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The antitumor properties of a nontoxic, nitric oxide–modified version of saquinavir are independent of Akt

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Abstract

Application of the HIV protease inhibitor saquinavir (Saq) to cancer chemotherapy is limited by its numerous side effects. To overcome this toxicity, we modified the original compound by covalently attaching a nitric oxide (NO) group. We compared the efficacy of the parental and NO-modified drugs *in vitro* and *in vivo*. The novel compound saquinavir-NO (Saq-NO) significantly reduced the viability of a wide spectrum of human and rodent tumor cell lines at significantly lower concentration than the unmodified drug. In contrast to Saq, Saq-NO had no effect on the viability of primary cells and drastically reduced B16 melanoma growth in syngeneic C57BL/6 mice. In addition, at the equivalent of the 100% lethal dose of Saq, Saq-NO treatment caused no apparent signs of toxicity. Saq-NO blocked the proliferation of C6 and B16 cells, up-regulated p53 expression, and promoted the differentiation of these two cell types into oligodendrocytes or Schwann-like cells, respectively. Although it has been well documented that Saq decreases tumor cell viability by inhibiting Akt, the anticancer properties of Saq-NO were completely independent of the phosphatidylinositol 3-kinase/Akt signaling pathway. Moreover, Saq-NO transiently up-regulated Akt phosphorylation, delivering a protective signal that could be relevant for primary cell protection and the absence of drug toxicity *in vivo*. It was unlikely that released NO was independently responsible for these drug effects because Saq-NO treatment increased intracellular and secreted NO levels only slightly. Rather, the chemical modification seems to have produced a qualitatively new chemical entity, which may have a unique mode of action against cancer cells. [Mol Cancer Ther 2009;8(5):1169–78]

Introduction

HIV protease inhibitors (HIV-PI) are antiretroviral agents that have been approved for human use since 1993. HIV-PIs are designed to selectively bind to the catalytic site of the HIV protease, thereby blocking replication and the production of infectious viral particles (1). Because proteases are not restricted to HIV but rather also participate in normal cell physiology, it is not surprising that these drugs also affect cellular processes such as angiogenesis, inflammation, the processing and presentation of antigens, cell survival, and tissue remodeling (2–5). Recent evidence has indicated that the potent anticancer effects of HIV-PIs, both *in vitro* and *in vivo*, are mediated primarily through the induction of apoptotic cell death (6–9). Although their mechanism of action against cancer cells is not completely understood, the potential targets of these drugs may include Akt, extracellular signal–regulated kinase, nuclear factor κB, signal transducers and activators of transcription 3, matrix metalloproteinase, basic fibroblast growth factor, and vascular endothelial growth factor (7, 10–13). In addition, these drugs have been shown to sensitize tumor cells to radiation, enhance the anticancer effects of other cytostatic drugs, and to inhibit the growth and invasion of angiogenic tumor cells in nude mice (10, 11, 14).

Unfortunately, HIV-PIs possess numerous adverse side effects such as hyperlipidemia or hypolipidemia, cardiovascular disease, diabetes, body fat redistribution, osteopenia, and osteoporosis (15). In studies of nonsteroidal anti-inflammatory drugs, the addition of a nitric oxide (NO) moiety through an aromatic spacer has been shown to both reduce their toxicity and enhance their pharmacologic efficacy (16). In the present study, we asked whether a similarly beneficial pharmacologic effect can be conferred on Saq by covalent attachment of NO. Directly comparison of the effectiveness and toxicity of unmodified Saq to that of Saq-NO, both *in vitro* and *in vivo*, has revealed several differences in their respective modulation of oncogenic pathways.

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Note: D. Maksimovic-Ivanic and S. Mijatovic contributed equally to this work.

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Materials and Methods

Reagents and Cells

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes. Inhibitor Akt VI was obtained from Calbiochem. All other chemicals were purchased from Sigma unless otherwise specified. Saquinavir (Saq; MW 670 g/L) and saquinavir-NO (Saq-NO; MW 715 g/L) were stored at −20°C at a concentration of 5 mg/mL in RPMI 1640 supplemented with 5% FCS and containing 25% DMSO. Drug samples were diluted in culture medium immediately before use. Control cell cultures were treated with an equivalent volume of DMSO. (*S*,*R*)-3-Phenyl-4,5-dihydro-5-isoxazole acetic acid-nitric oxide (GIT-27NO) was purchased from Ganial Immunotherapeutics.

Cell Lines

The C6 rat glioma cell line was a kind gift from Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The B16 murine melanoma and HeLa human adenocarcinoma cell lines were a kind gift from Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia). The HCC1419 human breast and PC-3 prostate cell lines were purchased from LGC Promochem srl. Primary mouse fibroblasts and rat astrocytes were prepared as previously described by Mijatovic et al. (17).

Cells were grown in HEPES-buffered RPMI 1640 supplemented with 5% FCS, 2 mmol/L glutamine, 0.01% sodium pyruvate, 5×10^{-5} mol/L 2-mercaptoethanol, and antibiotics (culture medium) at 37°C in a humidified atmosphere with 5% CO_2. After a conventional trypsinization procedure, cells were seeded on 96-well plates (10^4 per well), six-well plates (2×10^5 per well), or four-well chamber slides (3×10^4 per well). Cells were cultured overnight and then exposed to the drug. Inbred C57BL/6 mice, 2 to 3 mo of age, were obtained from our facility at the Institute for Biological Research "Sinisa Stankovic" and were kept under standard laboratory conditions (nonspecific pathogen-free) with free access to food and water. The handling of animals and the study protocol were in accordance with international guidelines and approved by the local Institutional Animal Care and Use Committee.

Saquinavir-NO Synthesis

Saq was purchased from Hoffman-La Roche. Saq-NO was purchased from Ganial Immunotherapeutics and was synthesized as follows (Supplementary Fig. S1).[7] Saquinavir (3 g, 4.48 mmol/L) in $CHCl_3$ (18 mL) was added to a stirring mixture of fuming nitric acid (>90% HNO_3, 1 mL, 23.6 mmol/L) and Ac_2O (3.5 mL, 37.1 mmol/L) at −10°C, and then slowly warmed up to room temperature over 2 h under nitrogen. The reaction mixture was quenched with ice-cold water and extracted with CH_2Cl_2. The extracts were washed with ice-cold, saturated $NaHCO_3$ and water, dried with $MgSO_4$, and filtered. The solvent was evaporated under pressure and the crude product was purified by FCC elution with 3:2 to 3:3 Hex/acetone. The resulting product was recrystallized from EtOAc/Hex to yield saquinavir-ONO_2 (1.7 g, 53%) as a white solid, and the purity was assessed using high-performance liquid chromatography and mass spectrometry (m/z 716.33 [M + H+]; Supplementary Figs. S2–S4).[7]

Cell Viability Determination by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and Crystal Violet

Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan depends on the mitochondrial activity of cultured cells, so that absorption of crystal violet dye is correlated with the number of adherent, live cells. The cells were seeded in flat-bottomed 96-well plates in a final volume of 200 μL culture medium containing different agents, and the assays were done after 24-h incubation as previously described (18). Mitochondria-dependent production of formazan and the intensity of absorbed crystal violet by adherent cells were assessed with an automated microplate reader at 570 nm.

Cell Cycle Distribution and Determination of Cell Proliferation

Analysis of the cell cycle was done as described elsewhere (17). The rate of cell proliferation was measured by flow cytometric analysis of cells labeled with CFSE (19). Briefly, detached cells were stained with 1.5 μmol/L CFSE for 15 min at 37°C, washed twice, seeded in six-well plates at 2×10^5 per well, and then exposed to drugs. After 24 and 48 h of cultivation, cells were trypsinized and washed twice. Finally, the cells were resuspended in PBS and analyzed by flow cytometry. Green fluorescence emission was measured using a FACSCalibur machine (BD) and analyzed using CellQuest software.

Measurement of NO Release and Nitrite Accumulation

Nitrite accumulation, as an indirect measure of NO release, was measured using the Griess reaction as described previously (20). For intracellular NO detection, the cells were stained for 1 h at 37°C with 2 μmol/L of the NO indicator DAF-FM-diacetate (Molecular Probes) in phenol red–free RPMI 1640. The cells were then washed and incubated for an additional 15 min at 37°C in fresh RPMI 1640 before drug treatment. After 2 h, the cells were trypsinized, washed, and finally resuspended in PBS and analyzed by FACSCalibur using CellQuest software.

Cell-Based ELISA

To measure the expression of galactocerebroside, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), cyclin D3, p53, and p-Akt, we used a slight modification of the method for cell-based ELISA (cELISA) described by Maksimovic-Ivanic et al. (20). Briefly, at the end of the cultivation period, cells were fixed in 4% paraformaldehyde, and the endogenous peroxidase was quenched with 1% H_2O_2 in PBS containing 0.1% Triton X-100 (PBST). Nonspecific binding of antibodies was then blocked by incubation in PBST solution containing 10% FCS. Primary mouse monoclonal antibodies specific for rat and mouse p-Akt (1:200; Santa Cruz Biotechnology), GFAP (1:200, BioYeda), galactocerebroside (1:100; Boehringer Mannheim), MBP (1:100, Boehringer Mannheim), cyclin D3 (1:750; Santa Cruz

[7] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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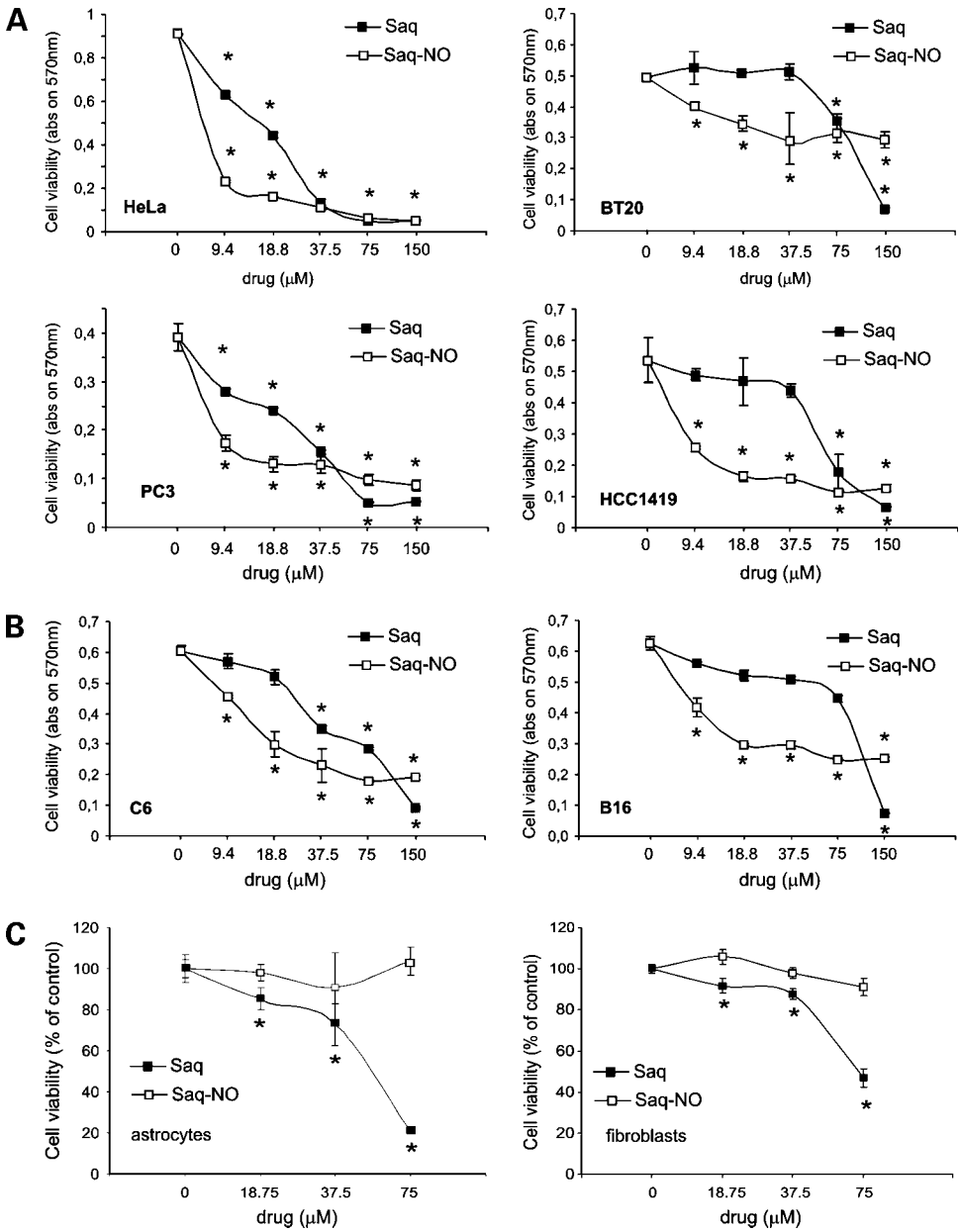

Figure 1. Cytotoxicity of Saq-NO versus Saq; Saq-NO reduces the viability of tumor but not primary cells. Human (**A**) and rodent (**B**) tumor cells (1×10^4 per well) and primary, nontransformed rodent cells (**C**; 3×10^4 per well) were exposed to varying concentrations of either Saq or Saq-NO for 24 h. Cell viability was then evaluated by crystal violet test. *Points,* mean from three independent representative experiments; *bars,* SD. *, $P < 0.05$, compared with untreated cultures.

Biotechnology), and p53 (1:250; Santa Cruz Biotechnology) were added in PBST supplemented with 2% bovine serum albumin (PBSTB), followed by secondary peroxidase-conjugated goat anti-mouse IgG (1:2,500 in PBSTB; GE Healthcare) for anti-GFAP and galactocerebroside or antirabbit IgG (1:2,500; GE Healthcare) for anti–p-Akt, anti–cyclin D3, and anti-p53. All incubations were done at 37°C for 1 h. Absorbance at 450 nm was then measured in an automated microplate reader 15 min after incubation with the tetramethylbenzidine peroxidase substrate and subsequently 0.1 mol/L HCl was added. To facilitate comparison between treatments, the measured absorbance of each sample was normalized to cell number, which was determined using crystal violet staining as described in the original protocol (17).

Tyrosinase Activity Assay and Melanin Determination

Tyrosinase activity was determined by measuring the rate of oxidation of 3,4-dihydroxy-L-phenylalanine. Briefly, subconfluent cultures in six-well plates were lysed in 100 μL phosphate buffer (pH 6.8) with 1% Triton X-100 and then centrifuged at 10,000 rpm for 5 min. A 40-μL aliquot of each extract was mixed with 100 μL of 3,4-dihydroxy-L-phenylalanine substrate solution (2 mg/mL). The enzymatic reaction was carried out at 37°C and the absorbance at 570 nm was read every 10 min for at least 1 h. The final activity was corrected for the total amount of protein, which was estimated by the Bradford assay. For melanin determination, cells were incubated in six-well plates for 24 h, trypsinized, counted, and then lysed in 100 μL of 1 mol/L NaOH. To this

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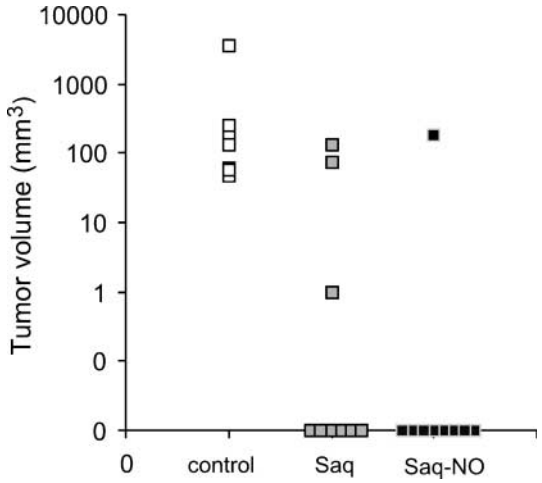

Figure 2. Saq and Saq-NO inhibit the growth of melanoma cells in C57BL/6 mice. Tumors were induced by s.c. implantation of 2.5×10^5 B16 melanoma cells, and either Saq or Saq-NO was injected i.p. for 15 consecutive days starting on day 10 following tumor implantation. Tumors were harvested, and tumor volumes calculated 30 d after tumor implantation.

lysis solution, 400 μL of distilled water were added and samples were incubated at 60°C for 1 h. Absorbance of the dissolved dye was measured at 492 nm.

Immunocytochemical Detection

The expressions of cyclin D3 and p53 were assessed immunocytochemically as previously described (18). The cells were cultivated on glass chamber slides (3×10^4 per well), and cyclin D3 and p53 expressions were detected with specific antibodies against cyclin D3 (1:1,000) or p53 (1:500; both from Santa Cruz Biotechnology). Bound primary antibodies were detected using the rabbit ExtrAvidin Peroxidase staining kit according to the manufacturer's instructions (Sigma) and using diaminobenzidine (R&D Systems) as a substrate. The cells were counterstained with Mayer's hematoxylin, and slides were mounted with glycergel mounting medium (Dako).

Western Blot Analysis

Cells (1×10^6) were seeded in flasks (25 cm^3), incubated in 0.5% FCS RPMI overnight, and subsequently treated with drugs for 30, 60, or 120 min. Whole-cell lysates were prepared in a solution containing 62.5 mmol/L Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 50 mmol/L DTT, and 0.01% (w/v) bromophenol blue, and were subjected to electrophoresis on 12% SDS-polyacrylamide gels. The samples were electrotransferred to polyvinylidene difluoride membranes at 5 mA/cm^2 using a semidry blotting system (Fastblot B43, Bio-Rad). The blots were blocked with 5% (w/v) nonfat dry milk in PBS 0.1% Tween 20 and probed with specific antibodies to p53, CD3, Akt, phosphorylated Akt, and β-actin (all were diluted 1:1,000; Akt and p-Akt were from Cell Signaling Technology; CD3, p53, and β-actin were from Santa Cruz Biotechnology), followed by incubation with secondary antibody (ECL donkey anti-rabbit horseradish peroxidase linked, GE Healthcare). Bands were detected by chemiluminescence (ECL, GE Healthcare).

Induction of Melanoma and Drug Treatment of Mice

To induce primary tumors, 2×10^5 B16 melanoma cells were injected s.c. in the dorsal right lumbosacral region of syngeneic C57BL/6 mice. Drug treatment with Saq and Saq-NO was started 10 d after implantation, and tumor growth was monitored daily. Both compounds, dissolved in 0.02% DMSO PBS, were injected i.p. at a dose of 10 mg/kg body weight, once daily for 15 consecutive days. Control animals were injected with diluent. Mice were sacrificed on day 30, at which time tumor growth was quantified by three-dimensional measurements of individual tumors from each mouse. Tumor volume was calculated according to the following formula: $0.52 \times a \times b^2$, where a is the longest diameter and b is the shortest diameter (20).

Acute Toxicity

To define the acute toxicity of Saq and Saq-NO, the compounds were administered i.p. to CD1 mice at single doses of 250, 500, 1,000, and 1,500 mg/kg. Control mice were each injected with 100 μL of the DMSO vehicle. Each dosage and control group consisted of 10 mice. Mortality was evaluated every hour for the first 4 h and then every 10 h until 14 d after dosing.

Statistical Analysis

The significance of differences between various treatments was analyzed by ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. $P < 0.05$ was considered significant.

Results

Addition of NO to Saq Enhances Its Antitumor Properties *In vitro* and *In vivo* and Neutralizes Its Toxicity

We first compared Saq and Saq-NO in terms of both their anticancer activity and their toxicity against primary cells. We measured the effect of both drugs on the viability of transformed human (HeLa cervix adenocarcinoma, BT20 and HCC1419 breast carcinoma, and PC-3 prostate carcinoma) and rodent (C6 rat astrocytoma and B16 mouse melanoma) cell lines, as well as on primary astrocytes and fibroblasts. As evidenced by crystal violet assay (Fig. 1A and B) and measurement of mitochondrial respiration (data not shown), both compounds possessed marked antitumor potential. However, Saq-NO was more potent than Saq at significantly lower doses, with a clear plateau effect evident in all cell lines tested. Similarly, the lowest dose of Saq that was highly efficient in suppressing tumor growth was also highly toxic against normal, healthy cells. In contrast, even the highest concentration of Saq-NO tested had no detectable effect on the viability of nontransformed primary astrocytes and fibroblasts (Fig. 1C).

To confirm these data *in vivo*, B16 tumor cells were inoculated s.c. into syngeneic C57BL/6 mice, which were then treated with i.p. injections of either Saq or Saq-NO. Drug treatment commenced 10 days after tumor induction and was given daily for 15 consecutive days. As shown in Fig. 2A, at the time of autopsy (day 30 after tumor challenge), all control animals

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had developed solid tumors. In the group treated with Saq, three of nine animals developed tumors, but their volume was significantly reduced compared with untreated animals. Remarkably, only one tumor was observed in the Saq-NO–treated group. Animals receiving Saq underwent as much as a 10% loss of their initial body weight, and four animals showed strong inflammation of the peritoneum. In contrast, no visible signs of toxicity were apparent in the Saq-NO–treated animals, whose body weight conversely increased by an average of 13% over initial values. A more detailed analysis of acute toxicity (Supplementary Tables S1 and S2)[7] revealed that a dose of Saq-NO corresponding to the 100% lethal dose of Saq (1,500 mg/kg) was absolutely non-toxic for animals as evidenced by lack of lethality and an incremental body weight gain through the 14-day observation period that was similar to that of vehicle-treated mice (Supplementary Table S2).[7] In contrast, the analysis of body weight gain in the groups of mice treated with doses of Saq that did not provoke 100% lethality (250, 500, and 1,000 mg/kg) revealed an apparent dose-dependent decrease or arrest of body weight gain during the first week after the injection, which was followed by recovery during the second week of observation (Supplementary Table S2).[7] These data clearly indicated that the attachment of a NO moiety to the Saq molecule considerably diminished its toxicity and intensified its tumoricidal activity.

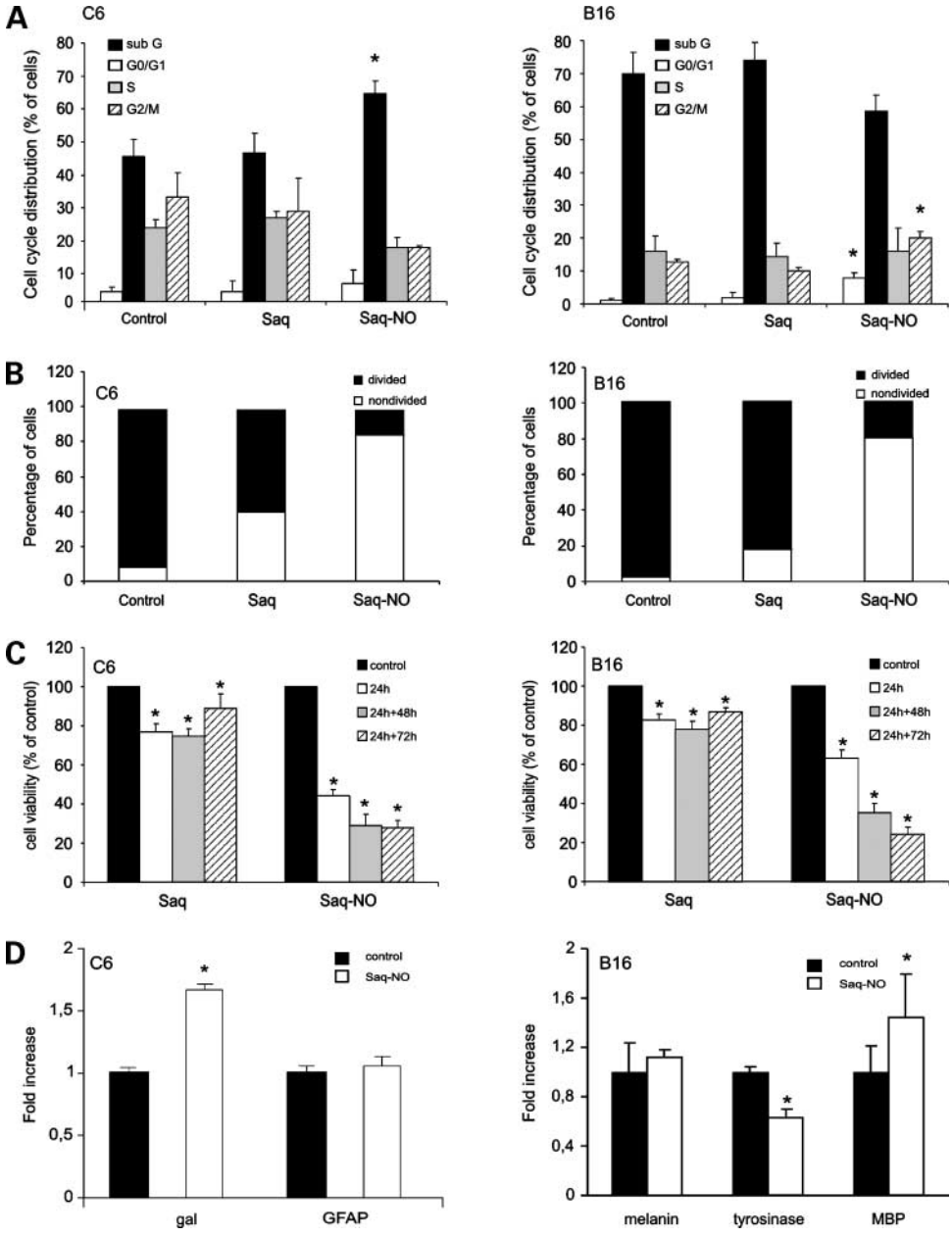

Figure 3. Saq-NO strongly inhibited cell proliferation and induced differentiation of C6 and B16 cells. **A,** tumor cells were incubated with 18.8 μmol/L of Saq or Saq-NO for 24 h, and then cells were stained with propidium iodide and analyzed by flow cytometry. **B,** cells were stained with CFSE and incubated for 48 h with 18.8 μmol/L of Saq or Saq-NO, and the rate of proliferation was determined by flow cytometry. **C,** cells were incubated with 18.8 μmol/L of Saq or Saq-NO for 24 h, at which point the drugs were removed from the cells and cell viability was determined by crystal violet test after 48 and 72 h of additional incubation. **D,** after 24-h incubation with 18.8 μmol/L Saq-NO, markers of differentiation of C6 and B16 cells, including galactocerebroside, GFAP, and MBP, were assessed by cELISA, whereas melanin and tyrosinase activity were assessed as described in Materials and Methods. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.05$, compared with untreated cultures.

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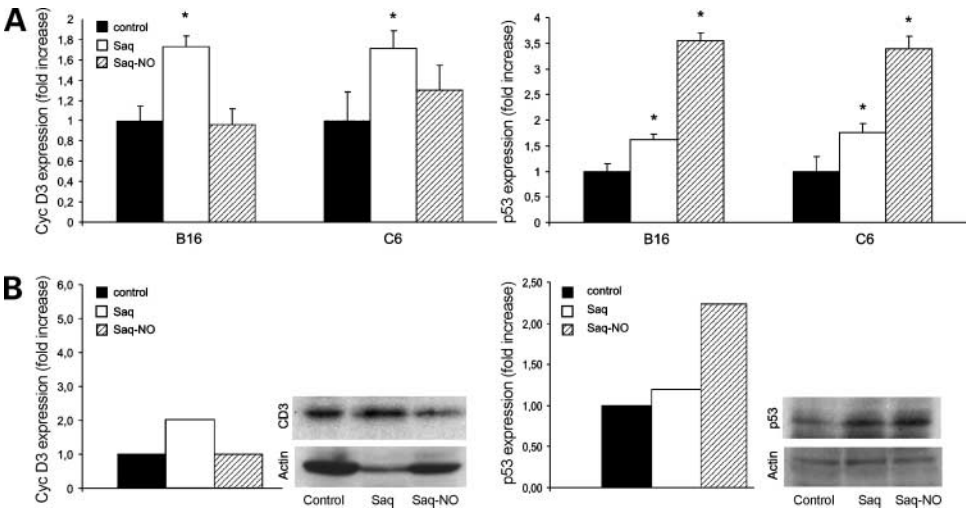

Figure 4. Saq-NO promoted strong up-regulation of p53 but not cyclin D3 expression. Cells were incubated with 18.8 μmol/L of Saq or Saq-NO for 24 h, and then cyclin D3 and p53 expressions were evaluated by cELISA (**A**) and Western blot (**B**). *Columns,* mean from three representative, independent experiments (two in the case of cELISA); *bars,* SD. *, $P < 0.05$, compared with untreated cultures.

The Antitumor Activity of Saq-NO Is Based Primarily on Its Cytostatic Effect

The observation that Saq-NO was effective at low doses, and that it showed a plateau effect, suggested that this drug was reducing the growth rate of tumor cells rather than inducing them to die. To more precisely define the antitumor mechanisms involved, we directly compared the effects of Saq and Saq-NO in inducing different types of cell death in two representative rodent cell lines, C6 and B16. We used each drug at a dose of 18.8 μmol/L because at this concentration Saq-NO had been shown to reduce cell viability to ~50% in C6 cells and 70% in B16 cells. During a 24-hour incubation with either Saq-NO or Saq, no significant percentage of cells was observed to die, either by necrosis or by type I or II apoptosis (data not shown). Whereas 18.8 μmol/L Saq did not promote significant changes in cell cycle distribution, Saq-NO induced a marked arrest of C6 cells in G_0-G_1 (44 ± 5.9 in controls versus 63 ± 4.0 in Saq-NO treated; Fig. 3A, *left*) and an accumulation of B16 cells in the G_2-M phase of the cell cycle (12.7 ± 0.95 in controls versus 20.1 ± 1.79 in Saq-NO treated; Fig. 3A, *right*).

These results suggested that the primary mechanism by which Saq-NO acts against tumor cells is by inhibiting proliferation rather than inducing cell death. We confirmed the cytostatic effect of Saq-NO by staining cells with the vital dye CFSE. CFSE is stable in the cytoplasm for more than 15 generations, but the intensity of its fluorescence declines predictably with each mitosis, allowing it to be used as a gauge to track cell division. Approximately 90% of control C6 (Fig. 3B, *left*) and B16 cells (Fig. 3B, *right*) divided after 48 hours. In contrast, fewer than 10% of cells of either type retained the ability to proliferate when treated with Saq-NO (Fig. 3B). In comparison with Saq-NO, the inhibition of proliferation observed in the presence of Saq was significantly lower, concordant with its weaker effect on cell viability (Fig. 1B). On the withdrawal of Saq, the viability of cells was almost restored after 72 hours. In contrast, removal of Saq-NO under the same experimental conditions was not affected, suggesting that the loss of proliferative capacity was permanent (Fig. 3C). In summary, these results strongly suggest that the cytostatic effect of Saq-NO is stronger that of Saq.

Inhibition of cellular proliferation following Saq-NO treatment was accompanied by morphologic transformation (Supplementary Fig. S5A)[7] and elevated expression of the oligodendrocytic marker galactocerebroside on C6 cell membranes (Fig. 3D, *left*). In B16 melanoma cells, Saq-NO unexpectedly led to a mild elevation of MBP expression, which was accompanied by a decrease in tyrosinase activity but no change in the quantity of melanin (Fig. 3D, *right*). Thus, exposure to Saq-NO seemed to have prompted C6 cells to assume some of the characteristics of oligodendrocytes, and B16 cells to assume Schwann cell–like properties. This phenomenon, termed transdifferentiation, has been observed in normal melanocytes as the final stage before involution (21). Because the type D cyclins, particularly cyclin D3, and the p53 tumor suppressor protein have been shown to be important in oligodendrocytes not only for regulation of the cell cycle but also for regulating the development of oligodendrocytes, we next investigated their expression in C6 and B16 cells exposed to Saq-NO. Both c-ELISA assay (Fig. 4A) and Western blot analysis (Fig. 4B and C), as well as immunocytochemistry (Supplementary Fig. S5B),[7] revealed up-regulation of p53 expression in both cell lines tested, whereas the expression of cyclin D3 was not changed. Having in mind that same dose of Saq promoted cyclin D3 accumulation, it is obvious that Saq and Saq-NO have different effects on the regulation of cell cycle.

Saq and Saq-NO Have Opposing Effects on the Akt Signaling Pathway

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is known to be one of the most important intracellular targets of Saq and other HIV-PIs. Inhibition of this pathway has been suggested both to be critical to their tumoricidal properties and to be responsible for the high toxicity of this family of drugs. Our observation that Saq-NO retained the antitumor properties of Saq while not retaining its toxicity prompted us to use cELISA and Western blots

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analysis to determine how this compound might affect the Akt signaling pathway in C6 and B16 cells. We found that, in sharp contrast to the ability of Saq to markedly abrogate p-Akt expression, Saq-NO induced considerable transient phosphorylation of Akt in both B16 and C6 cells (Fig. 5A and B). Cells that were additionally treated with either a specific inhibitor of upstream PI3K (3-MA) or an inhibitor of Akt (Akt VI) exhibited a further decrease in viability (Fig. 5C). These findings suggested that the antitumor properties of Saq-NO are not mediated by an effect on the Akt pathway.

Saq-NO Triggers the Release of a Negligible Amount of NO

The apparently contradictory findings that Saq-NO had retained the antitumor ability of Saq, but with an opposing effect on Akt activation and a loss of toxicity against primary cells, led us to question whether its effects might be mediated by its attached NO moiety. To answer this question, we first measured the intracellular accumulation of NO, as well as its liberation in cell culture medium, after 24 hours of cell incubation in the presence of the drug. Surprisingly, unlike other NO-modified compounds such as GIT-27NO (20), Saq-NO treatment caused only a minor intracellular release of NO in both B16 and C6 cells (Fig. 6A). In accordance with this, a negligible amount of NO was released into the culture medium (Fig. 6B). These data indicated that these minimal amounts of NO liberated from the drug could not be directly responsible for the efficiency of the drug against malignant cells.

Discussion

HIV-PIs have recently been shown to possess strong antitumor activity, in addition to their initially recognized ability to inhibit the HIV protease. HIV-PIs inhibit the growth of numerous types of tumor cell lines, including multiple myeloma, SW872 liposarcoma, T24 bladder carcinoma, A549 lung carcinoma, U373 glioblastoma, Jurkat leukemia cells, DU-145 and PC-3 prostate cancer cells, NB4 and HL-60 human myelocytic leukemia cells, and Kaposi's sarcoma (6, 7, 9, 12, 14). These drugs are capable of decreasing the incidence of Kaposi's sarcoma and promoting its regression, and of amplifying the therapeutic efficacy of radiotherapy and chemotherapy of head, neck, bladder, and prostate cancers (7, 8, 10, 11, 13, 14). However, long-term administration of HIV-PIs results in serious adverse side effects, including hyperbilirubinemia, hyperlipidemia or hypolipidemia, insulin resistance, and diabetes, among others (2).

Previous studies on nonsteroidal anti-inflammatory drugs had indicated that covalently attaching a NO moiety to the drug increased its potency while diminishing its toxicity. In the present study, we performed this modification on the HIV-PI drug Saq. We found that the product of this modification, Saq-NO, had a comparatively elevated antitumor activity against numerous rodent and human cell lines, whereas its toxicity to normal cells was abrogated. Although it has been suggested, in studies of other NO-donating compounds, that NO release itself is

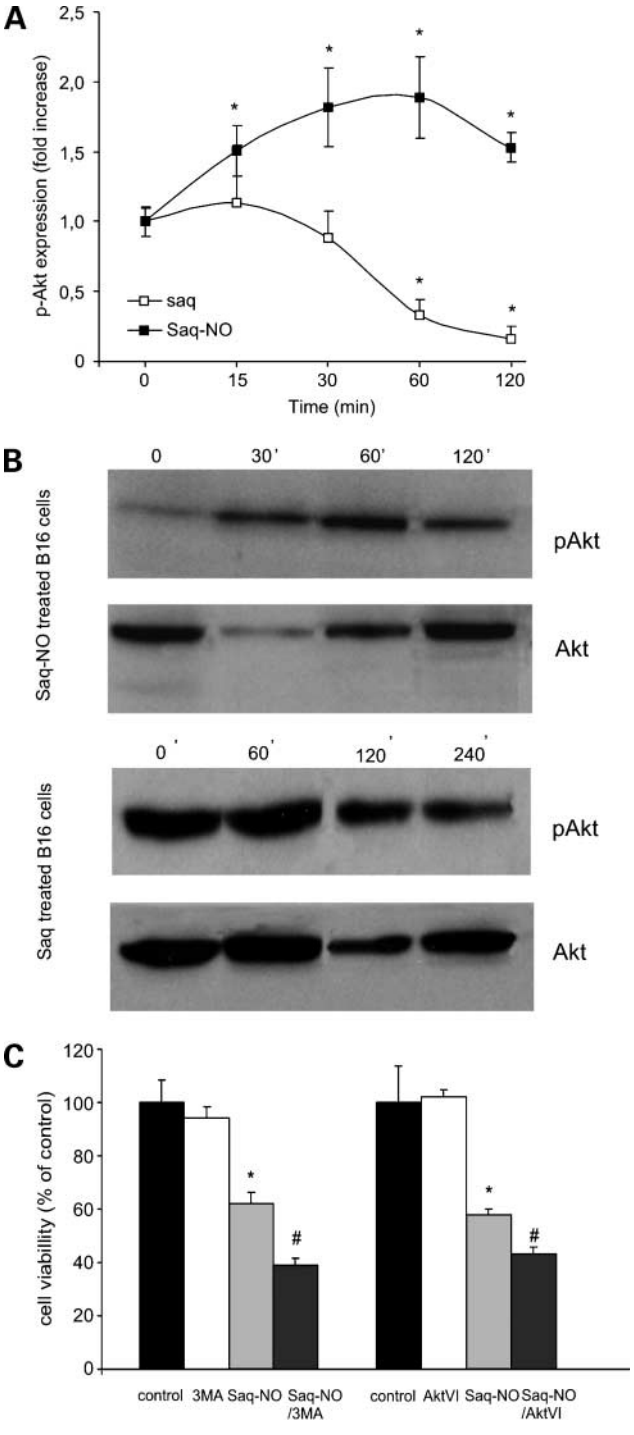

Figure 5. Saq and Saq-NO have opposing effects on Akt activity. **A,** B16 cells were incubated with 18.8 μmo/L of Saq or Saq-NO, and Akt activity was assessed by cELISA. *Points,* fold increase relative to values obtained in untreated control cultures; *bars,* SD. *, $P < 0.05$. **B,** effects were confirmed by Western blot at indicated time points. **C,** B16 cells (1×10^4 per well) were incubated for 24 h with 18.8 μmol/L Saq-NO, with or without the Akt inhibitor Akt VI (50 μg/mL) or 3-MA (1 mmol/L), and cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.05$, compared with untreated cultures.

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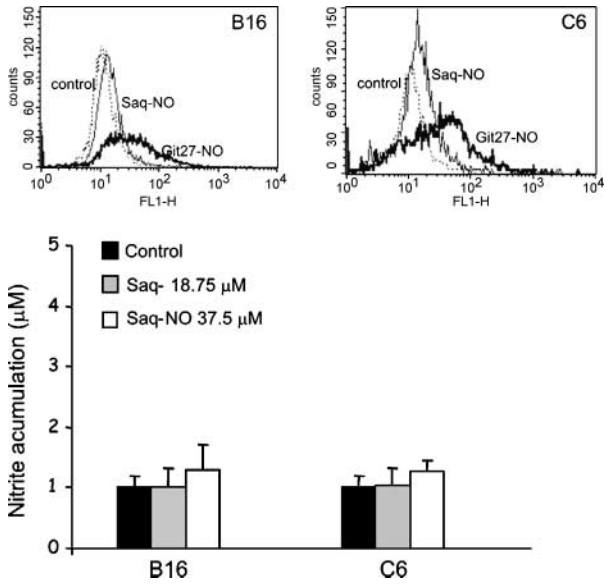

Figure 6. Tumor cell treatment with Saq-NO led to negligible NO release. **A,** intracellular NO was detected by flow cytometry of DAF-FM-diacetate–stained B16 and C6 cells after 24 h of incubation without (*Control*) or with Saq-NO (18.8 μmol/L). Cells treated with GIT-27NO (75 μmol/L) served as a positive control. **B,** accumulation of nitrites in B16 and C6 cell culture supernatants was detected after 24-h incubation of cells with the indicated concentrations of Saq-NO. *Columns,* mean from three independent representative experiments; *bars,* SD.

directly or indirectly responsible for their cytotoxicity (16), we found Saq-NO to liberate only a trivial quantity of NO, which was therefore unlikely to be responsible for its cytotoxic effects. Thus, the mechanism by which covalent attachment of NO to Saq achieves its effects remains unexplained. One possible explanation lies in the known ability of NO to reduce the activity of cytochrome *P*450, an enzyme responsible for the conversion of Saq to an inactive form (22, 23). In addition, it has been well documented that the release of NO and the subsequent activation of cyclic guanosine 3′,5′-monophosphate signaling pathways are fundamental for cell protection *in vitro* as well as *in vivo* (24). A cyclic guanosine 3′,5′-monophosphate–dependent prosurvival signal has been shown to be associated with reduced mitochondrial cytochrome *c* release, as well as translational and posttranslational modifications of caspase activity, including direct inhibition of caspases by S-nitrosylation of cysteine (25).

Taken together, these potential effects of NO might be responsible for the absence of Saq-NO toxicity. In parallel with its clearly apparent effect to decrease tumor cell viability, the observed cell cycle arrest and the CFSE staining data indicated that inhibition of proliferation is crucial for the anticancer activity of Saq-NO. Notably, withdrawal of Saq-NO failed to restore cell viability, suggesting that the loss of proliferative properties it induced was permanent.

It has recently been shown that certain HIV-PIs induce both preadipocyte and human myelocytic leukemia cells to differentiate (6, 8, 26). In agreement with this, we observed significant phenotypic transformations in C6 and B16 cells treated with Saq-NO. The capacity of C6 and B16 cells to differentiate is well documented. Depending on the stimulus, C6 cells are able to differentiate toward both astrocytes and oligodendrocytes (18, 27). B16 cells, which are derived from the same embryonic precursors as C6 cells, differentiate to acquire the characteristics of primary melanocytes when triggered by various stimuli (28–30). We found that treatment with Saq-NO prompted C6 cells to acquire some of the characteristics of oligodendrocytes. Unexpectedly, B16 cells treated with Saq-NO did not acquire the characteristics of mature melanocytes, apart from acquiring some of their morphologic features. However, it is known that B16 cells can be driven into a process known as transdifferentiation, in which they acquire a Schwann cell–like phenotype (31), which represents the end point of the process of involution of normal melanocytes (21). We observed in Saq-NO–treated B16 cells both down-regulation of melanocytic markers and elevated expression of the Schwann-cell marker MBP, suggesting that this treatment may have pushed the B16 cells directly into the end stage of the melanocyte life cycle.

Tokumoto et al. have previously established a connection between cyclin D3 expression and oligodendrocytic development when the latter is promoted by platelet-derived growth factor withdrawal or thyroid hormone treatment (32). Up-regulation of cyclin D3, the most widely expressed cyclin D family member in mammals, was observed in some myoblast cell lines and in the HL-60 human promyelocytic leukemia line during the process of differentiation (33–35). In addition, the maturation of multipotential stem cells toward oligodendrocytes when stimulated by thyroid hormone was found to be tightly coupled to up-regulation of cyclin D3, as well as to increased expression of p53 (33, 35). Cyclin D3 is believed to be the molecule primarily responsible for the differentiation of oligodendrocytes. Our observation that Saq-NO exposure of both cell lines studied led to a significant increase in cellular p53 protein, but with no change in the level of cyclin D3, is in agreement with a previously hypothesized pivotal role for p53 in neuronal tissue cell maturation (36).

It has been well documented that HIV-PIs interfere with the PI3K/Akt signaling pathway. For example, nelfinavir, amprenavir, and Saq have each been shown to inhibit Akt activity in numerous cell lines. However, the mechanism responsible for this inhibition is still not clear (11). In agreement with previous data, we observed that Saq decreased Akt phosphorylation in both C6 and B16 cells, and that this resulted in decreased activity of this kinase. The rate of Akt inhibition was correlated with the observed reduction of tumor cell viability, implicating an effect on this signaling pathway in the tumoricidal action of the drug. In contrast, treatment of B16 cells with Saq-NO induced only a transient activation of Akt. A similar pattern activation of Akt has been observed during differentiation of endothelial cells, osteoblasts, and myofibroblasts (36–38). Interestingly, cotreatment with an Akt inhibitor or an inhibitor of upstream PI3K resulted in a further decrease in cell viability. The data suggested that the antitumor properties

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we observed in Saq-NO were not based on the ability of Saq to modulate Akt phosphorylation.

It is possible that the observed absence of toxicity of Saq-NO to normal cells may have been a result of the drug delivering a protective signal to the cells through a powerful but transient stimulation of Akt. In agreement with this hypothesis, the neuroprotective role of NO has been shown to depend, at least partially, on its ability to promote Akt phosphorylation (39). In addition, Saq has been shown, in a manner dependent on its ability to down-regulate the PI3K/Akt pathway, to dramatically affect the propagation of signals triggered by insulin receptor binding, and thereby to trigger insulin resistance in treated cells (11). This and analogous events may be responsible for one or more of the serious side effects associated with Saq treatment. Conserved antitumor activity of Saq-NO without abrogated Akt signal, which is recognized as highly toxic for primary cells, may represent a considerable advance in cancer treatment. Performed structural modification of Saq seemed to have led to a qualitatively new drug that is quite different from other NO-donating compounds. In particular, its strong anticancer potential that we observed *in vitro* and *in vivo*, together with absence of toxicity, provides a powerful motive for further assessment of Saq-NO as a promising anticancer drug.

Disclosure of Potential Conflicts of Interest

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