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PRECLINICAL STUDIES

Lopinavir-NO, a nitric oxide-releasing HIV protease inhibitor, suppresses the growth of melanoma cells in vitro and in vivo

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Summary

We generated a nitric oxide (NO)-releasing derivative of the anti-HIV protease inhibitor lopinavir by linking the NO moiety to the parental drug. We investigated the effects of lopinavir and its derivative lopinavir-NO on melanoma cell lines in vitro and in vivo. Lopinavir-NO exhibited a twofold stronger anticancer action than lopinavir in vitro. These results were successfully translated into syngeneic models of melanoma in vivo, where a significant reduction in tumour volume was observed only in animals treated with lopinavir-NO. Both lopinavir and lopinavir-NO inhibited cell proliferation and induced the trans-differentiation of melanoma cells to Schwann-like cells. In melanoma cancer cell lines, both lopinavir and lopinavir-NO induced morphological changes, minor apoptosis and reactive oxygen species (ROS) production. However, caspase activation and autophagy were detected only in B16 cells, indicating a cell line-specific treatment response. Lopinavir-NO released NO intracellularly, and NO neutralization restored cell viability. Treatment with lopinavir-NO induced only a transient activation of Akt and inhibition of P70S6 kinase. The results of this study identify lopinavir-NO as a promising candidate for further clinical trials in melanoma and possibly other solid tumours.

Keywords HIV protease inhibitors · Lopinavir · Nitric oxide · Trans-differentiation · Melanoma · Schwann-like cells

Introduction

HIV protease inhibitors (HIV-PIs) are synthetic peptides that mimic the peptide bond between phenylalanine and proline or tyrosine and proline, thereby inhibiting the activity of the HIV aspartyl protease, a key protein in the assembly of viral particles [1]. The anticancer properties of HIV-PIs were empirically noted in patients treated with HIV-PIs, as the incidence rate of HIV-associated malignancies, including Kaposi's sarcoma and non-Hodgkin lymphoma, was observed to regress in these patients [2, 3]. Initially designed to target HIV proteases, these compounds can interact with mammalian proteins through the inhibition of cytochrome P450 and the P-gp transporter [4, 5], impairment of antigen presentation and inhibition of the proteasome, leading to apoptosis [6, 7].

During the last 10 years, several independent preclinical and clinical studies conducted by our group and others have proven that beyond their primary pharmacological profile, HIV-PIs possess pharmacological properties that qualify them as potential anticancer agents. Although drug-to-drug differences in the anticancer properties of this class of drugs have been reported, most common anticancer effects of HIV-PIs stem from their capacity to inhibit angiogenesis, reduce tumour invasiveness and block matrix metalloproteinase activation [8, 9]. In addition, HIV-PIs may sensitize tumours to radiotherapy [7, 10]. Cytotoxic chemotherapies such as docetaxel and imatinib have been successfully combined with HIV-PIs, suggesting that HIV-PIs have properties that may

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synergize with those of conventional cancer therapeutics [11, 12].

A downside of HIV-PI therapy is the low potency of these drugs; concentrations higher than 10 μM are required for their chemotherapeutic activity [13]. Additionally, HIV-PIs cause numerous adverse effects, such as dyslipidaemia (hypercholesterolaemia and hypertriglyceridaemia), cardiovascular diseases, diabetes, body fat distribution abnormalities, osteopaenia, and osteoporosis [14]. Different modifications of HIV-PIs aimed at increasing the potency and subsequently reducing the doses and limiting the side effects and toxicity of these drugs are being considered.

The packaging of HIV-PIs into nanostructured lipid carriers enhances their oral bioavailability and overcomes P-gp-mediated drug efflux [15]. Another modification incorporates saquinavir-loaded folic acid into polyethylene glycol (PEG) nanoparticles, thereby increasing cytotoxicity and cellular uptake via a folate receptor-mediated endocytosis mechanism [16]. In agreement with previous studies indicating that the addition of a nitric oxide (NO) moiety to parental drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) increases the anticancer potential of the base drug, we generated NO-derived HIV-PIs such as saquinavir-NO and demonstrated that this addition markedly reduces the toxicity and enhances the anticancer activity of several different parental compounds [17–19].

NO is a highly reactive molecule implicated in numerous physiological and pathological processes [20]. In addition, it plays an important role in tumour cell growth and survival [21]. NO can interact with other molecules by covalent reactions or noncovalent ligand-receptor interactions. By acting as an electron donor or electron acceptor, this molecule can react with proteins, modifying their enzymatic and transcriptional activities [22, 23]. Under oxidative stress conditions, NO can function as an antioxidant against reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-), creating reactive nitrogen species (RNS); these molecules are responsible for DNA nitrosation, nitration and deamination, which lead to DNA instability [24, 25].

NO can act as either a pro- or antitumourigenic compound. These dual functions of NO are governed by its concentration and redox status, along with the cell type [26]. Physiologically low concentrations of NO can induce proliferation and angiogenesis, whereas high concentrations can be cytotoxic and apoptotic [27]. Hence, NO-donating compounds are promising cancer therapeutics. The in vivo use of NO and NO-generating donors has been shown to increase the anticancer efficacy of dendritic cells, significantly reducing tumour growth [28]. Administration of various NO donors, such as N-(acetyloxy)-3-nitrosothiovaline (SNAP), *S*-nitrosoglutathione (GSNO) and 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN), to doxorubicin-resistant HT-29 cells partially reversed the resistance to doxorubicin [29]. The NO donor diethylenetriamine (DETA)-NONOate stimulates the S-nitrosylation of the NFκB p50 subunit, thereby inhibiting its activity [30]. The addition of NO to NSAIDs, including aspirin and indomethacin has been demonstrated to induce potent anticancer activity of the parental drug in vitro and in vivo. In addition, we showed that the addition of an NO moiety to the isoxazoline compound VGX-1027 (previously GIT-27) induces de novo acquisition of the anticancer activity of the compound [31–33].

We have repeatedly demonstrated via independent validation of our in vitro data at the National Cancer Institute (NCI) [34] that the HIV-PI saquinavir-NO possesses pleiotropic anticancer activities superior to those of saquinavir, including a reduction in the viability of adherent tumour cells in vitro and the inhibition of melanoma, prostate, and colon cancer growth in vivo [17, 18, 35, 36]. The antitumour effect of saquinavir-NO occurs through apoptosis, inhibition of cancer cell proliferation and reversion to a normal phenotype through the processes of differentiation or trans-differentiation.

Trans-differentiation of malignant cells could be a nonaggressive approach in cancer treatment [37]. This type of process is observed in the differentiation of melanocytes to a glial cell phenotype in which the loss of melanogenesis is accompanied by a change in cell morphology and the expression of gene markers of early Schwann cells [38]. The maturation of melanocytes to Schwann-like cells is marked by the disappearance of early markers, including box 3, and the induction of myelin genes, such as those encoding myelin basic protein (MBP) and protein zero (P0) [39, 40].

Following this line of research, we also synthesized the NO-derivative of another HIV-PI, lopinavir, and previously demonstrated that this compound has more powerful chemotherapeutic activity than the parental compound on controlling the growth of different blood cancer cells [19]. These data prompted us to evaluate the effects of lopinavir-NO in solid tumours, and we carried out a head-to-head comparison of the effects of lopinavir-NO vs. those of lopinavir on the growth of melanoma cancer cell lines in vitro and in vivo. The data demonstrate that lopinavir-NO is an NO-releasing compound with more powerful anticancer potential than lopinavir and that it is endowed with a unique mechanism of anticancer action that entails cytostatic effects on solid and metastatic melanoma and the trans-differentiation of melanoma cells to Schwann-like cells.

Material and methods

Reagents and cells

Foetal calf serum (FCS), RPMI-1640 medium, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), carboxy-PTIO, chloroquine diphosphate, and N-acetyl-L-

cysteine were obtained from Sigma (St. Louis, MO, USA). SIN and SNAP were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lopinavir and lopinavir-NO were purchased from OncoNOx. The B16, B16F10 and A375 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS and 2 mM L-glutamine, 0.01% sodium pyruvate, 100 U/ml penicillin 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Animals

Six- to eight-week-old C57BL/6 mice were obtained from the facility at the Institute for Biological Research "Sinisa Stankovic", University of Belgrade (Belgrade, Serbia). The mice were kept under standard laboratory conditions (nonspecific pathogen-free), with free access to food and water. The animal studies were performed in accordance with local guidelines and approved by the local Institutional Animal Care and Use Committee (IACUC), approval nr. 01–08/17.

Determination of cell viability by crystal violet (CV) and MTT assays

A total of 3000 cells/well were seeded in 96-well plates overnight, treated with the tested compounds for 48 h, fixed and stained with 1% CV (Mol, Belgrade, Serbia) for 15 min at room temperature (RT). Stained cells were washed and dried, and the dye was dissolved in acetic acid. The absorbance of the dissolved dye was measured at 540 nm. Cell viability was calculated as a percentage of control. Mitochondrial dehydrogenase activity was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cells were treated with 0.5 mg/ml MTT (Sigma, St. Louis, MO, USA) and incubated at 37 °C until the colour of the solution changed from yellow to brown. DMSO was added to dissolve the formazan, and the absorbance was measured at 540 nm. Cell viability was calculated as a percentage of control (untreated cells), which was arbitrarily set to 100%.

Annexin V/propidium iodide (PI) staining

Melanoma cells were seeded in 24-well plates (20,000 cells/well) and treated with HIV-PIs, stained with 1 mg/ml annexin V-FITC (BD Pharmingen, San Diego, CA, USA) and 1 mg/ml PI (Sigma, St. Louis, MO, USA), and analysed via flow cytometry using a CyFlow Space (Partec, Muenster, Germany).

Caspase activation assay

Twenty thousand cells per well were seeded in 24-well plates and treated with HIV-PIs. The pan-caspase inhibitor ApoStat (R&D Systems, Minneapolis, MN, USA) was added to trypsinized cells and incubated for 30 min at 37 °C. Cells were washed with PBS, resuspended, and analysed by flow cytometry.

Detection of cell proliferation

Prior to treatment, cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma, St. Louis, MO, USA) to a final concentration of 1 μM and incubated for 20 min at 37 °C. Next, cells were washed, seeded in 24-well plates (20,000 cells/well), treated with HIV-PIs for 48 h, and analysed by flow cytometry.

Measurement of ROS/RNS generation

The production of ROS/RNS was detected by measuring the intensity of green fluorescence emitted by the redox-sensitive dye dihydrorhodamine 123 (DHR), Thermo Fisher Scientific, Waltham, MA, USA) upon excitation at 488 nm. Cells were incubated with DHR for 20 min at 37 °C, seeded in a 24-well plate (20,000 cells/well) and treated for 48 h, followed by washing with PBS, trypsinization and flow cytometric analysis.

Measurement of intracellular NO and nitrite accumulation

After 48 h of treatment in 24-well plates, 20,000 cells/well were stained with 2 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C in phenol red-free RPMI-1640 medium supplemented with 10% FCS, washed with PBS, resuspended and analysed by flow cytometry or by a microplate reader. Nitrite accumulation, an indirect measure of NO release, was assessed in cell culture supernatants by the Griess reaction. Equal amounts of cell culture supernatants and Griess reagent (N-(1-naphthyl) ethylenediamine and sulfanilamide) were mixed and incubated for 10 min at RT, and the absorbance at 514 nm was measured.

Detection of autophagy

A total of 20,000 cells/well were seeded in a 24-well plate and treated with HIV-PIs. Post incubation, cells were stained with 1 μg/ml acridine orange (Lab Modena, Paris, France) and incubated for 15 min at 37 °C. Acridine orange was removed by washing with PBS. Cells were resuspended in PBS and analysed by flow cytometry in the green and red channels.

Tyrosinase activity and melanin production

Tyrosinase activity was determined by measuring the rate of oxidation of 3,4-dihydroxy-L-phenylalanine (L-DOPA, Sigma, St. Louis, MO, USA). A concentration of 2 mg/ml L-DOPA was added to 1.5 million cells/sample and incubated for 15 min at 37 °C in Falcon tubes. Supernatants were transferred into a 96-well plate, and the absorbance at 570 nm was measured. Cells were treated for 48 h, trypsinized, counted and adjusted to 1.5 million cells/sample. Sodium hydroxide was added to lyse cells, and cells were incubated for one hour at 60 °C. Supernatants were transferred into 96-well plates, and the absorbance at 492 nm was measured.

Immunocytochemical detection

Cells were cultivated on glass chamber slides and treated with HIV-PIs, and the expression of MBP was detected by a specific antibody (#808401, Biolegend, San Diego, CA, USA). After washing and incubation with the biotinylated secondary antibody, cells were incubated with ExtrAvidin peroxidase (ExtrAvidin peroxidase staining kit, Sigma, St. Louis, MO, USA) for 30 min. Diaminobenzidine (Liquid Plus Substrate Chromogen system, Agilent, Santa Clara, CA, USA) was used as the substrate, and this step was followed by counterstaining with Mayer's haematoxylin (Sigma, St. Louis, MO, USA) and mounting with glycerine gel (Agilent, Santa Clara, CA, USA).

Western blot analysis

Cell lysates (20 μg) were separated by 12% SDS-PAGE, electroblotted onto PVDF membranes (Merck, Burlington, MA), blocked with 5% BSA in TBS-Tween (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.05% Tween 20), and probed with primary antibodies overnight at 4 °C. After washing, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactive bands were visualized by an ECL detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Primary antibodies against the following proteins were used: phospho-P70S6K (Cell Signaling Technology #9205), P70S6K (Cell Signaling Technology #9202), phospho-S6 (Cell Signaling Technology #2215, 1:1000), S6 (Cell Signaling Technology #2217), phospho-Akt (Cell Signaling Technology #4058), Akt (Cell Signaling Technology #9272), phospho-Erk (Cell Signaling Technology #4370), and Erk (Cell Signaling Technology #4695).

Induction of melanoma and experimental treatment

Tumours were induced by subcutaneous implantation of 300,000 B16 melanoma cells in the dorsal right lumbosacral region of syngeneic female C57BL/6 mice. For tumour inoculation, animals were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. When the tumours were palpable—11 days after tumour implantation—the animals were randomly allocated to three groups and treated daily for 9 consecutive days with an intraperitoneal (i.p.) injection of 10 mg/kg body weight lopinavir or lopinavir-NO. HIV-PIs were prepared by diluting the stocks dissolved in DMSO with PBS; the final DMSO concentration was 4%. The control group was treated with 4% DMSO in PBS. Tumours were harvested 20 days after tumour implantation, and their volumes were calculated by the following formula: (length x width^2) × 0.52.

Statistical analysis

We used Statistical Package for the Social Sciences (SPSS, IBM, Armonk, NY, USA) for data analysis. Student's t test and ANOVA (with post hoc Tukey's test) were performed as parametric tests, and Mann-Whitney and Kruskal-Wallis tests were performed as non-parametric tests. To examine whether variables were normally distributed, the Kolmogorov-Smirnov test was used.

Results

Lopinavir-NO affects the viability of melanoma cells more potently than the original drug both in vivo and in vitro

Mouse solid melanoma (B16), mouse metastatic melanoma (B16F10) and human melanoma (A375) cells were treated with various concentrations of lopinavir and lopinavir-NO for 48 h. As shown in Fig. 1, both compounds significantly diminished cell viability in a dose-dependent manner, as determined by CV and MTT assays. However, lopinavir-NO was more effective than the parental drug, with IC_{50} levels approximately two times lower than those of the parental drug in the B16F10 and A375 cell lines (Table 1). In the B16 cell line, which is derived from a less-invasive form of melanoma, the IC_{50} values of the NO-modified and parental drugs were similar. To confirm these data in vivo, we used a syngeneic model of B16 melanoma in C57BL/6 mice. Despite the very similar anticancer efficacy of lopinavir and lopinavir-NO in B16 cells in vitro, at the time of autopsy—20 days after tumour challenge—only the mice treated with lopinavir-NO had a significantly lower mean tumour volume than the controls (Fig. 2).

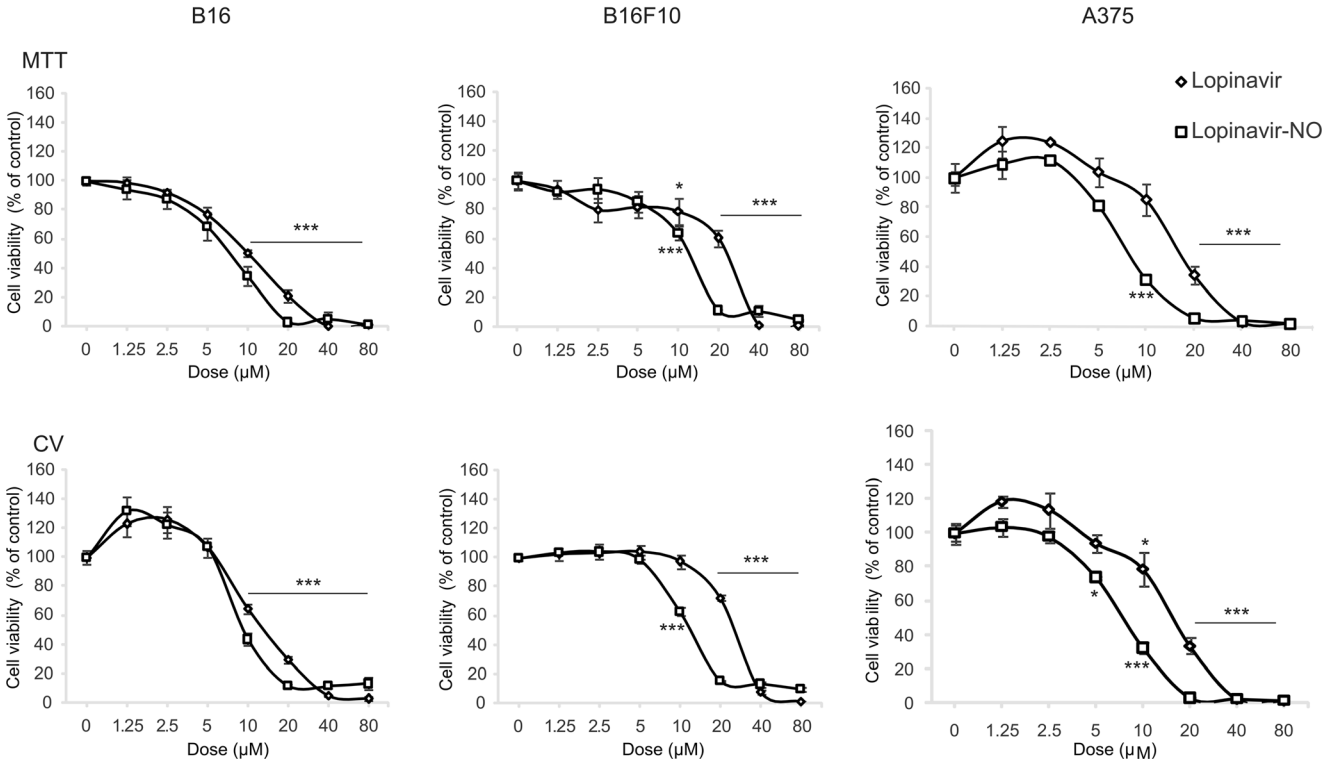


Fig. 1 Lopinavir and lopinavir-NO reduce the viability of melanoma cells in a dose-dependent manner. Mouse solid melanoma (B16), mouse metastatic melanoma (B16F10) and human melanoma (A375) cells were treated with a range of concentrations of lopinavir and lopinavir-NO for 48 h. Cell viability was determined by MTT and CV assays. The data are presented as the percentage of control ± SD from one representative experiment of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ refer to untreated cultures

The antitumour activity of lopinavir and lopinavir-NO is based primarily on their cytostatic effect

CFSE is a dye stable in the cytoplasm for more than 15 generations, but its fluorescence intensity declines with each mitosis. Therefore, this dye is used to track cell division. Staining of B16 cells with CFSE revealed strong inhibition of cell proliferation (60%) after treatment with lopinavir-NO (Fig. 3a), compared to the cell proliferation inhibition of only 20% achieved with lopinavir treatment. However, both compounds inhibited cell proliferation by 60% in the metastatic melanoma cell line B16F10. Inhibition of cellular

Table 1 IC_{50} values of Lopinavir and lopinavir-NO in melanoma cell lines

Cell line	Assay	IC_{50} (μM)	
		Lopinavir	Lopinavir-NO
A375	MTT	13.04 ± 3.8	6.68 ± 0.9
	CV	14.07 ± 2.15	6.76 ± 0.52
B16	MTT	11.9 ± 1.91	7.97 ± 0.67
	CV	13.75 ± 0.39	8.24 ± 0.52
B16F10	MTT	22.82 ± 0.56	12.84 ± 0.96
	CV	24.57 ± 2.3	12.33 ± 0.45

Data are presented as the means ± SEMs of three independent experiments

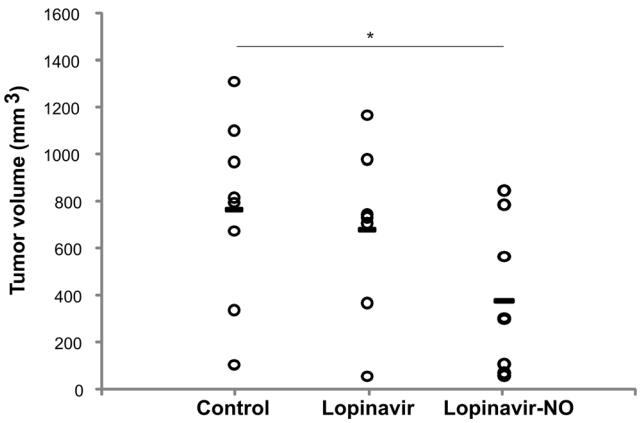
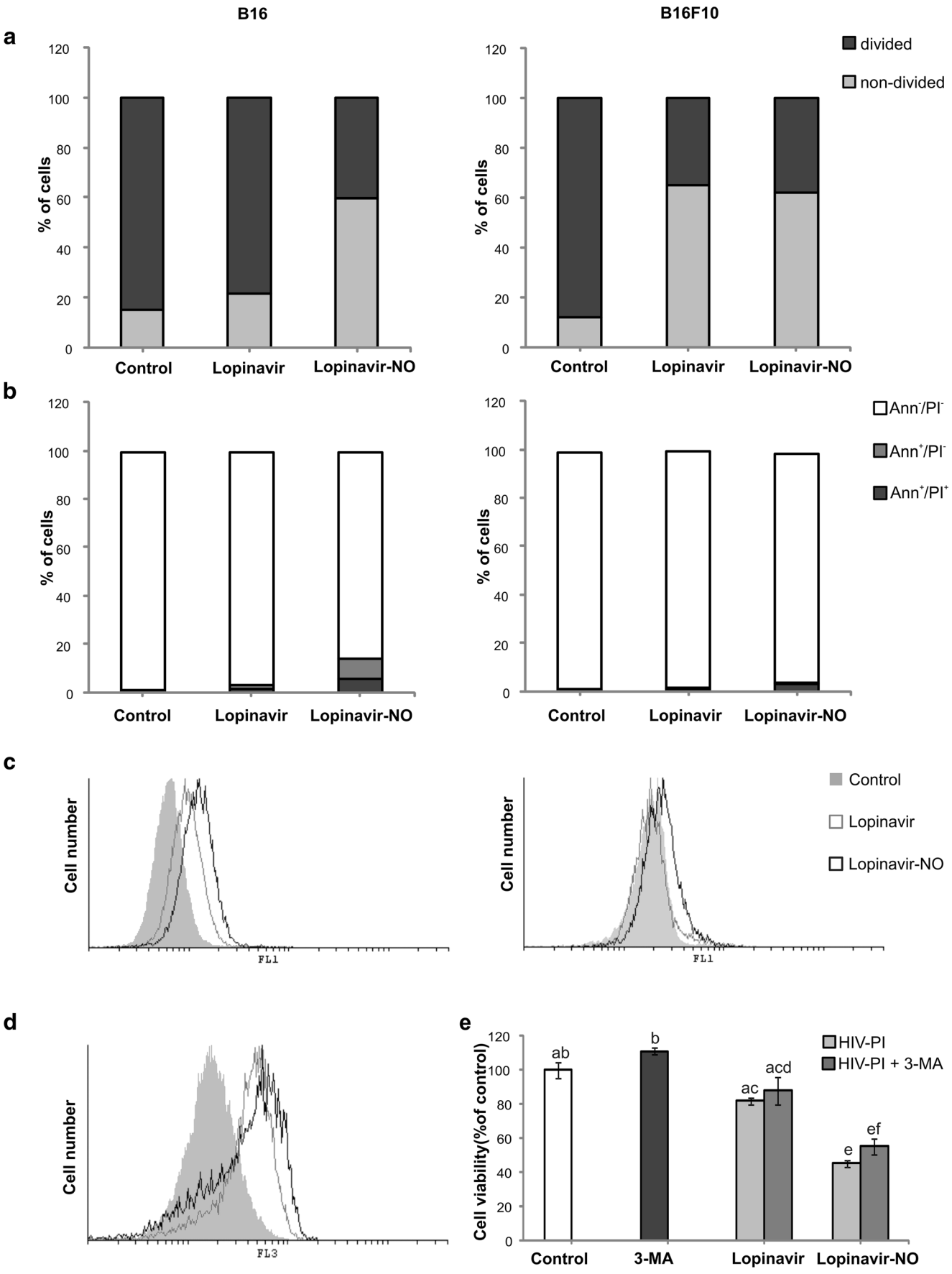


Fig. 2 Lopinavir-NO treatment contributes to tumour volume reduction. Tumours were induced by the implantation of B16 cells in C57BL/6 mice. Tested compounds were administered by i.p. injection for 9 consecutive days starting on day 11 after tumour implantation. Tumours were harvested 20 days after implantation, and their volumes were calculated by the following formula: (length x width^2) × 0.52. *$p = 0.028$ for lopinavir-NO vs. control, which was the only statistically significant difference between the groups. The solid line represents the median tumour volume for each group. Number of animals per group: control, $n = 8$; lopinavir, $n = 7$; and lopinavir-NO, $n = 8$


B16
B16F10
a
divided
non-divided
% of cells
Control
Lopinavir
Lopinavir-NO
b
Ann-/PI-
Ann+/PI-
Ann+/PI+
c
Cell number
Control
Lopinavir
Lopinavir-NO
FL1
d
FL3
e
Cell viability(%of control)
HIV-PI
HIV-PI + 3-MA
3-MA
ab
b
ac
acd
e
ef

◀ **Fig. 3 HIV protease inhibitors induce proliferation inhibition and minor apoptosis in both cell lines but induce caspase activation and autophagy only in B16 cells.** B16 (left) and B16F10 (right) cells were treated with IC_{50} concentrations of the tested compounds for 48 h and then subjected to **a** CFSE, **b** annexin/PI, **c** ApoStat and **d** acridine orange staining. Cells were analysed by flow cytometry, and the results of one representative experiment of three experiments are shown. **e** B16 cells were treated with IC_{50} concentrations of the tested compounds combined with the autophagy inhibitor 3-MA (1 mM). The data are presented as the percentage of control ± SEM from three independent experiments. Bars not sharing a common letter indicate significant differences, $p < 0.05$

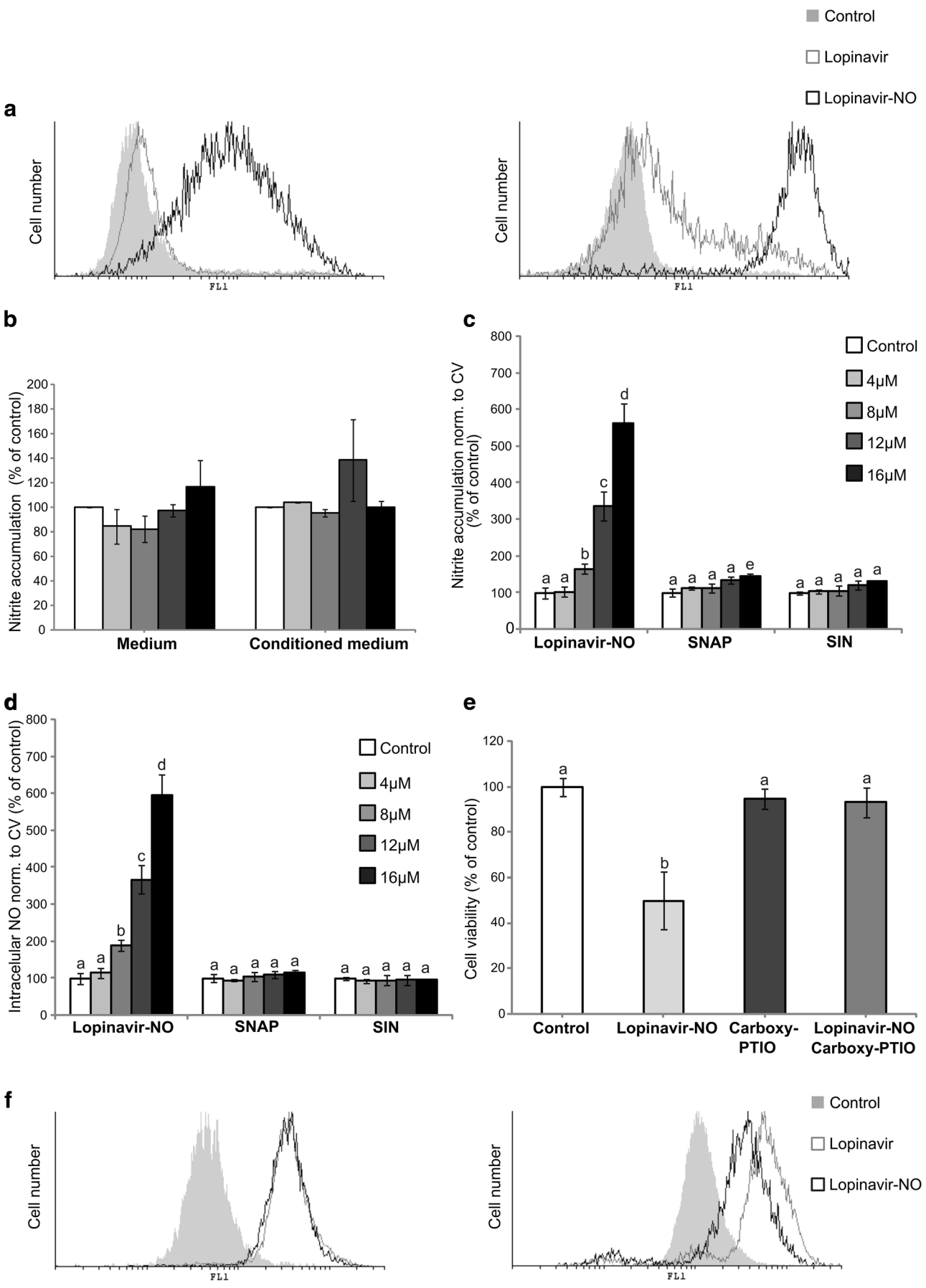
proliferation was accompanied by morphological transformation, possibly indicating a differentiation effect of the tested compounds (Supplement 1). Double staining with annexin V/PI did not reveal a substantial presence of apoptotic or necrotic cells (Fig. 3b). Staining of nuclei using PI in cells treated with either lopinavir or lopinavir-NO revealed that the majority of nuclei were large and euchromatic and that few segmented nuclei typical of apoptotic nuclear destruction were present (Supplement 2). These data suggest that apoptosis is a minor anticancer effect of lopinavir and lopinavir-NO and that the primary mechanism by which these compounds act against tumour cells is the inhibition of proliferation rather than the induction of cell death. Caspase activation was detected in the less-aggressive B16 subclone upon treatment with either compound (Fig. 3c). In the metastatic B16F10 subclone, caspase activity was detected solely after exposure to lopinavir-NO and was, in general, less profound than in B16 cells, underlining the differences between metastatic vs. primary tumour-derived cells. The development of acidic autophagosomes as a hallmark of autophagy was identified by flow cytometric analysis of acridine orange-stained cells. The fluorescence intensity was significantly increased in the B16 cell line relative to that in the B16F10 cell line (Fig. 3d). However, in metastatic melanoma B16F10 cells, treatment with neither compound induced autophagy (data not shown). We further investigated the role of autophagy in the response of the less-invasive B16 subclone to lopinavir or lopinavir-NO exposure by using specific autophagy inhibitors such as chloroquine. Chloroquine acts by changing the lysosomal pH, thereby inhibiting autophagic degradation in lysosomes [41]. As assessed by the results of the CV assay (Fig. 3e), the viability of lopinavir- and lopinavir-NO-treated cells was unaltered in the presence of the autophagy inhibitor, thereby excluding autophagy as a mediator of the anticancer mechanism of the compounds.

Lopinavir-NO is an NO donor

Intracellular NO was detected by DAF-FM diacetate fluorescence. The results suggested that lopinavir-NO releases substantial amounts of NO intracellularly (Fig. 4a). Since the release of NO can occur extracellularly, spontaneously or by contact with the cell membrane or cellular products, the conditions under which NO release was detectable were investigated. Pure medium and conditioned medium were supplemented with IC_{25}, IC_{50}, IC_{75}, and lethal concentrations of lopinavir-NO (4 μM, 8 μM, 12 μM, and 16 μM, respectively). Conditioned medium was medium collected from nontreated cell cultures after 72 h of cultivation and could contain cell fragments or released products. After 48 h of exposure to lopinavir-NO, nitrite accumulation, an indicator of NO release, was measured by the Griess reaction. A minimal amount of nitrates was released in culture or conditioned culture medium (Fig. 4b). However, nitrite accumulation in the supernatants of cells treated for 48 h showed a concentration-dependent increase (Fig. 4c). These data, with the detection of intracellular NO release, indicate the involvement of cell-specific enzymes or cell membranes as the trigger of NO release from the compound. The kinetics of NO release revealed that the intracellular NO levels increased significantly after 16 h (Supplement 3). The nitrite accumulation in cell supernatants was perfectly synchronized with the rate of intracellular liberation of this free radical under the same conditions. Importantly, in the same time frame and concentration range, the extracellular and intracellular release of NO by the two conventional NO donors SNAP and SIN was negligible (Fig. 4c and d). Lopinavir-NO released from two to six times more intracellular NO (depending on the dose) than the control, implying that lopinavir-NO is a suitable intracellular donor of NO. Finally, the contribution of NO to the antitumour effect of lopinavir-NO was investigated. Cells were concomitantly cultured in the presence of the drug and the NO scavenger carboxy-PTIO. The viability of the cells treated with the combination of lopinavir-NO and the scavenger was almost completely restored (Fig. 4e), indicating that NO release strongly impacts cell viability. As NO can react with ROS, amplifying cell damage through the generation of the most aggressive free radical molecule, peroxynitrite, the possible generation of ROS/RNS in lopinavir-NO-treated cells was measured by the intracellular DHR fluorescence. The cumulative intracellular production of ROS/RNS was markedly elevated for both components in both cell lines (Fig. 4f). In contrast to the ROS neutralization by the NO scavenger carboxy-PTIO, ROS neutralization by the antioxidant N-acetylcysteine did not improve the viability of treated cells, implying that ROS play a limited role in the mechanism of action of lopinavir-NO (Supplement 4).

Melanoma cell lines differentiate towards Schwann-like cells

The inhibition of cellular proliferation following lopinavir and lopinavir-NO treatment was accompanied by the morphological transformation of B16 and B16F10 melanoma cells (Supplement 1). Treatment with either compound markedly


a
Control
Lopinavir
Lopinavir-NO
Cell number
FL1
b
Nitrite accumulation (% of control)
Medium
Conditioned medium
c
Nitrite accumulation norm. to CV (% of control)
Control
4μM
8μM
12μM
16μM
Lopinavir-NO
SNAP
SIN
d
Intracelular NO norm. to CV (% of control)
e
Cell viability (% of control)
Control
Lopinavir-NO
Carboxy-PTIO
Lopinavir-NO Carboxy-PTIO
f

◀ **Fig. 4 NO release and ROS production in cells treated with the tested HIV-PIs.** After treatment with IC_{50} concentrations of lopinavir and lopinavir-NO for 48 h, cells were subjected to **a** DAF-FM diacetate staining and flow cytometric analysis. One representative experiment of three experiments is shown (B16-left, B16F10-right). **b** Nitrite accumulation was measured by the Griess reaction. **c** Cells were treated with a range of concentrations of either lopinavir-NO, SNAP or SIN. Nitrite accumulation was measured by the Griess reaction and normalized to the number of viable cells. **d** Intracellular NO release was measured by DAF-FM diacetate fluorescence and normalized to the number of viable cells. **e** B16 cells were treated with lopinavir-NO and/or carboxy-PTIO (20 μM) for 48 h and subjected to CV staining. **f** DHR staining and analysis by flow cytometry (B16-left, B16F10-right). The data are presented as the means ± SEMs of three independent experiments. Bars not sharing a common letter indicate significant differences, $p < 0.05$

altered the cell shape and size; cells became elongated with tiny, tapering dendrites resembling a glial-like phenotype. To delineate the possible underlying mechanism, differentiation markers of melanocytes and Schwann-like cells were analysed. Compared to control cells, treated cells exhibited no significant changes in tyrosinase activity or melanin production (Fig. 5a). However, treatment with either lopinavir or lopinavir-NO increased MBP expression (Fig. 5b). These data implicate the induction of a trans-differentiation phenomenon directed towards Schwann-like cells, not melanocytes, in response to HIV-PIs.

Lopinavir-NO transiently activates Akt and permanently inhibits P70S6 kinase

To better decipher the molecular mechanism underlying the anticancer activity of lopinavir-NO and its parental compound, we studied the effects of these compounds on two major signalling pathways involved in tumour cell survival and proliferation: the PI3K/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway. Lopinavir-NO transiently increased Akt phosphorylation, while lopinavir maintained Akt phosphorylation at relatively stable low levels for up to 48 h (Fig. 6). In addition, Erk phosphorylation was affected inversely from Akt activation. Transient upregulated expression was found after lopinavir treatment but did not occur in lopinavir-NO treated cells. In addition, lopinavir-NO induced of P70S6K inhibition, but surprisingly, S6 phosphorylation was unaltered, suggesting that an additional signalling mechanism was involved in S6 protein regulation.

Discussion

In this study, we show for the first time the in vitro and in vivo proof-of-concept preclinical data that support the possible use of lopinavir-NO in melanoma therapy. A strong sensitivity of melanoma cells to treatment with lopinavir-NO was demonstrated, and the antitumour potential of lopinavir-NO was significantly stronger than that of the parental drug. Additionally, we gained new insights into the anticancer mechanism of action of lopinavir-NO.

The antitumour potential of HIV-PIs has been demonstrated in numerous cell lines and in vivo models [34]. The majority of data are related to saquinavir and nelfinavir; few studies have described the efficacy of lopinavir alone or in combination with other drugs. In primary effusion lymphoma, a type of non-Hodgkin lymphoma, lopinavir induced caspase-dependent apoptosis and diminished NF-κB activity, and its efficacy was also observed in a xenograft mouse model [42]. Johnson et al. investigated the influence of lopinavir on primary cultures of meningioma cells isolated from foetal or adult tumours of different grades and found that this drug inhibits meningioma growth by inducing cell cycle arrest [43]. Lopinavir has been reported to display synergistic anticancer activity with another protease inhibitor, ritonavir, and the proteasome inhibitor carfilzomib in renal and bladder cells, respectively [44]. Combined with ritonavir, which interferes with the metabolic pathways of the drug, therefore enhancing its blood levels, lopinavir sensitized head and neck cells to irradiation through inducing endoplasmatic reticulum stress [45]. Through a similar mechanism, HIV-PIs triggered significant cell death in cultures of several bladder cancer cell lines [46]. Additionally, HIV-PIs interfere with the activity of membrane transporters. Atazanavir, lopinavir, and ritonavir function as inhibitors of the MDR1 P-gp, MRP1 and BCRP but not as substrates for ATP-binding cassette (ABC) transporters [47]. Based on the constitutive anticancer properties of lopinavir and the demonstrated ability of the NO linkage to enhance the baseline anticancer activity or achieve de novo quality, we synthesized lopinavir-NO and generated preliminary in vitro data. These results demonstrated the higher efficacy of lopinavir-NO than the parental compound in blood cancer cell lines such as Raji, Jurkat, and K-652 through caspase-dependent apoptosis [19]. The IC_{50} values in haematological malignancies were moderately higher than those found in this study in melanoma cell lines. Since the cell lines used in this study differ in their invasive potential and metastatic ability, the capacity of lopinavir-NO to nonselectively suppress their growth in vitro is of major interest [48], as one of the major problems in chemotherapy is the absence of drug activity in high-grade tumours [49]. We previously reported that saquinavir-NO but not saquinavir exerted antitumour activity through the permanent inhibition of cell division [18, 36]. Similarly, in this study, both lopinavir and lopinavir-NO suppressed proliferation in metastatic cell lines, but only lopinavir-NO inhibited the growth of less-aggressive cell lines, implying that the parental drug was inferior in inhibiting the proliferation of non-metastatic cells.

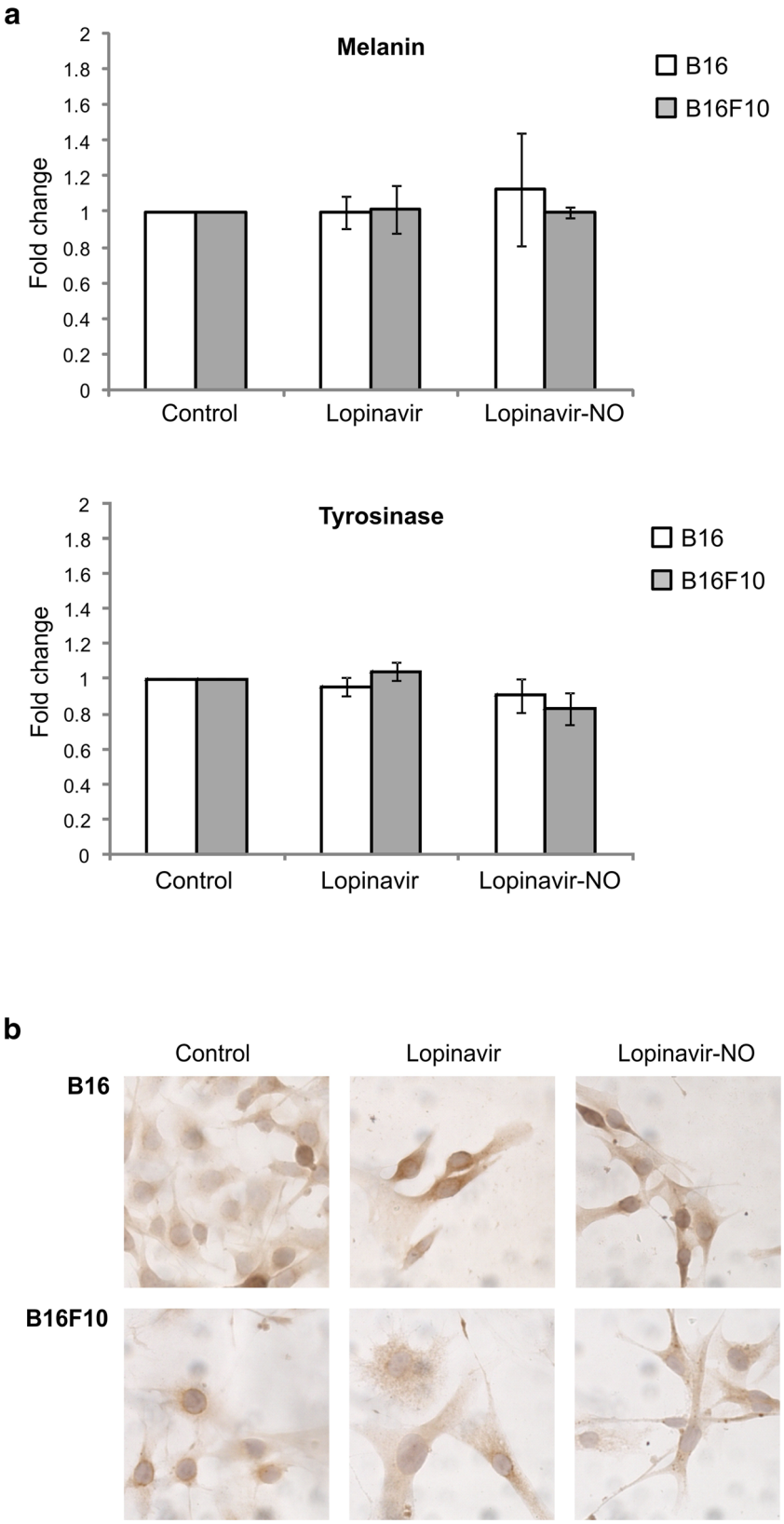


Fig. 5 Differentiation of melanoma cells is directed towards Schwann-like cells, not melanocytes. a Melanin production and tyrosinase activity were measured as markers of melanocyte differentiation. The data are presented as the means ± SEMs of three independent experiments. **b** Immunocytochemical staining of MBP. One representative staining sample of three replicates is shown; 400X magnification

Analysis of the annexin V-PI double staining results showed that lopinavir and its modified counterpart did not trigger substantial apoptosis. Morphological characterization of nuclei by PI staining and microscopy confirmed these findings. The literature refers to apoptosis as one of the main mechanisms of action of HIV-PIs;

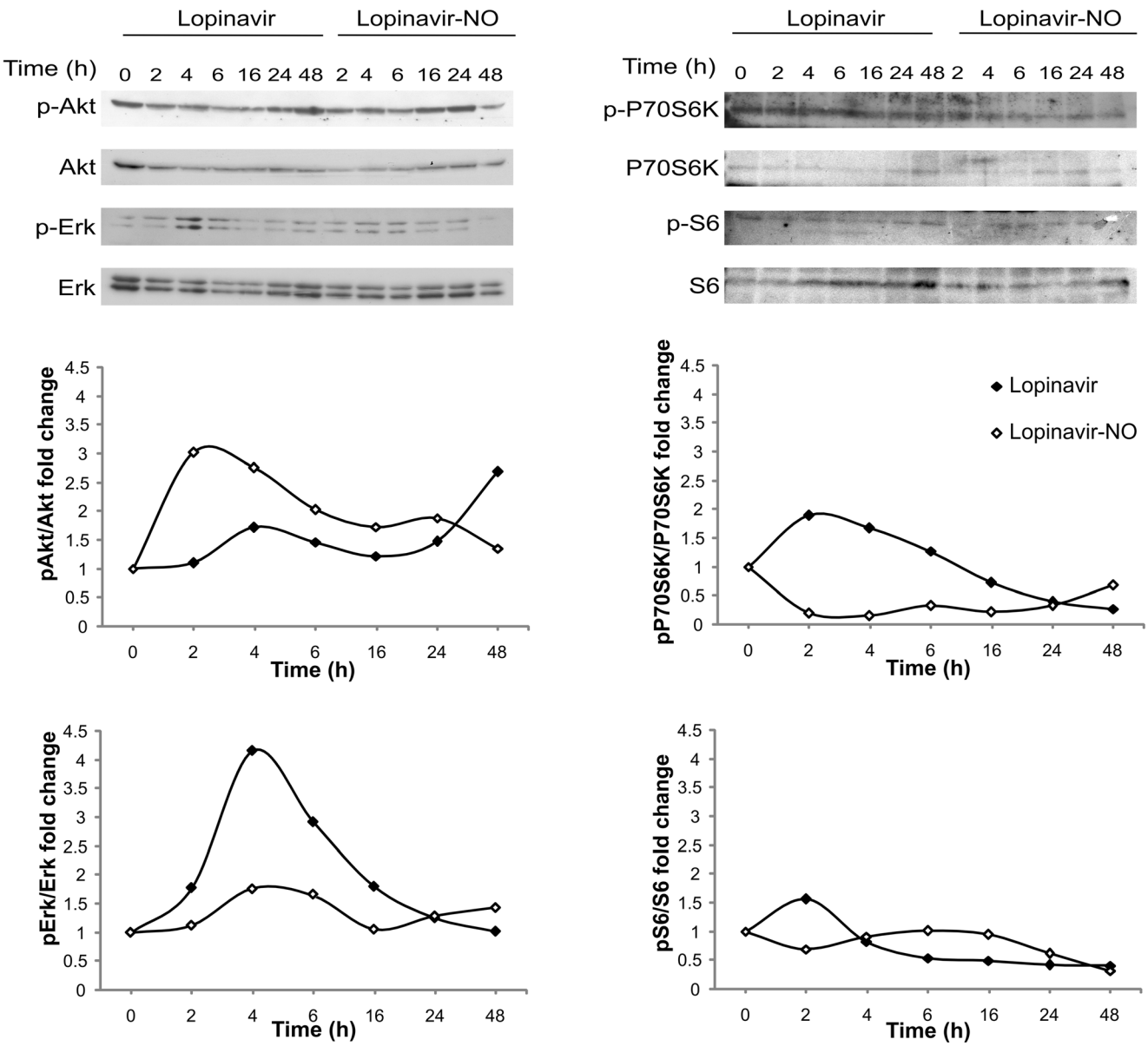


Fig. 6 Lopinavir-NO treatment activates Akt and inhibits P70S6K. B16 melanoma cells were treated with 12 μM lopinavir and 8 μM lopinavir-NO for the indicated durations, and protein expression was analysed by western blotting. The results of the densitometric analysis of the data are presented as the fold change relative to the control. The results of one representative experiment of three experiments are shown

this effect has been shown in a wide range of cancer types [42, 50, 51]. However, here, the effect of lopinavir and lopinavir-NO on melanoma cells was cytostatic rather than cytotoxic, revealing a different anticancer mechanism. These findings correlate with those in a study of lopinavir-treated meningioma [43]. Moreover, recent data confirmed that metastatic melanoma frequently relapses after cytotoxic therapy since tumour repopulation might occur as a response to the induction of cell death [49]. Thus, the ability of lopinavir and lopinavir-NO to inhibit cell division but induce only a minor amount of cell death might overcome a serious disadvantage of cytotoxicy-based strategies in tumour treatment.

Caspase activation and autophagy were detected only in the cell line derived from solid tumours, not the cell line derived from metastatic tumours. This observation indicates another difference in the characteristics of metastatic and primary tumour cells, offering an explanation for the different responses of developed metastases compared to those of primary tumours [52, 53]. The B16F10 cell line originates from the B16 cell line but is highly metastatic and possesses a different set of activated genes, proteins, and signalling pathways, resulting in a different therapeutic response [54, 55].

The antitumour effects of lopinavir and lopinavir-NO caused by proliferation inhibition were followed by trans-differentiation. Certain HIV- PIs have been shown to induce cell

differentiation to oligodendrocytes and Schwann-like cells [18]. In response to either lopinavir or lopinavir-NO, B16 and B16F10 cells acquired flattened morphologies with long, tiny dendrite-like structures. The detailed analysis excluded the acquisition of the characteristics of mature melanocytes. Instead, trans-differentiation into a Schwann cell-like phenotype [39] was confirmed by the elevated expression of the Schwann cell marker MBP. These findings suggest that treatment induced melanoma cells to differentiate into a glial phenotype [40, 56]. MBP expression was more intense in the less-aggressive subclone of mouse melanoma cells and was clearly independent of NO. A possible explanation for the stronger differentiation in cultured B16 cells could be the increase in caspase activation upon treatment. Specifically, ApoStat detects all activated caspases whose activation can be attributed to the differentiation process in an environment with minimal apoptosis. The role of caspases is complex and connected with processes that further influence the fate of cells in the surrounding tumour, such as phenotypic transformation, apoptosis/autophagy interplay and intercellular communication [49, 57]. Forcing melanoma cell trans-differentiation into a neuronal phenotype due to the depletion of cancer stem cells can be promising in cancer therapy [58].

The NO-donating ability of lopinavir-NO provides an additional mechanism for its antitumour activity. This compound releases higher amounts of NO than the exogenous NO donors SNAP and SIN at equivalent doses. The role of intracellular NO was proven by co-treatment with the NO scavenger carboxy-PTIO, which restored cell viability, confirming the crucial role of released NO in mediating drug-mediated cytotoxicity. Considering that NO is a short-lived and highly reactive molecule, the benefit of using lopinavir-NO as a chemotherapeutic agent that selectively releases NO intracellularly is clear. The main mechanisms by which NO induces the inhibition of cell proliferation are the activation of soluble guanylyl cyclase (sGC) [59, 60] and the inhibition of cyclin-dependent kinase (CDK) [61, 62]. Moreover, NO reacts with superoxide radicals to form peroxynitrites. Both NO and peroxynitrites can alter cell signalling pathways important for the regulation of proliferation, differentiation, and cell death [25, 63, 64].

One of the major limitations of the clinical use of NO-donating compounds is their spontaneous NO release, which impedes NO from reaching the intracellular target. Therefore, we refer to lopinavir-NO, which releases NO only in contact with cells, as a "smart NO-donating compound". In contrast, saquinavir-NO could release only trivial amounts of NO both extracellularly and intracellularly, so its ability to affect tumour proliferation and alter cell phenotype appeared to be minimally, if at all, dependent on NO. Independent of the ability of HIV-PIs to release NO upon NO linkage, this applied intervention markedly improved their antitumour potential [34].

Additionally, and by means unrelated to NO, the antitumour potential of HIV-PIs can be connected to the induction of oxidative stress. Lopinavir in combination with other protease inhibitors such as carfilzomib promoted apoptotic cell death at least partly by upregulating ROS production [44]. In this study, oxidative stress followed treatment with both compounds, but its abrogation did not affect their efficacy, suggesting that the antitumour activity of lopinavir-NO is not explicitly related to these reactive species in this experimental setting.

HIV-PIs interfere with the PI3K/Akt signalling pathway [65, 66]. Nelfinavir, lopinavir, ritonavir, and saquinavir inhibit Akt activity via a mechanism independent of proteasome inhibition [67]. Here, we found that treatment with lopinavir-NO induced only transient activation of Akt. In addition, 3-methyladenine (3-MA) treatment did not affect the antitumour efficacy of lopinavir-NO, implying that the suppressive effects of lopinavir-NO on malignant cell proliferation occur via an Akt-independent mechanism [43, 68]. In addition, lopinavir-NO suppressed P70S6 kinase activity without the consequent inhibition of S6 protein activy, suggesting that S6 protein activation is not regulated solely by P70S6 kinase. Hence, lopinavir-NO-mediated signalling alterations differ from those of saquinavir-NO in terms of S6 protein activation [19]. In parallel, Erk was strongly activated only with lopinavir treatment, demonstrating an inverse activation pattern of the PI3K/Akt and MAPK pathways in cells treated with lopinavir-NO and its parental drug.

Finally, our in vivo study indicated the growth inhibition of tumours induced by B16 melanoma cell implantation in C57BL/6 mice upon treatment with lopinavir-NO. Treatment with the original drug was not efficient; thus, the NO modification was responsible for the observed in vivo effect. Taken together, the data presented here will aid in understanding the molecular mechanisms underlying the antitumour activity of HIV-PIs. Lopinavir-NO is a new, improved HIV-PI and a promising candidate for future clinical studies. In particular, these data are of immediate translational relevance for the use of lopinavir-NO in melanoma treatment. Major therapeutic advancements that have greatly improved the course of metastatic melanoma have been made. Small molecules targeting BRAF and MEK inhibitors that block the MAPK pathway in melanoma patients with BRAF V600 mutations and monoclonal antibodies against the immune checkpoint mediators cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) have been found to be beneficial. However, the major limitations of these treatments persist, since as many as 50% of patients achieve limited benefits from the standard-of-care treatments.

In agreement with its previously described dual role in oncogenesis, the role of NO in melanoma is controversial;

most data suggest a pro-oncogenic role for endogenous NO in melanoma and other malignancies, indicating the protective role of this molecule. As in other forms of cancer, the pro- or anti-oncogenic action of NO in melanoma depends on several factors, including the concentration and site of action of NO. For example, nitrite levels have been correlated with the worst prognosis in melanoma patients, and NO has been shown to impair the antigen-presenting function of dendritic cells and to favour the activity of myeloid-derived suppressor cells [2]. However, other data have indicated that NO confers therapeutic activity to dendritic cells in a mouse model of melanoma and boosts the chemotherapeutic action of cisplatin [3, 4]. In addition, in vivo treatment with NO donors increases the efficiency of cytostatic therapy and slows the development of drug resistance [5].

Consistent with the demonstrated lack of pro-oncogenic effects of exogenous NO on melanoma cell growth in vivo, we showed that in vivo treatment with the NO donor DETA NONOate did not influence the in vivo growth of A375 melanoma cells [69] and that the intracellular NO donor GIT-27NO reduces the syngeneic growth of B16 cells in vivo [31].

Of particular relevance for the treatment of metastatic melanomas that metastasize most frequently to the brain, NO donors have been demonstrated to increase the efficiency of cytostatic therapy and slow the development of drug resistance. Indeed, the metastasis inhibition index of B16 melanoma with cyclophosphamide monotherapy increased from 50% to over 80% upon the addition of an NO donor. Potentiating effects of NO donors in combination with cytostatic drugs were also observed in intracerebral leukaemia P388 cell transplantation. Comparative studies of an NO donor (organic nitrate) and a similar compound in which ONO(2) moieties were replaced by OH groups demonstrated that the presence of NO(2) is required for the adjuvant activity of compounds and confirmed that NO modifies the antitumour effects of cytostatics. In addition, NO donors have been shown to slow the development of cyclophosphamide resistance [70].

In summary, on the basis of these data, we propose that lopinavir-NO may represent a first-in-class compound endowed with a pleiotropic chemotherapeutic mechanism of action that entails the simultaneous capacity to induce transdifferentiation and donate NO into the tumour. We believe that these data warrant additional studies aimed at evaluating the possible use of lopinavir-NO in combination with immune checkpoint inhibitors and small molecules used for the treatment of melanoma [28, 70–73].

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Compliance with ethical standards

Conflicts of interest Svetlana Paskas declares that she has no conflicts of interest. Emanuela Mazzon declares that she has no conflicts of interest. Maria Sofia Basile declares that she has no conflicts of interest. Eugenio Cavalli declares that he has no conflicts of interest. Yousef Al-Abed is a cofounder and shareholder of OncoNOx, which has outlicensed lopinavir-NO to Inflamalps.

Mingzhu He declares that she has no conflicts of interest. Sara Rakocevic declares that she has no conflicts of interest. Ferdinando Nicoletti is a cofounder and shareholder of OncoNOx, which has outlicensed lopinavir-NO to Inflamalps. Sanja Mijatovic declares that she has no conflicts of interest. Danijela Maksimovic-Ivanic declares that she has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal studies were performed in accordance with local guidelines and approved by the local Institutional Animal Care and Use Committee (IACUC), approval nr. 01–08/17.

Informed consent For this type of study, formal consent is not required.

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