNA was evaluated by SYBR Green (Applied Biosystems) real time RT-PCR. PCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 25 μL containing 1× SYBRGreen PCR master mix with AmpErase UNG (Applied Biosystems), 0.5 μmol/L primers and cDNA template (20 ng of RNA converted to cDNA) at cycle conditions: 2 min at 50 °C for dUTP activation, 10 min at 95 °C for initial denaturation and Taq polymerase activation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Primer pairs (Sigma) were: for DR5 5′-TGC AGC CGT AGT CTT GAT TG-3′ and 5′-GCA CCA AGT CTGCAAAGTCA-3′ and for GAPDH 5′-CATCCAT-GACAACTTTGG TAT CG-30 and 5′-CCA TCA CGC CAC AGT TTC C-3′. The experimental threshold was calculated from the mean baseline fluorescence signal from cycle 3 to 15, plus 10 standard deviations. The point at which the amplification plot crosses this threshold is defined as Ct. No template control was used in each run. Each sample was run in triplicate and a mean value of each Ct triplicate was used for further calculation. A reference, endogenous control, was included in every analysis to correct the differences in inter-assay amplification efficiency, and the expression of each gene was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. Quantification was performed by the $2^{-\Delta\Delta Ct}$ method (25). The results obtained were analyzed by RQ Study Add ON software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% ($p<0.05$).

DR5 membrane expression

The expression of DR5 was assessed by flow cytometry as previously described (24). After the 24 h of treatment with 18.8 μmol/L, Saq-NO cells were detached with PBS-EDTA (1 mmol/L), incubated in PBS–10% FCS for 1 h for membrane reconstitution, and then stained with anti-DR5 antibody (mouse anti-human DR5, Biosource International, Camarillo, CA) 45 min at RT. After washing in PBS–0.5% BSA, cells were incubated for 30 min at RT with secondary TRIC-conjugated goat anti-mouse IgG (ZymedTM, Invitrogen) washed twice with PBS–0.5% BSA and finally resuspended in PBS. Analysis was performed on a FACS Calibur flow cytometer using CellQuest software.

Rho123 accumulation assay

Rho123 accumulation was analyzed by flow cytometry utilizing the ability of Rho123 to emit fluorescence. The intensity of the fluorescence was proportional to Rho123 accumulation. Studies were per-

formed with dex-VER and Saq-NO. NCI-H460/R cells were grown to 80% confluence in 75 cm^2 flasks, trypsinized and resuspended in 10 mL centrifuge tubes in a Rho123-containing medium. Then, the cells were treated with Saq-NO and VER (18.8 μmol/L) and incubated at 37 °C in 5% CO_2 for 30 min. At the end of the incubation period, the cells were pelleted by centrifugation, washed with PBS and placed in cold PBS. The samples were kept on ice in the dark until analysis on a FACS calibur flow cytometer (Becton Dickinson, Oxford, United Kingdom). The fluorescence of Rho123 was assessed on fluorescence channel 2 (FL2-H) at 530 nm. A minimum of 10,000 events were assayed for each sample. The differences in curve shape were quantified using a Kolmogorov–Smirnov nonparametric statistic. P values were calculated (available on request) in CellQuest Pro and run on a Macintosh computer.

Statistical analysis

The results are presented as mean ± SD of triplicate observations from the representative of three experiments, except if indicated otherwise. The significance of the difference between treatments and control was analyzed by ANOVA followed by Student–Newman–Keuls test. $P<0.05$ was considered significant.

Results

Saq-NO decreased the viability of both doxorubicin sensitive and resistant NSCLC cells

To investigate the effects of Saq-NO and Saq on the viability of sensitive NSCLC cells (NCI-H460) and compare it with their potential to inhibit the growth of doxorubicin resistant NSCLC cells (NCI-H460/R), cells were exposed to a wide range of doses for 48 h when the number of viable cells was measured by both MTT and CV assays. As can be seen in *Figure 1*, Saq-NO decreased the mitochondrial respiration (left panel) and the viability (right panel) of NCI-H460 cells more potently than its parental drug, Saq. Importantly, the same pattern of action of the HIV protease inhibitor and its NO-modified form was observed in the doxorubicin resistant form of this cell line. The results obtained from the viability assays clearly indicated that NO-modified Saquinavir possesses stronger potential to suppress the growth of doxorubicin sensitive and resistant non-small lung carcinoma cells than the original drug (*Figure 1A*). It was previously observed that Saq-NO acted basically through decreasing their dividing potentials. To determine the reason for the abrogated viability observed in NCI-H460 or NCI-H460/R cells after exposure to Saq-NO, cell cycle distribution was estimated. For that purpose, NCI-H460 and NCI-H460/R cells were incubated for 48 h with doses at the IC_{50} level. Flow cytometric analysis revealed a remarkable G_0/G_1 arrest in cultures exposed to the drug independently from their sensitivity to doxorubicin. In parallel with this, an insignificant amount of hypodiploid cells was detected upon the treatment indicating that inhibition of proliferation rather than apoptosis was responsible for the diminished number of cells observed after the treatment with Saq-NO. Ann/PI double staining confirmed the absence of both early and late apoptotic cells in NCI-H460 and NCI-H460/R cells (data not shown) confirming the nonaggressive suppression of tumor cell growth. In summary, overexpression of P-gp in NSCLC cells did not alter the capacity of Saq-NO to downregulate the rate of cell division and decrease their malignant potential.

Saq-NO increased the sensitivity of NSCLC cells to doxorubicin

Since we observed that Saq-NO was equally efficient against doxorubicin sensitive and resistant cells, it was of interest to evaluate its capacity to provoke or to revert the sensitivity to the chemotherapeutic drug. NCI-H460 and NCI-H460/R cells were treated with Saq-NO for 4 h prior to doxorubicin application. Cell viability was measured by the CV test after the 48 h. As shown in *Figure 2A*, Saq-NO amplified the toxicity of doxorubicin in both sensitive and resistant cells under these experimental conditions. These effects were observed when the cells were exposed to both treatments simultaneously. The viability of cells simultaneously treated with Saq-NO and doxorubicin was significantly lower in comparison to their viability when treated with doxorubicin alone and the effect was more obvious on NCI-H460/R cells, as expected (*Figure 2B*). Importantly, the chemosensitizing capacity of Saq-NO was more potent than the one observed with the P-gp inhibitor dex-verapamil, or original drugs in the same doses (4.5 left or 9 μmol/L, right) (*Figure 2C*). Taken together, Saq-NO is able to sensitize the resistant cells to one of the most frequently used chemotherapeutic drugs, doxorubicin, and to decrease the efficient dose in sensitive clones.

Saq-NO blocked the P-gp pumps

The ability of Saq-NO to sensitize the resistant NCI-H460/R cells to doxorubicin indicated the possibility that the target for its action are P-gp pumps which are overexpressed in these cells. To evaluate this hypothesis, the cells were exposed to 18.8 μmol/L of either Saq-NO or dex-verapamil for 30 min and then the capacity of the cells to export the Rho123 stain was measured by flow cytometric analysis (*Figure 3*). Cells exposed to Saq-NO exhibited increased Rho123 fluorescence, confirming that Saq-NO blocked the P-gp pumps more potently than the commercial P-gp inhibitor dex-verapamil.

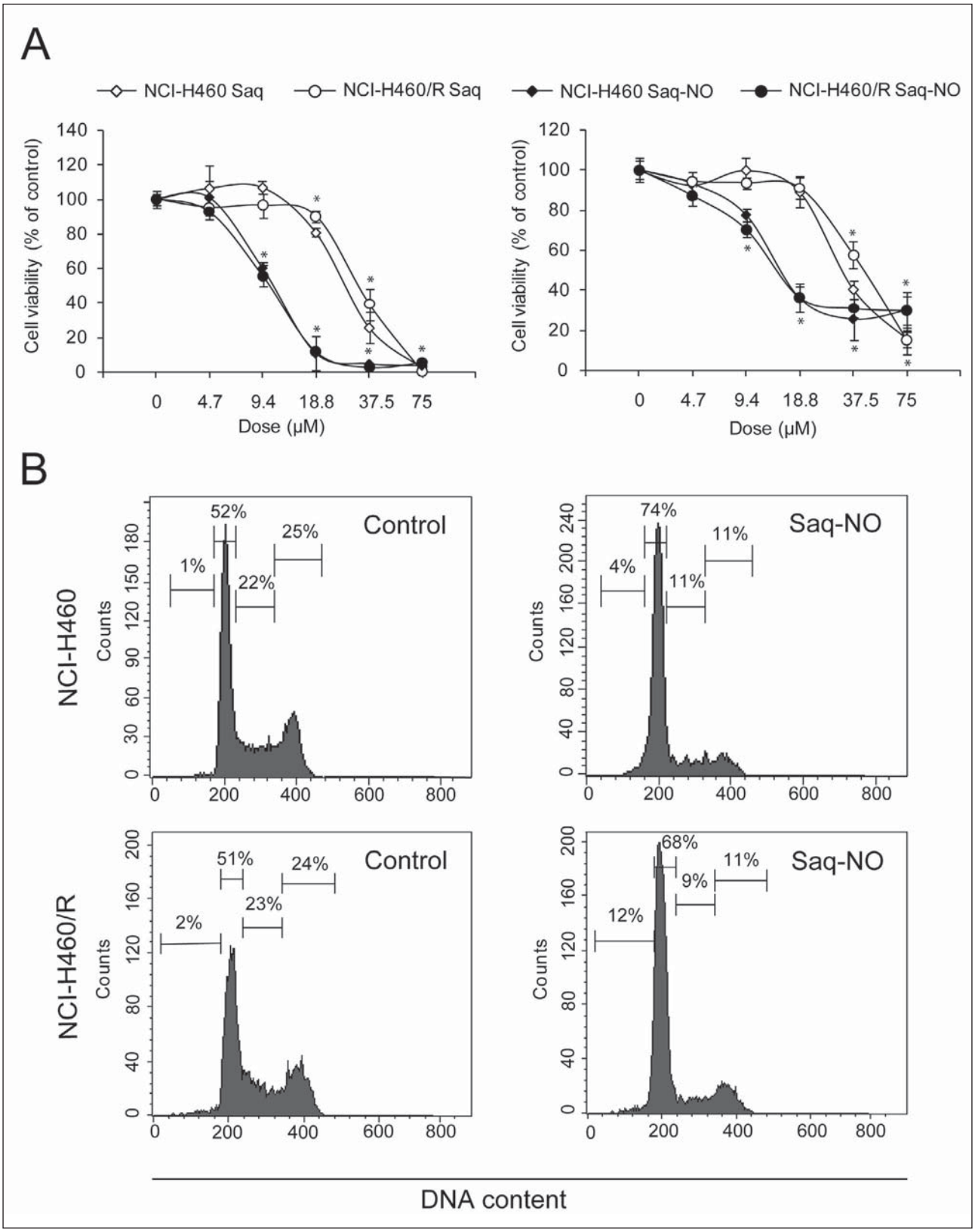

Figure 1 Saq-NO decreased viability of both DOXO-sensitive and DOXO-resistant human NSCLC cell lines. A, NCI-H460 and NCI-H460/R cells (4×10^3 cells/well) were treated with a range of concentrations of Saq or Saq-NO for 48 h after which cell viability was estimated by MTT (left panel) and CV (right panel) tests. The data are presented as the mean ± S.D. from a representative of three independent experiments. *, $p < 0.05$, refers to untreated cultures. B, Cells were treated with IC50 dose (18.8 μM) of Saq-NO or left untreated (control) and cell cycle analysis was performed by flow cytometry after 48 h.

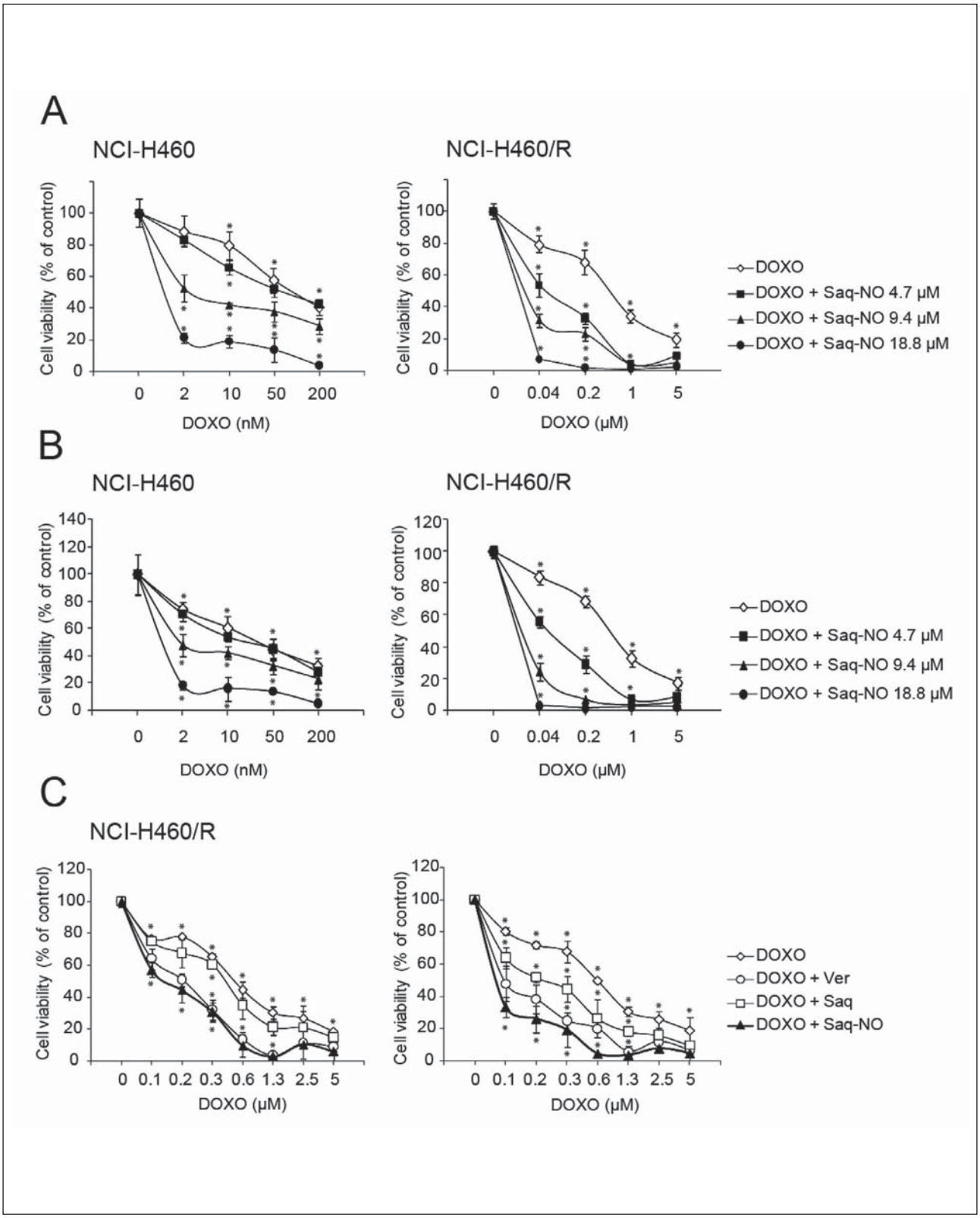

Figure 2 Saq-NO is more potent than dex-verapamil in sensitizing human NSCLC cells to DOXO. NCI-H460 and NCI-H460/R cells (4×10^3 cells/well) were pretreated with various doses of Saq-NO for 6 h (A) or simultaneously treated with a range of DOXO concentrations (B). After 48 h of DOXO treatment, cell viability was estimated by CV. C, NCI-H460/R cells (4×10^3 cells/well) were simultaneously treated with 4.7 μmol/L (left panel) or 9.4 μmol/L (right panel) of dex-ver, Saq or Saq-NO and with a range of concentrations of DOXO and after 48 h cell viability was evaluated by CV. The data are presented as the mean ± S.D. from a representative of three independent experiments. *, $p < 0.05$, refers to untreated cultures.

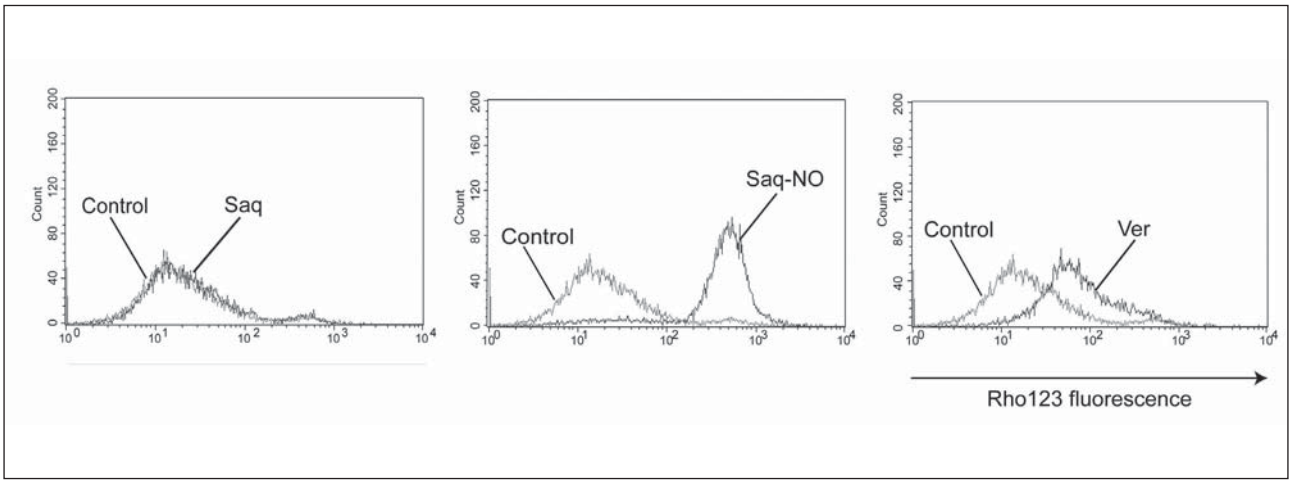

Figure 3 Saq-NO inhibited P-gp activity. NCI-H460/R cells were treated with Saq, Saq-NO or dex-ver (18.8 μmol/L) or left untreated (control) for 30 min and Rho123 accumulation was analyzed by flow cytometry.

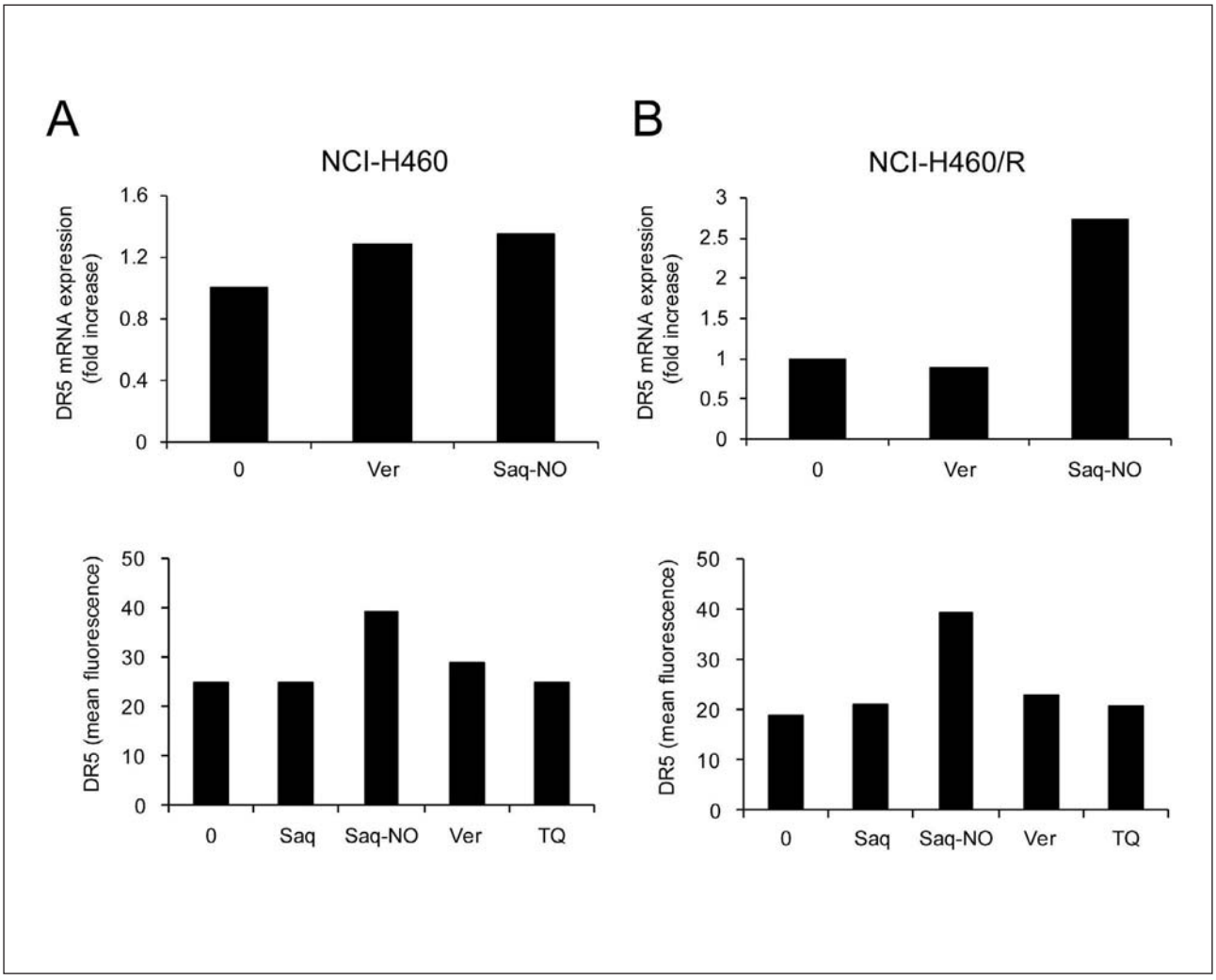

Figure 4 SSaq-NO upregulated DR5 expression at mRNA and membrane level. NCI-H460 (A) and NCI-H460/R (B) cells were treated with Ver or Saq-NO (18.8 μmol/L for 24 h and qRT PCR analyses for DR5 mRNA was performed (upper panels) or cells were treated with Saq, Saq-NO, dex-Ver or TQ (18.8 μmol/L) for 24 h after which DR5 membrane expression was analyzed by flow cytometry (lower panels).

Saq-NO increased the expression of death receptor 5 (DR5)

One of the main characteristics of NSCLC, which is often associated with the resistance to apoptosis induced by chemotherapy, is its loss of membrane death receptors able to bind the TRAIL molecule, one of the most potent products of the immune system in elimination of cancer cells (26, 27). To evaluate the possible influence of Saq-NO on DR5 gene expression, cells were exposed to 18.8 μmol/L of either Saq-NO or dex-verapamil and the expression of DR5 was examined by real-time RT-PCR. Both drugs elevated DR5 mRNA expression in sensitive NCI-H460 cells, while in NCI-H460/R cells significant gene expression of this receptor was observed under the Saq-NO but not dex-verapamil treatment (*Figure 4* upper panel). DR5 expression correlated with its presence on the membrane of cells (*Figure 4* lower panel). While the competitive inhibitor, dex-verapamil, and the noncompetitive inhibitor, tariquidar (TQ), did not alter the presence of DR5 on the membrane of either NCI-H460 or NCI-H460/R cells, the expression of this receptor was elevated after the exposure to Saq-NO.

Saq-NO antagonized the TRAIL activity

The potential of Saq-NO to elevate the expression of DR5 indicated its possibility to reverse the sensitivity of these cells to selective antitumor response driven by ligation of TRAIL to this receptor. To clarify this possibility, NCI-H460 and NCI-H460/R cells resistant to TRAIL were treated in parallel with this molecule and Saq-NO (*Figure 5*). Cell viability was estimated by MTT tests and isobologram curves were created. Unfortunately, interaction between Saq-NO and TRAIL was antagonistic suggesting that, apart from Saq-NO potential to amplify the DR5 expression, it was not able to exclude blockage at the intracellular level which may be responsible for the ineffective response to TRAIL.

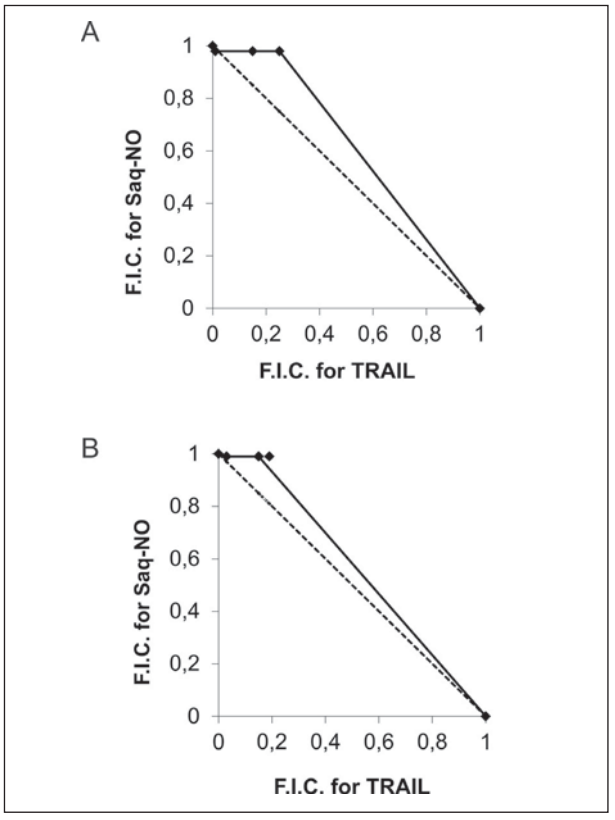

Figure 5 Lack of Saq-NO sensitization of human NSCLC cells to TRAIL-mediated apoptosis. NCI-H460 (A) and NCI-H460/R (B) cells were treated with IC30 dose of Saq-NO (9.4 μmol/L) for 6 h after which various concentrations of human recombinant TRAIL were added and further incubated for 18 h. Viability of cells was accessed by CV and isobologram curves from representative of three experiments are presented. Fractional inhibitory concentration (F.I.C.) > 1 is considered antagonistic.

Discussion

Numerous studies performed during the last decade indicate that antiinflammatory or antimicrobial drugs could target cancer cells (28). One of the promising candidates are the HIV protease inhibitors which, due to the inhibition of PI3K/Akt signaling pathway and proteasome activity, suppress the growth of not just HIV-related malignancies but also various types of tumors (4–12). Poor pharmacological profile of these drugs as well as high toxicity seriously compromised their utilization and indicated the need for their modification (13). In order to improve its quality, we attached a NO moiety to Saq (14). Recently, we found that NO-modified Saq efficiently inhibited the progression of tumor cells derived from rodent or human origin *in vitro* as well as in syngeneic and xenograft models (14–19). An absence of Saq-NO toxicity was observed on primary cells *in vitro* and was further confirmed by studies of acute and subacute toxicity (14). The results revealed the impressive fact that the NO-modified compound was completely innoxious when it was applied in a dose corresponding to a 100% lethal dose of parental drug. A major aspect of Saq-NO action was its capacity to stop the tumor growth in a nonaggressive manner. Rodent melanoma, glioma cells and colon cancer cells underwent the process of differentiation/transdifferentiation after time-limited exposure to Saq-NO (14, 19). Loss of their malignant potential and metastatic capacities were accompanied by chemosensitization as well as reconstitution of their sensitivity to the TRAIL-mediated immune response (15, 18). Moreover, its efficacy was impressive even in multidrug (MDR) resistant forms of prostate cancers (20). NSCLC is one of the most aggressive forms of tumor which during its progression loses the sensitivity to death receptor-mediated antitumor activities and response to chemotherapy. NSCLC rapidly progresses to the apoptotic resistant form (2). Here, we have

compared the capacity of Saq-NO to downregulate the growth of two phenotypes of NSCLC, sensitive and resistant to doxorubicin. As previously observed in a panel of cell lines, Saq-NO exerted tumoricidal effects in lower concentrations than Saq which was independent from the expression of P-gp pumps (14–20). Similarly to our previous observations, Saq-NO induced cell cycle arrest in the G_0/G_1 phase and therefore decreased the proliferation of NSCLC. The ability to stop the growth of both types of NSCLC, sensitive and resistant, is very important to determine the role of P-gp in cancer cells that limit the application of chemotherapy. ATP-dependent pumps are responsible for the transfer of substances across biological membranes and, therefore, drugs across tissue barriers in cancer cell chemoresistant phenotypes (29–32). Saq-NO application 4 h before or simultaneously with DOXO decreased the efficient dose of this chemotherapeutic drug in sensitive cells and, more importantly, sensitized the resistant cells to this chemotherapeutic drug. Recently, it was observed that Saq can be a substrate of P-glycoprotein and also an inhibitor of the breast cancer resistance protein (BCRP1, gene product of ABCG2) (33–36). On the other hand, Rotwailer et al. demonstrated that Saq-NO sensitized P-gp–, MRP1-, or BCRP1-expressing prostate cancer cells to chemotherapy. Similarly, in NSCLC cells, Saq-NO inhibited the P-gp more effectively than the competitive inhibitor dex-verapamil (20). This is important due to the known toxicity of competitive ATP-Binding Cassette (ABC) transporter inhibitors and, therefore, the development of new less toxic drugs with the capacity to inhibit ABC transporters could be beneficial (29, 32, 37, 38). Namely, simultaneous administration of anticancer drugs and compounds that can impede the efflux of chemotherapeutic agents by ABC transporters can concomitantly modulate various cytochrome P450 (CYP450) enzymes, consequently influencing their anticancer drug metabolism. This can further result in unfavorable drug–drug interactions and altered pharmacokinetic properties of the applied anticancer drugs with additional adverse cytotoxic side effects (39). Thus, the first generation of P-gp inhibitor drugs confirmed the effect of a P-gp modulation to overcome MDR, but most of these drugs had limited clinical implementation because the administered doses were either noneffective or toxic for the patient. The second generation of P-gp inhibitors had reduced primary toxicity, however, they showed important pharmacological interactions which caused disturbed metabolism and accumulation of chemotherapeutic drugs, what finally lead to exaggerated toxicity as well. Finally, the third generation of inhibitors was designed for low pharmacokinetic interactions and high transporter affinity, but their efficacy has not yet been confirmed by clinical evidence summarized in Szakács et al. (29).

In addition to its potential to restore sensitivity to chemotherapy through drug pump inhibition, equal efficacy of Saq-NO against the sensitive and resistant type of NSCLC cells indicated that transporter proteins are not the barrier for its intrinsic capacity to effectively stop the division of non-small lung cancer cells. Moreover, strong antitumor potential observed *in vitro* was not accompanied by the induction of cell death. This is a very important feature of the Saq-NO mode of action, if it is well defined that induction of apoptosis is an undesirable way to treat the cancer in highly aggressive forms, such as this type of cancer. Having in mind that NSCLC rapidly adopts an apoptotic resistant phenotype in response to chemotherapy, it seems that efficacy of Saq-NO could be ascribed to its potential to inhibit the proliferation of these cells rather than kill them (2).

Development of an apoptotic resistant phenotype is often correlated with the loss of sensitivity to death signals triggered by immune cell products such as TRAIL (26, 27). This molecule is one of the most selective mediators of tumor cell death able to induce the apoptosis of transformed but not normal cell after ligation to its specific receptor. Cancer cells evade this through the lack of expression of its receptors or development of intracellular blockage in delivering the death signal triggered upon ligation. Saq-NO was able to upregulate the expression of DR5 in both DOXO sensitive and resistant cancer cells. In spite of this, TRAIL was not able to induce the apoptotic process. It is obvious that intracellular blockade responsible for apoptotic resistance was not eliminated by Saq-NO application. It was previously observed that Saq-NO efficiently restores the TRAIL-mediated antitumor response not just through upregulated expression of DR5 but also through a decrease in the expression of natural caspase-3 inhibitor XIAP in prostate cancer (18). The explanation of the unsuccessful immunosensitization by Saq-NO under these circumstances could be related to the described feature of NSCLC cells to be unable to mediate the posttranslational modifications of DRs and/or to bring to the membrane their nonfunctional forms (26, 27). These data are in disharmony with the previously found enhancement of TRAIL-induced apoptosis in P-gp-overexpressing leukemia and breast cancer cells, indicating that the intrinsic characteristics of cancer cells are crucial for the outcome of the treatment (40, 41). The capability of Saq-NO to function in a slow cytostatic and differentiating manner, and its potential to nonselectively target various types of tumors, even the most aggressive MDR forms, draw attention to the importance of further analysis of this class of drugs as antineoplastic agents.

Acknowledgement. This work was partly supported by the Serbian Ministry of Science, Technology and Development of the Republic of Serbia (Grants No 173013 and III41031).

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: August 6, 2013

Accepted: September 6, 2013