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In vitro and in vivo anticancer action of Saquinavir-NO, a novel nitric oxide-derivative of the protease inhibitor saquinavir, on hormone resistant prostate cancer cells

Marco Donia,[1] Danijela Maksimovic-Ivanic,[2] Sanja Mijatovic,[2] Marija Mojic,[2] Djordje Miljkovic,[2] Gordana Timotijevic,[3] Paolo Fagone,[1] Salvatore Caponnetto,[1] Yousef Al-Abed,[4] James A. McCubrey,[5] Stanislava Stosic-Grujicic[2] and Ferdinando Nicoletti[1,*]

[1]Department of Biomedical Sciences; University of Catania; Catania, Italy; [2]Department of Immunology; Institute for Biological Research "Sinisa Stankovic"; Belgrade University; and [3]Institute of Molecular Genetics and Genetic Engineering; Belgrade University; Belgrade, Serbia; [4]Laboratory of Medicinal Chemistry; North Shore Long Island Jewish Health System; New York, NY; [5]Department of Microbiology and Immunology; Brody School of Medicine at East Carolina University; Greenville, NC USA

Key words: prostate cancer, nitric oxide modified saquinavir, apoptosis, immunosensitization, chemosenzitization

Abbervations: NO, nitric oxide; Saq, saquinavir; Saq-NO, nitric oxide modified saquinavir; TRAIL, TNF related apoptosis inducing ligand; DR5, death receptor 5; ROS, reactive oxygen species; RNS, reactive nitrogen species; YY1, ying-yang 1; NFκB, nuclear factor κB

The NO-derivative of the HIV protease inhibitor saquinavir (Saq-NO) is a nontoxic variant of the parental drug with enhanced anticancer activity on several cell lines. However, it is still unclear whether the p53 status of the target cell might influence the sensitivity to Saq-NO. In this study we evaluated the in vitro and in vivo activity of Saq-NO on the p53-deficient hormone resistant prostate cancer PC-3 cells. We demonstrate that the absence of functional p53 is not essential for the capacity of Saq-NO to reduce prostate cancer cell growth. In contrast to its previously described cytostatic action in B16 and C6 cell lines, Saq-NO exerted cytotoxic effects in PC-3 cells leading to dominant induction of apoptosis and enhanced production of proapoptotic Bim. In addition, differently from saquinavir, Saq-NO restored TRAIL sensitivity that was correlated with increased expression of DR5 independent from ROS/RNS production and YY1 repression. NFκB activation may be responsible of the Saq-NO induced DR5 expression. Moreover, Saq-NO but not saquinavir, exerted synergistic activity with conventional cytostatic therapy. In agreement with these in vitro studies, Saq-NO inhibited the in vivo growth of PC-3 cells xenotransplants to a greater extent than the parental compound. Taken together, these data indicate that Saq-NO possesses powerful and suitable in vitro and in vivo chemotherapeutic potential to be further studied as a novel drug for the treatment of prostate cancer in the clinical setting.

Introduction

Prostate cancer is the second leading cause of cancer-related death in Europe among men and is the most commonly diagnosed cancer in European males.[1] Most prostate cancer related deaths are due to advanced disease and androgen deprivation therapy is considered the primary approach in the treatment of symptomatic advanced prostate cancer.[2] However, this treatment is palliative rather than curative and although it can slightly improve the likelihood of survival, virtually all patients progress to hormone-refractory prostate cancer.[3] Although encouraging results were recently obtained with docetaxel based chemotherapy,[4,5] therapeutic options for patients with hormone-refractory prostate cancer are still limited, with lack of evidence for long-term survival.

The antitumor properties of HIV protease inhibitors have been firstly investigated because of their success in treating HIV-related Kaposi's sarcoma through immune-independent mechanisms.[6,7] In particular saquinavir (Saq), the first FDA approved HIV protease inhibitor, promoted regression of human Kaposi's sarcoma in nude mice by blocking cell invasion via inhibition of matrix metalloprotease 2 at very low concentrations (0.1–1 umol/l) while at higher concentrations (50–100 μmol/l) it inhibited 20 S and 26 S proteasome activity, increased apoptosis and radiosensitised non-HIV-associated cancers, including prostate cancer, lymphoblastoid leukaemia and glioblastoma.[8-10] Moreover, Saq may radiosensitize H-ras mutated bladder cancer, epidermal growth factor receptor mutated head and neck cancer, and K-ras mutated pancreatic cancer.[11] In chemosensitive and chemoresistant ovarian cancer cells, Saq induces endoplasmic

*Correspondence to: Ferdinando Nicoletti; Email: ferdinic@unict.it
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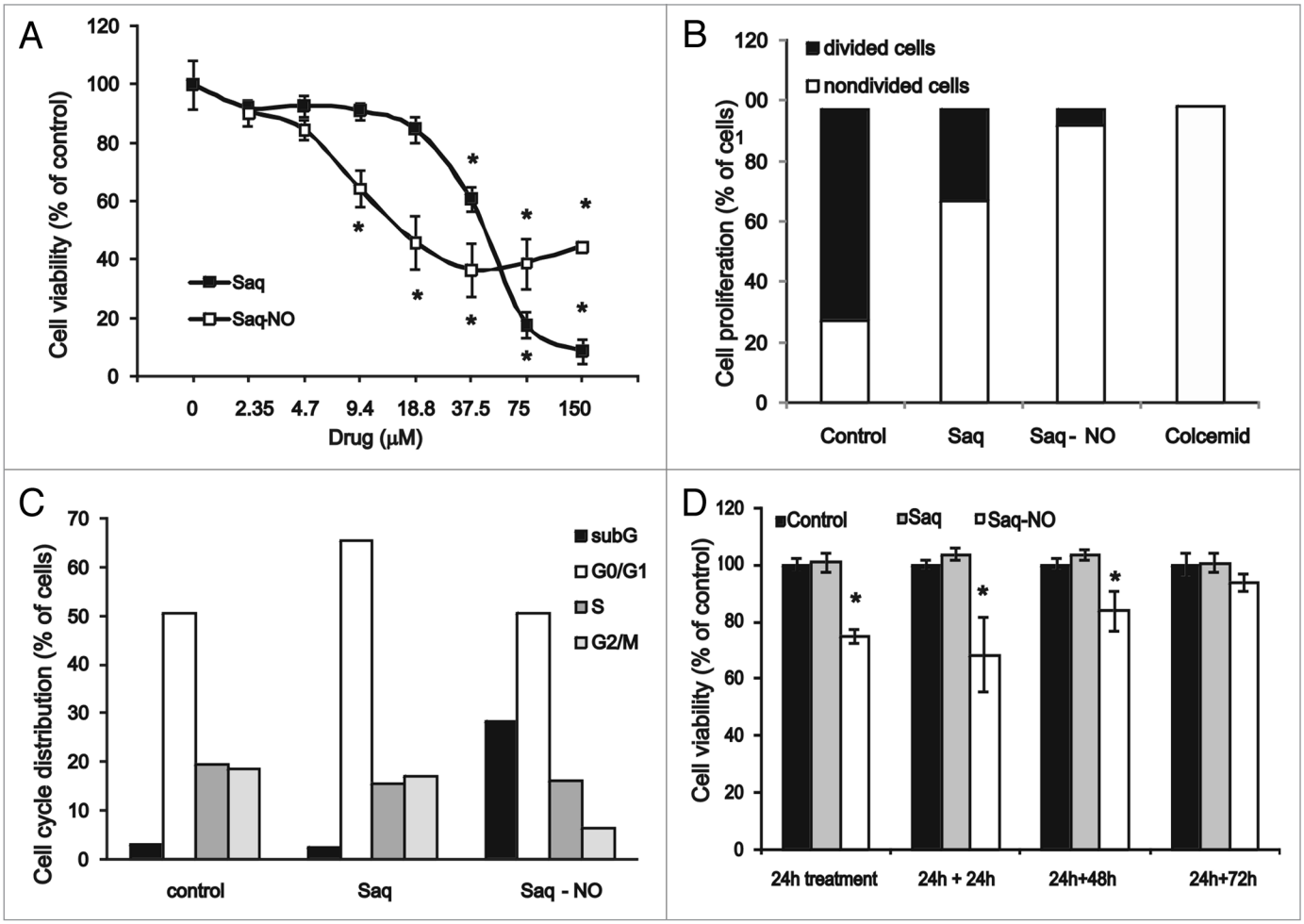

Figure 1. Saq-NO decreased the viability of PC-3 cells through inhibition of cell proliferation and subsequent induction of cell death. (A) Cells were treated with a range of concentrations of Saq or Saq-NO for 24 h, after which cell viability was determined by CV assay. The data are presented as mean ± SD from representative of three independent experiments. (B) CFSE stained cells were treated with 18.8 μM of Saq or Saq-NO and cell proliferation was measured after 96 h. (C) Cell cycle analysis of PI stained cells was performed by flow cytometry after 24 h. (D) Cells were treated with Saq or Saq-NO (18.8 μM) for 24 h and cell viability was measured during next 72 h. *p < 0.05, refers to untreated cultures.

reticulum stress, autophagy and apoptosis while in imatinib-resistant chronic myelogenous leukemia it may synergize with tyrosine kinase inhibitors.[12,13]

Previous studies by us and others have shown that the chemical modification based on the addition of a nitric oxide (NO) donating group reduces the toxicity and enhances the anticancer activity of several different parental compounds.[14-19] Along this line of research we have recently proposed a modified version of Saq named saquinavir-NO (Saq-NO), in which a NO moiety has been covalently attached to the original structure.[20] This new chemical entity (NCE) was shown to exert potent cytostatic, AKT-independent anticancer effect both in vitro and in vivo at significantly lower doses than the parental compound.[20] Moreover, Saq-NO did not exert toxic effects to primary cells or to normal healthy mice at any of the doses tested.[20] On the other hand, even the simple release of NO by classical NO-donors has recently shown promise in the treatment of prostate cancer. In a phase II study, treatment with a low dose transdermal glyceryl trinitrate patch was safe and effective in the prolongation of the prostate-specific antigen (PSA) doubling time in men with biochemical recurrence of prostate cancer after primary therapy.[21]

In light of this, we have tested the effects of Saq-NO on the in vitro and in vivo growth of the human androgen independent PC-3 prostate cancer cell line. Our results clearly show that Saq-NO is significantly more effective than the parental compound in reducing the viability of prostate cancer cells, with the additional capacity to synergize with chemo- or immuno-therapy.

Results

Saq NO affected viability of p53-deficient PC 3 cells more potently than the original drug. To evaluate the sensitivity of PC-3 cells to Saq vs. Saq-NO, the cells were exposed to wide range of doses of both compounds and cell viability was assessed by CV test after 24 h. The results presented in **Figure 1A** clearly showed that antitumor capacity of Saq-NO is significantly intensified, in particular at so low concentrations. Cultivation of CFSE-stained cells in the presence of Saq or Saq-NO, as well as

colcemid as positive control, revealed remarkable inhibition of cell division in Saq-NO treated cultures in comparison to Saq-exposed or nontreated cells (**Fig. 1B**). As judged by PI staining and subsequent cell cycle distribution analysis, notable amounts of hypodiploid cells were detected only in Saq-NO treated cultures (**Fig. 1C**). Removal of Saq-NO after 24 h and further cultivation of cells for 72 h indicated that inhibition of proliferation was reversible (**Fig. 1D**). Taken together, these data indicate that Saq-NO downregulated the number of viable cells through drug-dependent suppression of proliferation and subsequent apoptosis.

Saq-NO disturbed the balance of pro/anti-apoptotic molecules. To assess the molecular basis of differential response of PC-3 cells to Saq or Saq-NO, the cells were treated with 18.8 μM that reduced cell viability approximately 10 and 50%, respectively, and pro- and anti-apoptotic members of Bcl family were evaluated by western blot. As shown in **Figure 2A**, Saq treatment resulted in insignificant upregulation of Bcl-2 and specially Bim, while Bc-XL was not changed. On the other hand, Saq-NO upregulated the expression of all tested molecules-Bcl-2, Bc-XL and Bim (**Fig. 2B**). However, Bim expression was significantly elevated even after 6 h of incubation with Saq-NO reaching the approximately 11 times higher values than in control. Therefore, Bim could be responsible for the massive apoptosis observed.

Saq-NO sensitized PC-3 cells to TRAIL-mediated toxicity. It is well documented that PC-3 cells are resistant to TRAIL-mediated apoptosis. To investigate the possible influence of both compounds on the sensitivity to TRAIL-mediated cell death, cells were treated with 4.7, 9.4 or 18.8 μM of Saq or Saq-NO for 6 h and further exposed to different range of concentrations of recombinant TRAIL. After 24 h, cell viability was determined by CV test and evaluated by isobologram analysis. Results presented in **Figure 3A and B** show that unlike the parental drug, Saq-NO sensitized the PC-3 cells to TRAIL-mediated cell death. This phenomenon was associated with increased expression of DR5 gene (**Fig. 3C**).

In order to determine the possible causes of DR5 upregulation, we first measured the production of NO and ROS with specific indicators. The quantity of these reactive species in cultures of cells treated by Saq-NO was not different in comparison to controls indicating their irrelevance for Saq-NO-induced sensitization (**Fig. 3D**). Keeping in mind that the transcription factor YY1 is a repressor of DR5, we next evaluated its expression in cells exposed to Saq-NO. Western blot analysis revealed that YY1 expression was markedly elevated after the 24 h of treatment (**Fig. 3E**), indicating that upregulated DR5 expression was not mediated by YY1 inactivation. Since it is known that NFκB exerts a pleiotropic role in cell physiology and that there is a NFκB binding site located in the first intron of DR5 gene, we next explored the expression of pIκB protein that correlates with the level of free NFκB. Results presented in **Figure 3F** revealed a strong and continuous upregulation of pIκB protein expression reaching the values approximately 20 times higher than nontreated control. These data suggest that, DR5 upregulation is probably mediated by augmented NFkB transcriptional activity.

Saq-NO enhanced the effectiveness of chemotherapeutic drugs. To estimate the eventual interaction between low toxic doses of both drugs with conventional chemotherapeutics, the cells were treated with 4.7, 9.4 or 18.8 μM of Saq or Saq-NO for 2 h and then were exposed to different concentrations of doxorubicin, paclitaxel and cisplatin. Saq treatment in this range of doses did not affect the efficacy of applied treatments (not shown). In contrast, Saq-NO potentiated the influence of all tested drugs on cell viability. Isobologram analysis classified these interactions as synergism (**Fig. 4**).

Effect of Saq-NO vs. Saq on tumor growth in nude mice xenografted with PC-3 cells. The mice were treated i.p. with Saq-NO or Saq at the dose of 0.2 mg/mouse for 19 consecutive days starting from about 18 days after xenograft. The tumor volumes of the mice treated with Saq-NO were significantly reduced ($p < 0.05$) already starting from 6 days after the beginning of the treatment (Day 24) until the end of the observational period. The parental compound Saq started to show significant effects after 15 days of treatment until the end of the study (**Fig. 5**). As shown in **Figure 5**, from day 18 to 37, the tumor volumes in the control group achieved a 7.1-fold increase, whereas tumor volumes in Saq-NO treatment groups reached a 4.3-fold increase. It is worth noting that the inhibitory effect on tumor growth was higher than its parental compound Saq and comparable to that of cisplatin that was used as positive control drug (**Fig. 5**).

Discussion

Covalent attachment of NO to Saq improved the original drug in several important aspects including reduction of the toxicity and remarkable increase of antitumor action that allowed Saq-NO to exert its anti-cancer action at very low doses. Importantly, the original anti-HIV property of the parental compound was preserved. Treatment of mouse melanoma B16 and rat astrocytoma C6 induced differentiation or transdifferentiation process, respectively that were associated with transient upregulation of Akt activity and marked augmentation of p53 expression.[20] In addition, we have demonstrated that p53 mutation or depletion and expression of P-gp, MRP1 or BCRP1 did not influence the anti-cancer activity of Saq-NO. Moreover, Saq-NO sensitised P-gp, MRP-1 or BCRP1 expressing cancer cells to chemotherapy. We have also demonstrated that Saq-NO is a substrate of P-gp as well as of MRP1.[22]

In the present study, we aimed at elucidating the importance of p53 in the tumoricidal action of Saq-NO. We have shown that the drug was equally effective in PC-3 prostate carcinoma cells that lack functional p53. Whereas Saq-NO treatment of the previously mentioned melanoma and astrocytoma cells induced permanent changes that persisted even in the absence of the drug,[20] inhibition of PC-3 proliferation was temporary. Removal of Saq-NO led to resumption of proliferative ability of PC-3 cells. Most of the cells underwent apoptosis after 24 h of treatment. It is clear that the absence of functional p53 could be responsible for conversion of cytostatic to cytotoxic action of Saq-NO and more than that was not a barrier for the realization of apoptosis triggered by the drug. Observed expression of Bc-XL and Bcl-2 was neutralized by the dominant expression of their

natural antagonist Bim. Bim was considered as a key molecule for realization of apoptotic process and its deficiency led to abrogation of apoptosis.[23-24] This molecule attaches to Bcl-2 and Bc-XL attenuating their cytoprotective functions and subsequently liberates proapoptotic Bax.[25,26]

The present study also reveals that beside direct cytotoxicity, Saq-NO also possesses indirect antitumor action. It is known that PC-3 cells are insensitive to TRAIL-mediated antitumor response.[27] Induction of apoptosis is triggered upon ligation of TRAIL expressed on numerous immune cells such as cytotoxic lymphocytes, dendritic and NK cells to DR5 receptor which existed on malignant but not normally adult tissues.[28] PC-3 cells protect themselves from this destructive mechanism through inhibition of expression of membrane DR5.[27] The reduced expression of DR5 may be a consequence of defective function in these cells of p53 that is one of the crucial transcription factor that regulated DR5 gene independently or in conjunction with NFκB.[29] Many other relevant genes for apoptosis are also under the control of this transcription factor.[30] We have shown that whilst the treatment with Saq did not sensitize the PC-3 cells to TRAIL, the same concentrations of Saq-NO significantly augmented the transcription of the DR5 gene and rendered them susceptible to the action of recombinant TRAIL with significant reduction of cell viability. Our study indicates that promotion of DR5 expression on PC-3 cells by Saq-NO did not depend on ROS and RNS-dependent pathways or on the inhibition of the expression of DR5 repressor YY1. In fact, at the IC_{50} dose Saq-NO did not alter the quantity of endogenous NO and therefore the consequent production of ROS and RNS and the expression of YY1 repressor was not modified until 24 h of exposure to the drug and thereafter it was unexpectedly increased from Saq-NO. It is known that binding activity, as well as, the expression of YY1 is sensitive to NO and thus to variation of its concentration. The lack of impact on early YY1 expression from PC-3 in response to Saq-NO and the increased expression at later stage of the culture period provides additional indirect evidence of lack of notable NO release by the drug. On the other hand, Saq-NO potentiated the activity of another transcription factors (e.g., NFκB) that plays a complex and contradictory role in DR5 expression.[29]

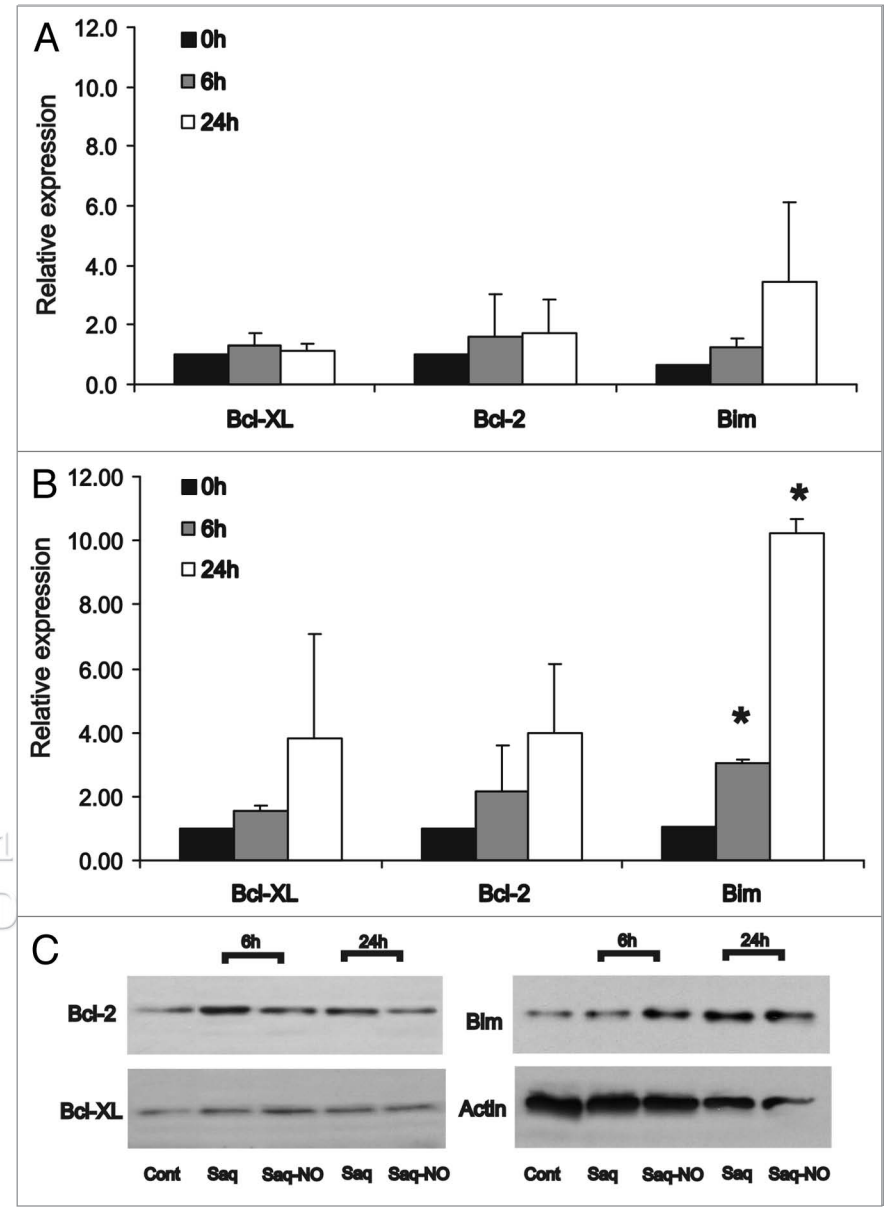

Figure 2. Saq-NO promoted apoptosis through dominant upregulation of Bim. Cells were incubated with 18.8 μM of Saq (A) or Saq-NO (B) for 6 and 24 h and expression of antiapoptitic Bcl-2 and $BclX_L$ as well as apoptotic Bim was evaluated by western blot. Densitometric analysis of data from representative of three experiments was presented as fold increase relative to control. Photographs from representative experiment was presented (C). *$p < 0.05$, refers to untreated cultures.

NFκB controls the expression of both antiapoptotic and proapoptotic genes.[31] The mechanism of NFκB-induced DR5 expression is unknown. It has however been shown that beside direct regulation of DR5, NFκB also influenced its expression through modulation of other molecules responsible for DR5 expression including upregulation of YY1.[32] On the other hand NFκB has been reported to downregulate stimulators of DR5 expression such as CCAAT/enhancer-binding protein-homologous protein (CHOP).[33] Although we have not presently studied the impact of Saq-NO on CHOP expression, our results seems to suggest that the net impact of NFκB upregulation induced by Saq-NO has ultimately resulted in enhanced DR5 expression. Taken together, nonfunctional p53, unchanged level of ROS and elevated expression of YY1 are not probably mediators of

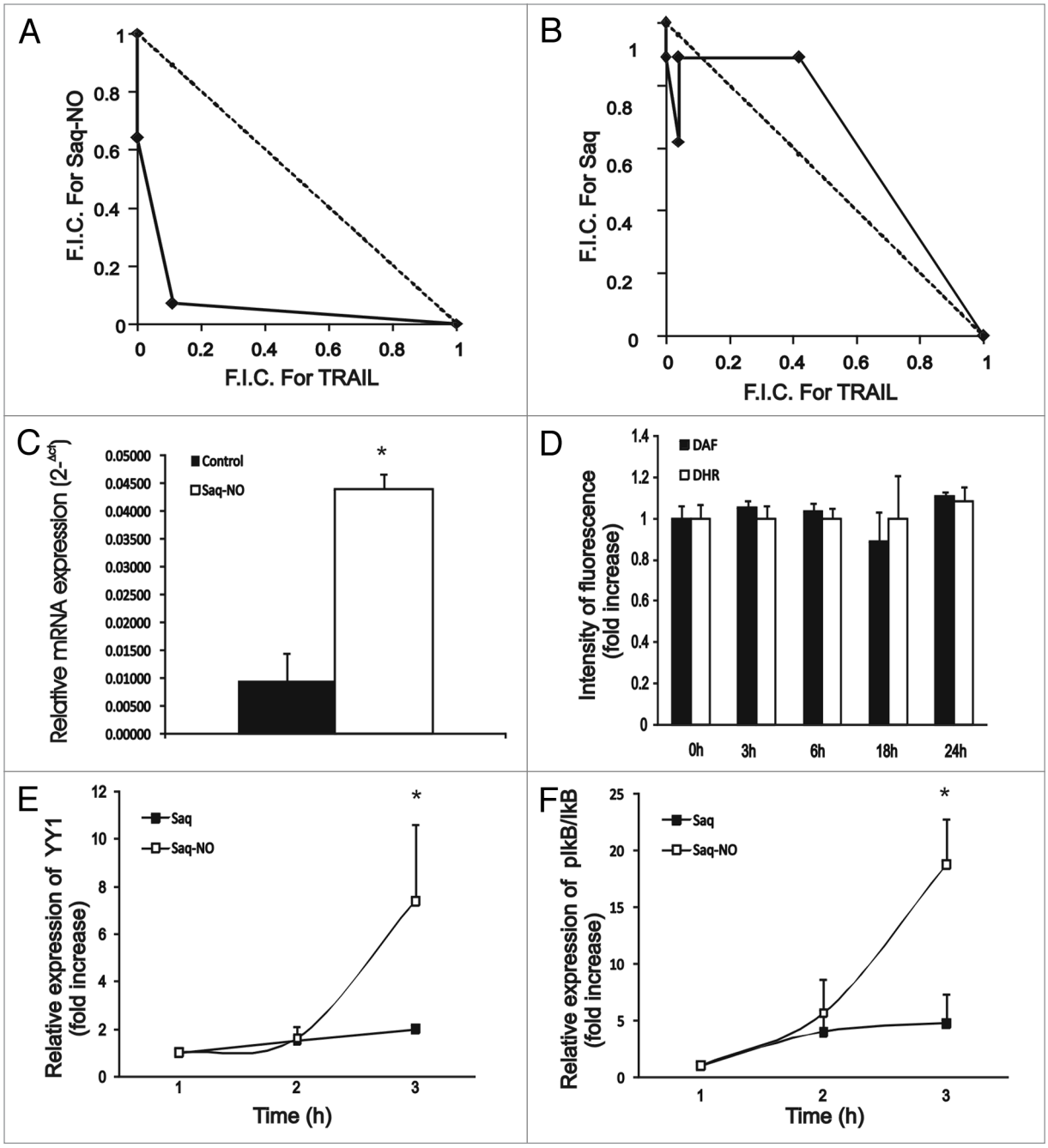

Figure 3. Saq-NO sensitized PC-3 cells to TRAIL-mediated cell death. Cells were treated with Saq-NO (A) or Saq (B) (4.7–18.8 μM) for 6 h. After that, various concentrations of TRAIL were added and further incubated for 18 h. Cell viability was determined by CV assay and isobologram curves from representative of three independent experiments were presented. (Fraction inhibitory concentration (F.I.C): concentration of each agent in combination/concentration of each agent alone, F.I.C <1 is considered synergistic; F.I.C >1 is considered antagonistic). (C) RT-PCR for DR5 was performed after 24 h and data were presented as relative expression of mRNA. (D) DAF-FM or DHR indicators were added to detect intracellular production of NO and ROS/RNS, respectively and fluorescence was determined by Chameleon multiplate reader. Cells were treated for indicated time points with 18.8 μM of Saq-NO or Saq and YY1 (E), pIκB and IκB (F) expression was analyzed by western blot. Densitometric analysis of data from representative of three experiments was presented as fold increase relative to control. *p < 0.05, refers to untreated cultures.

immunosensitization process. On the other hand, enhanced activity of NFκB suggested its essential role in sensitization of PC-3 by Saq-NO.

In vivo study in nude mice xenografted with PC-3 cells showed that monotherapeutic regime with this drug was at least as effective as common and more toxic anticancer agent such as cisplatin. These findings warrant additional studies for the use of Saq-NO in different cancer conditions and do not limit its application in a combination setting. In addition, the synergistic action of Saq-NO with conventional cytostatic drugs represents a

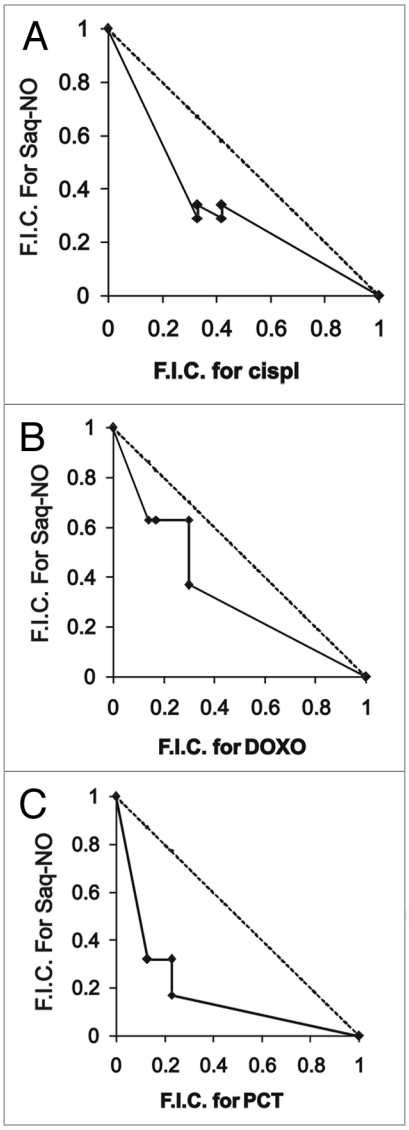

Figure 4. Saq-NO potentiated the responsiveness of PC-3 cells to various chemotherapeutic agents. Cells were treated with different doses of Saq-NO and subsequently cisplatin (A), doxorubicin (B) or paclitaxel (C) were added. After 24 h of cultivation CV was performed and isobologram curves from representative of three independent experiments were presented. (F.C.I.<1 is considered synergistic).

remarkable advantage of the modified drug that may permit the application of low toxic doses of both compounds and therefore avoiding multiple unwanted side effects.

Materials and Methods

Animals. BALB/c female athymic nude mice 5 to 6 weeks old were purchased from Harlan-Nossan. The mice were kept under standard laboratory conditions (non specific pathogen free) with free access to food and water. The animals used in the experiments were protected in accordance with Directive 86/609/EEC. The animal studies were carried out in accordance to local guidelines and approved by the local Institutional Animal Care and Use Committee (IACUC).

Reagents and cells. Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat#:M2128), doxorubicin (Cat#:D1515), paclitaxel (Cat#:T7402) and propidium iodide (PI, Cat#:81845) were obtained from Sigma. Recombinant human TRAIL/Apo2L was purchased from Peprotech (Cat#:310-04). Saq was purchased from Roche. Saq-NO was bought from GaNiAl Immunotherapeutics and was synthetized as review in reference 20.

Human androgen-independent prostate cancer PC-3 cell line was from American Type Culture Collection. Cells are regularly kept in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate, 5 x 10^{-5} M 2-mercaptoethanol and antibiotics (culture medium) at 37°C in a humidified atmosphere with 5% CO_2. After standard trypsinization, cells were seeded at 1 x 10^4/well in 96-well plates for viability determination, 2.5 x 10^5/well in 6-well plate for flow cytometry and real-time PCR and 1 x 10^6/25 cm^3 flask for western blot analysis.

Determination of cell viability by crystal violet (CV) assay. Cell viability was determined by crystal violet assay that is based on the property of viable cells to absorb the dye. 1 x 10^4 cells/well were cultivated in the presence of the different doses of drugs for 24 h, than fixed with methanol, stained with 1% crystal violet and viability was estimated. Tests were done as described elsewhere and cell viability was calculated as percentage of the control (untreated cells), that was arbitrarily set to 100%.

Cell cycle analysis. For cell cycle analysis the cells were fixed in 70% ethanol at 4°C for 30 min, washed twice in PBS and resuspended in PBS containing propidium iodide (20 μg/ml) and RNase (0.1 mg/ml). Cells were stained in the dark at 37°C for 30 min and analyzed with FACS Calibur flow cytometer (BD). Cell distribution among cell cycle phases was determined with Cell Quest Pro software (BD).

Detection of cell proliferation. For detection of proliferation the cells were stained with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, 1 μM, Sigma, Cat#:21888) 10 min at 37°C, washed twice and then treated with 18.8 μM of Saq or Saq-NO and colcemid at the dose of 0.2 μg/ml as positive control. After 96 h cells were detached, washed and analyzed by FACS Calibur flow cytometer.

Measurement of intracellular NO and ROS. The cells were treated with Saq-NO or Saq and intracellular NO was detected with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate, Molecular Probes, Cat#:D-23842). At the end of incubation period, the cells were stained with 2 μM of DAF-FM in phenol red free RPMI-1640-10% FCS for 1 h at 37°C, washed and additionally incubated in fresh RPMI-1640. For ROS detection, before treatment the cells were stained with dihydrorhodamine 123 (DHR, 4 μM, Molecular Probes, Cat#:D-632) for 20 min. At the end of the incubation period, stained cells were washed, resuspended in PBS and analyzed by fluorimeter (Chameleon multiplate reader, Hidex Oy).

Immunoblot analysis of Bcl, Bc-XL, YY1 and pIκB. PC-3 cells (1 x 10^6) were seeded in flasks (25 cm^3), treated with 18.8 μM the Saq or Saq-NO for the indicated times. At the end of

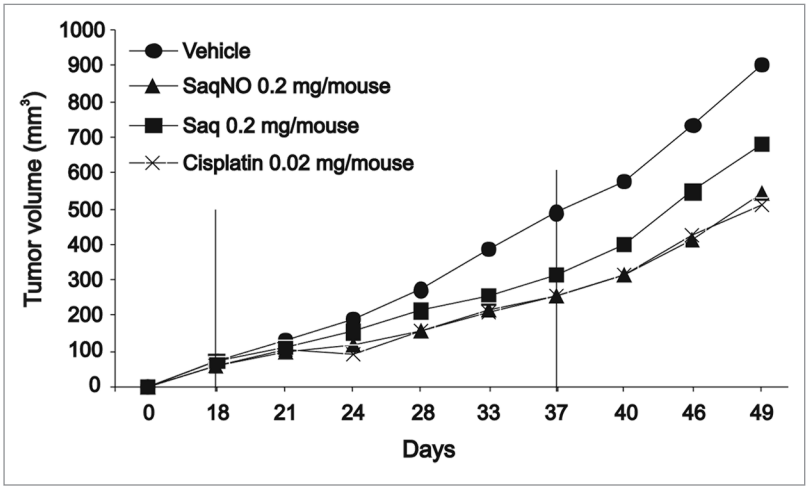

Figure 5. Saq-NO inhibited the growth of PC-3 cells in nude mice. Tumors were induced by subcutaneous implantation of 5 x 10^6 PC-3 cells and either Saq or Saq-NO at the dose of 0.2 mg/mouse for 19 consecutive days starting from approximately 18 days after xenograft. Tumor volumes were calculated two times a week until 49 days after tumor implantation.

incubation period, the cells were lyzed in buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT and 0.01% w/v bromophenol blue and subjected to electrophoresis on 12% SDS-polyacrylamide gels. Electrotransfer to polyvinylidene difluoride membranes at 5 mA/cm^2 was performed with a semi-dry blotting system (Fastblot B43, Biorad). As blocking reagent 5% w/v nonfat dry milk in PBS with 0.1% Tween-20 was used and blots were probed with specific antibodies to Bc-XL and Bcl-2 (eBioscience, Cat#:14-6994, Cat#:BMS1029), YY1 (Active Motif, Cat#:39071), pIκB and IκB (Santa Cruz Biotechnology, Cat#:sc101713, Cat#:sc371) at 4°C over night, followed by incubation with secondary antibody 1 h at room temperature (ECL donkey anti-rabbit HRP linked, GE Healthcare, Cat#:NA934). Bands were visualized using chemiluminescence detection system (ECL, GE Healthcare). Densitometry was performed by Scion Image (Scion Corp.).

RNA isolation and RT-PCR of DR5. Total RNA from PC-3 cells was isolated with an RNA Isolator (Metabion) according to the manufacturer's instructions. Reverse-transcription was done with Moloney leukemia virus reverse transcriptase and random primers (both from Fermentas, Cat#:EPO442, Cat#:SO142). PCR amplification of cDNA (1 μl per 20 μl of PCR reaction) was performed in a real-time PCR machine ABI Prism 7000 (Applied Biosystems) with SYBRGreen PCR master mix (Applied Biosystems) as indicated: 2 minutes at 50°C for dUTP activation, 10 minutes at 95°C for initial denaturation of cDNA, followed by 40 cycles, each consisting of 15 s of denaturation at 95°C and 60 s at 60°C for primer annealing and chain extension. Primer pairs (Sigma) for DR5 were: 5'-TGC AGC CGT AGT CTT GAT TG-3' and 5'-GCA CCA AGT CTG CAA AGT CA-3' and GAPDH, 5'-CAT CCA TGA CAA CTT TGG TAT CG-3' and 5'-CCA TCA CGC CAC AGT TTC C-3'. The expression level of each gene was calculated according to the formula $2^{-(Cti-Cta)}$ where Cti is the cycle threshold of the gene of interest and Cta is the cycle threshold value of GAPDH. The efficiency of real time PCR was in the optimal range of 90–110% (slope of standard curves 3·1–3·6) for the primer pairs used.

Isobologram analysis. Isobologram analysis was performed as previously described.[34] To establish the mode of interaction between Saq or Saq-NO and recombinant human TRAIL or cytostatic drugs, the cells were treated with different concentration of Saq or Saq-NO (4.7; 9.4; 18.8 μM) and TRAIL (1.25–10 ng/ml), doxorubicin (0.12–1 μM), paclitaxel (3–25 μM) or cisplatin (3.75–30 μM). Combinations attaining 30% of cytotoxicity were expressed as concentration of single agent alone producing this amount of toxicity. Analysis was done on the basis of dose-response curves of cell viability treated with Saq-NO, TRAIL or cytostatic drugs alone or their combination for 24 h and isobolograms were created.

Tumor induction and protocol of the drug treatment. Tumors were induced in athymic nude mice by injection of cultured PC-3 cells. The cells were dispersed by trypsin, washed (twice) in serum-free medium RPMI-1640 (10 min centrifugation, 200 x g), resuspended at the concentration of 2.5 x 10^7 cells/ml in the same medium and injected (0.2 ml) s.c. between the shoulder blades of each mouse using a 0.6 mm needle. Tumor growth was observed daily and measured with callipers (2 perpendicular diameters), and the tumor volume was calculated using the formula 0.52 x a x b^2, where a is the longest and b is the shortest diameter.

Three independent experiments were performed and each group consisted of 7–8 mice. Treatment with Saq or Saq-NO started when the tumors were already palpable with a range volume of 60–70 mm^3 and the mice were randomly assigned to each experimental group. Post randomization analysis revealed no significant differences in tumor volumes at the beginning of the treatment among the different groups. Saq or Saq-NO were prepared immediately before treatment and they injected intraperitoneally (i.p.) at a dose of 0.2 mg/mouse for 19 consecutive days. A group of mice was treated with the vehicle (DMSO 20%), and another group with cisplatin at the dose of 0.02 mg/mouse as positive control. The animals were observed for further 16 days after the interruption of the treatment.

Statistical analysis. The results are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results, unless indicated otherwise. Student's t-test was used to determine statistical significance. Values of $p < 0.05$ were considered to be statistically significant.

Acknowledgements

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Conflict of Interest

Y.A.A. and F.N. are shareholders of Ganial.

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