

***In vitro* anti-neuroblastoma activity of saquinavir and its association with imatinib**

FABIO TIMEUS¹, NICOLETTA CRESCENZIO², ALESSANDRA DORIA², LUISELDA FOGLIA²,
SARA PAGLIANO², EMANUELA RICOTTI², FRANCA FAGIOLI¹, PIER ANGELO TOVO²
and LUCA CORDERO DI MONTEZEMOLO²

¹Paediatric Haematology-Oncology, Regina Margherita Children's Hospital; ²Department of Paediatrics, University of Turin, Regina Margherita Children's Hospital, Piazza Polonia 94, I-10126 Turin, Italy

Received September 28, 2011; Accepted October 27, 2011

DOI: 10.3892/or.2011.1582

Abstract. Neuroblastoma (NB) has a poor prognosis when in advanced stages, highlighting the need for new therapeutic options. The human immunodeficiency virus (HIV) protease inhibitor saquinavir is active *in vitro* against chronic myeloid leukaemia cells, in synergy with the tyrosine kinase inhibitor imatinib. Here, we evaluated the effects of saquinavir, alone or in association with imatinib, on cell proliferation (count of viable cells after trypan blue exclusion), apoptosis (Annexin V binding) and invasion (through a transwell membrane coated with Matrigel) in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE NB lines, all expressing c-kit and PDGF-R (determined by flow cytometry). Saquinavir showed a dose-dependent anti-proliferative and anti-invasive activity on NB lines, increased by the association with imatinib when the two drugs were utilized at clinically attainable concentrations. The same low saquinavir concentrations inhibited in NB cells the nuclear activation of NF- κ B (Western immunoblotting for nuclear NF- κ B p50 and p65). Saquinavir at high concentrations also exerted a pro-apoptotic activity on NB lines, significantly increased by the association with imatinib. In conclusion, saquinavir and imatinib are both drugs utilized for long-term therapies, with good oral bioavailability and a well-known toxicity profile. The anti-NB activity of saquinavir and of its association with imatinib suggests a potential usefulness in the treatment of NB, particularly for remission maintenance.

Introduction

Human immunodeficiency virus (HIV) protease inhibitors (PIs) are widely utilized antiretroviral drugs (1) which also show anti-cancer activity. Since the initial observation that they promote regression of Kaposi sarcoma by inhibiting angiogenesis (2), their direct antitumour effects have been documented on various tumour cell lines, such as human prostate carcinoma, glioblastoma, leukaemia, non-small cell lung cancer and melanoma (3-6). This antitumour activity has been related to impaired proteasome function, inhibition of cyclin-dependent kinase (CDK) 2 and a decrease in growth factor-induced or endogenous Akt-driven signalling (6-10). In other experimental models, PIs at therapeutic dosages have been shown to affect the proteolytic activity of purified human proteasomes and to prevent NF- κ B activation (11). According to the above reports, we previously demonstrated that the PI saquinavir exerts inhibitory and pro-apoptotic activity on chronic myeloid leukaemia (CML) cells, in synergy with imatinib, with a stronger effect on the imatinib-resistant CML cell lines (12). These saquinavir-mediated effects were associated with a reduced NF- κ B nuclear translocation. Imatinib, also defined as STI571, was initially described to inhibit the *in vitro* growth of Bcr-Abl-positive CML cells through selective inhibition of the Abl tyrosine kinase, and then proved to exert inhibitory activity on tyrosine kinase receptors such as c-kit and platelet-derived growth factor receptor (PDGF-R) (13).

Neuroblastoma (NB) is the third most common paediatric cancer. It is an embryonic cancer derived from the peripheral sympathetic nervous system with a heterogeneous clinical course ranging from spontaneous regression to very aggressive forms. In advanced disease stages, NB has, in spite of aggressive multimodal therapy (14), a poor prognosis, with a long-term survival of <40% in high risk patients, thus highlighting the need for new therapeutic options (15-19). A number of observations indicate that c-kit, PDGFR and their ligands play a substantial role in the survival and proliferation of NB cells (20-24). Imatinib is active against NB cells *in vitro* and in xenografts (24-27), but it shows little or no activity as a single agent in children with relapsed or refractory NB (28).

Correspondence to: Dr Fabio Timeus, Paediatric Haematology-Oncology, Regina Margherita Children's Hospital, Piazza Polonia 94, I-10126 Turin, Italy
E-mail: fabio.timeus@unito.it

Abbreviations: CML, chronic myeloid leukaemia; NB, neuroblastoma

Key words: saquinavir, imatinib, HIV protease inhibitors, neuroblastoma cell lines

The aim of the present study was to investigate the effects of saquinavir alone or in combination with imatinib in NB cell lines, in order to evaluate a possible additive antitumor activity of the two drugs.

Materials and methods

Saquinavir and imatinib stock solutions. Saquinavir (Roche, UK) and Imatinib (STI571; Novartis Pharmaceuticals, Basel, Switzerland), were solubilized in 100% dimethyl sulfoxide (DMSO) at final concentrations of 40 and 1 mM, respectively, and used as the stock solution for all experiments.

Cell cultures. The NB cell lines SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE (29-31) were maintained in monolayer cultures in RPMI-1640 medium supplemented with 10 or 1% fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin at 37°C in a 5% CO₂ humidified atmosphere.

Flow cytometry for PDGF-R β . SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cells (5x10⁵) were incubated with an anti-PDGF-R β chain monoclonal antibody (R&D Systems, Minneapolis MN, USA) for 20 min at 4°C. After washing, the cells were incubated with PE-conjugated goat anti-mouse secondary antibody (BD Pharmingen, San Diego, CA, USA) for 30 min and then analyzed by a BD FACSCanto (BD Pharmingen). The osteosarcoma cell line SJSA-1 (obtained from the American Type Culture Collection) was utilized as a positive control.

Cell proliferation assays. The exponentially growing SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cell lines were cultured in the presence or absence of saquinavir from 0.1 up to 40 µM, imatinib from 0.001 to 50 µM or the association of saquinavir 5 µM with increasing imatinib concentrations for 24, 48 or 72 h. Following exposure to the drugs or DMSO (control), the cells were trypsinized, harvested and stained with trypan blue for their viability evaluation. Cellular proliferation was determined by quantifying viable cells manually using a haemocytometer. Inhibiting concentrations of 50% (IC₅₀) were calculated by nonlinear regression analysis using the SPSS 11.5 software package (SPSS, Inc., Chicago, IL, USA). Experiments were performed in triplicate.

Because there is a reported interference of imatinib with the most commonly utilized MTT test which can lead to erroneous results (32), this technique was avoided.

Apoptosis assays. Following 24, 48 and 72 h exposure of SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cells to various concentrations (Fig. 3) of saquinavir, imatinib, imatinib plus saquinavir 5 µM or DMSO (control) in the presence of 1 or 10% FCS, the cells were trypsinized and harvested. Apoptosis was evaluated by two colour-flow cytometry, using Annexin V-FITC conjugates (apoptosis detection kit, R&D Systems), according to the manufacturer's instructions, with an EPICS XL2 Coulter flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). This method evaluates cells in early (Annexin V⁺/propidium iodide⁻) and late apoptosis (Annexin V⁺/propidium iodide⁺). Experiments were performed in triplicate.

Invasion assays. Cell migration was evaluated in SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cell lines using 24-well transwell plates (6.5-mm diameter) with an 8-µm pore size polycarbonate membrane (Corning Costar, MA, USA) coated with Matrigel (BD Pharmingen) 1.25 µg/µl. The lower compartment was filled with 600 µl of IMDM containing 10% FCS as a chemoattractant. NB cells (2x10⁴) resuspended in 100 µl of IMDM containing 1% FCS in the presence of saquinavir 5, 10, 20 µM, imatinib 0.01 µM alone or imatinib 0.01 µM plus saquinavir 5 µM were added into the upper well and incubated at 37°C. After 24 h incubation, the cells on the upper side of the membrane were removed with a cotton swab and the cells on the lower side of the membrane were fixed and stained with crystal violet. The cells on the entire lower surface of the membrane, visualized with an inverted microscope, were counted in 5 fields under a x400 magnification. The experiments were carried out in triplicate.

Preparation of nuclear extracts. NB cells treated with or without 5 µM saquinavir for 16 h were stimulated with PMA (phorbol 11-myristate-12-acetate, Sigma-Aldrich, St. Louis, MO, USA) 10 ng/ml and calcium ionophore (Sigma-Aldrich) 500 ng/ml for 15 min. After incubation, cells were washed twice with ice-cold phosphate-buffered saline. Pellets were resuspended in buffer A (HEPES 10 mM pH 7.9, KCl 10 mM, PMSF 1 mM, DTT 1 mM) and cells were homogenized using insulin syringes. After 45 min incubation at 4°C samples were centrifuged at 12,000 rpm for 10 min. Nuclei were extracted in buffer C (HEPES 20 mM pH 7.8, MgCl₂ 1.5 mM, NaCl 420 mM, EDTA 0.2 mM, PMSF 1 mM, DTT 1 mM, and 25% glycerol) for 30 min on ice, then cleared by centrifugation (12,000 rpm for 30 min at 4°C). Protein concentration was determined by the Micro BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA), following the manufacturer's instructions.

Western blot analysis. Samples containing equal amounts of protein (20 µg) were resolved on 11% SDS-polyacrylamide gels (SDS-PAGE). Resolved proteins were electrotransferred onto PVDF membrane, blocked overnight with 5% w/v BSA (Sigma-Aldrich) in TBS (Tris 20 mM pH 7.6, NaCl 140 mM) containing 0.1% Tween-20 (blocking solution) and probed with primary antibodies anti-p65 and anti-p50 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Immunoreactive bands were detected by ECL. Blots were stripped (glycine 200 mM, SDS 0.1%, Tween 1% pH 2.2) and reprobed with anti SP-1 (Santa Cruz Biotechnology) and detected by ECL. Relative band intensities were quantified using Quantity one image analysis software (Bio-Rad, CA, USA).

Statistical analysis. Drug concentrations causing a 50% inhibition of cellular growth (IC₅₀) were calculated by nonlinear regression analysis using the SPSS 11.5 software package (SPSS, Inc.). Student's t-tests were used to analyse the drug effects on cell proliferation, apoptosis and migration. P-values <0.05 were considered statistically significant.

Results

NB cell lines express PDGF-R β . Flow cytometry demonstrated in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines the

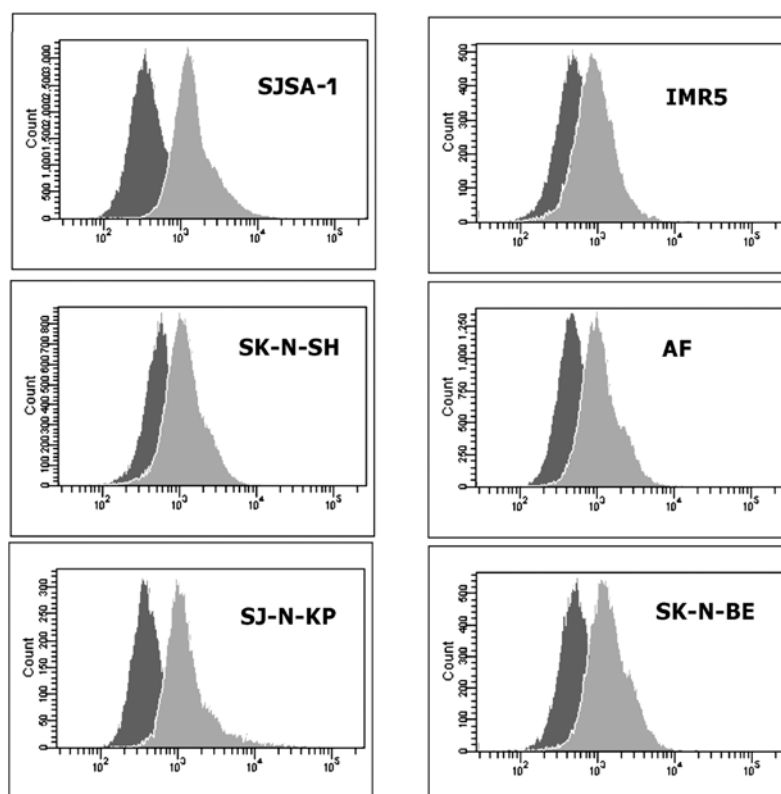


Figure 1. SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE neuroblastoma cell lines express membrane PDGF-R β . Flow cytometric analysis was performed by an anti-PDGF-R β chain monoclonal antibody on cells cultured in RPMI-1640 plus 10% FCS. The osteosarcoma cell line SJSA-1 was utilised as a positive control.

Table I. IC₅₀ of saquinavir and imatinib in NB cell lines.

	24 h			48 h			72 h		
	Saq	Im	Saq + Im	Saq	Im	Saq + Im	Saq	Im	Saq + Im
SJ-N-KP	6.96	2.01	0.02	3.17	2.70	0.27	3.80	1.85	0.34
IMR5	6.91	4.01	0.07	3.96	2.14	0.26	3.62	1.36	0.29
AF-8	6.56	4.54	0.63	3.72	1.07	0.08	4.44	0.99	0.08
SK-N-SH	6.54	1.79	0.10	3.33	0.63	0.02	2.49	0.76	0.02
SK-N-BE	4.78	2.63	0.41	4.73	1.27	0.18	3.67	0.98	0.03

IC₅₀ (expressed in μ M, calculated by non-linear regression analysis) of saquinavir (Saq), imatinib alone (Im) and imatinib in association with saquinavir 5 μ M (Im + Saq) in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines.

expression of membrane PDGF-R β at comparable levels (Fig. 1). The expression of c-kit in the same lines has been previously described by our group (22). PDGF-R β and c-kit expression provide the rationale for the use of imatinib in our study.

Saquinavir alone and in combination with imatinib inhibits NB cell line proliferation. Proliferation assays were carried out on SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines exposed for 24, 48 or 72 h to saquinavir or imatinib or the two drugs in combination. As single agent saquinavir demonstrated a concentration-dependent antiproliferative effect. The association of saquinavir 5 μ M with various concentrations of imatinib caused a significant increase of the imatinib antiproliferative activity (considering all cell lines taken together: saquinavir

5 μ M plus imatinib from 0.001 to 10 μ M vs. imatinib alone at the same concentrations; $P < 0.01$ after 24, 48, 72 h exposure). Proliferation assay results and saquinavir IC₅₀ values are shown in Fig. 2 and Table I).

Saquinavir alone and in combination with imatinib inhibits NB cell invasion through the matrigel matrix. Cell invasion assays showed a significant inhibitory effect of saquinavir at 5, 10 or 20 μ M on cell invasion in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines ($P < 0.0001$ vs. control, RPMI-1640 plus DMSO) (Table II). Imatinib alone at 0.01 μ M also inhibited cell migration. The association of imatinib 0.01 μ M with saquinavir 5 μ M significantly increased the anti-invasive activity of the single compounds (saquinavir 5 μ M

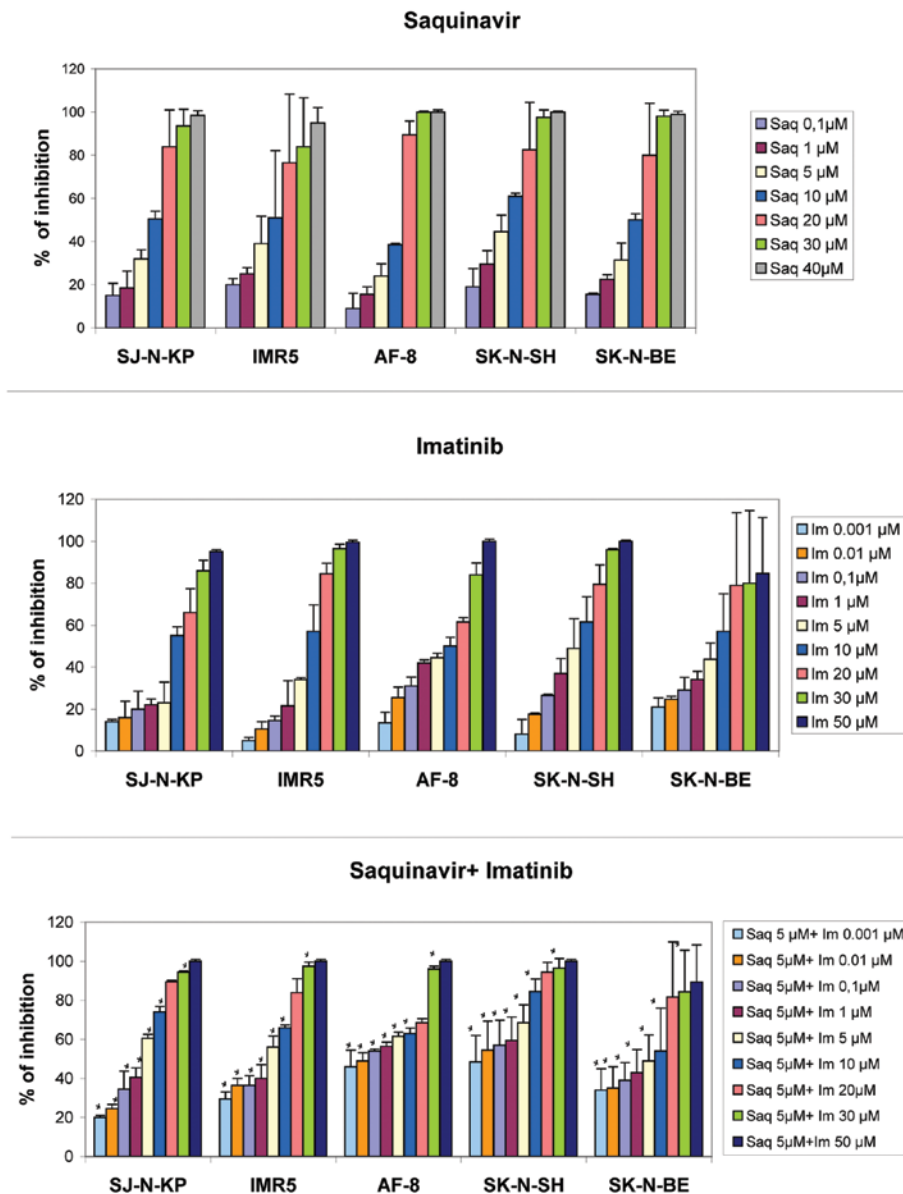


Figure 2. Saquinavir, imatinib and their combination inhibit neuroblastoma SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell line proliferation in a dose-dependent manner. The figure shows the percentage of inhibition of cell proliferation compared to DMSO (drug vehicle) after 72 h exposure to the drugs alone or in combination. Data are the mean \pm SD of three independent experiments and are expressed as % of inhibition in treated cells. Statistical significance of the combination of saquinavir 5 μ M with various concentrations of imatinib compared to imatinib alone, * $P < 0.01$.

Table II. Inhibition of NB cell invasion in the presence of saquinavir and imatinib.

	SJ-N-KP (%)	IMR5 (%)	AF-8 (%)	SK-N-SH (%)	SK-N-BE (%)
5 μ M Saq	39 \pm 8.5	48 \pm 4.9	35 \pm 12.0	33 \pm 7.1	33 \pm 9.2
10 μ M Saq	46 \pm 9.9	59 \pm 6.4	55 \pm 0.2	46 \pm 0.5	55 \pm 11.3
20 μ M Saq	67 \pm 4.9	72 \pm 15.5	71 \pm 7.1	69 \pm 12.7	75 \pm 8.5
0.01 μ M Im	54 \pm 9.9	52 \pm 8.5	35 \pm 1.0	45 \pm 7.1	31 \pm 16
0.01 μ M Im + 5 μ M Saq	67 \pm 9.2	66 \pm 11.3	63 \pm 12.0	56 \pm 12.7	65 \pm 3.5

Evaluation of cell invasion through transwell plates with 8- μ m pore size polycarbonate membrane in SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cell lines. Data are expressed as percentages of inhibition of migration compared to the control after 24 h-exposure to saquinavir alone at various concentrations, imatinib 0.01 μ M or saquinavir 5 μ M plus imatinib 0.01 μ M. Values are the mean \pm standard deviation of three independent experiments. The association of imatinib 0.01 μ M with saquinavir 5 μ M significantly increased the anti-migratory activity of saquinavir 5 μ M alone ($P < 0.0001$). Saq, saquinavir; Im, imatinib.

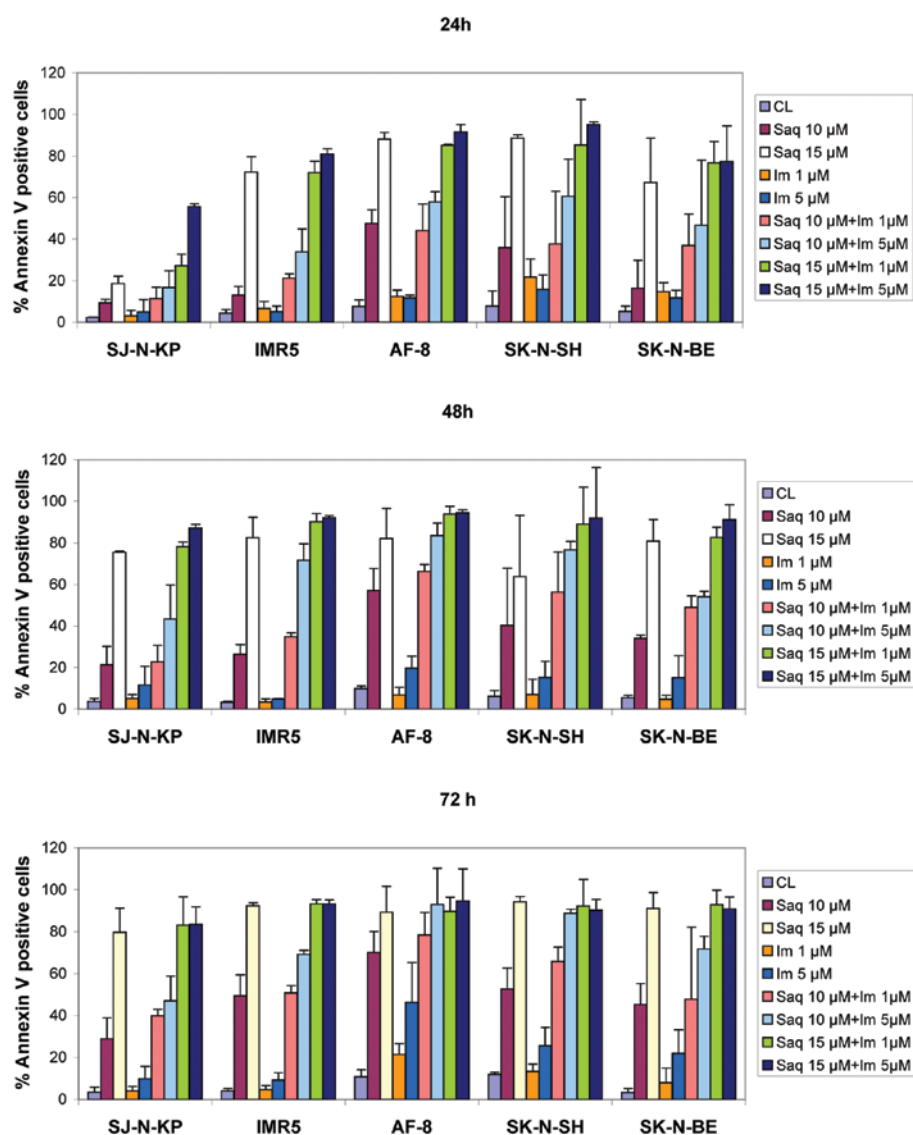


Figure 3. Saquinavir exerts a pro-apoptotic activity on neuroblastoma SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines in a dose-dependent manner. The figure shows the percentage of apoptotic cells after exposure to saquinavir alone (Saq), imatinib alone (Im) or the combination of various concentrations of saquinavir and imatinib. Cultures were carried out for 24, 48, 72 h in the presence of 1% FCS. The association with imatinib significantly increased the pro-apoptotic activity of saquinavir (saquinavir 10 μ M in combination with imatinib 5 μ M vs. saquinavir 10 μ M: $P<0.05$ after 24 h, $P<0.001$ after 48 h, $P<0.001$ after 72 h). The data express the mean percentage \pm SD of three independent experiments.

plus imatinib 0.01 μ M vs. saquinavir 5 μ M alone $P<0.001$; saquinavir 5 μ M plus imatinib 0.01 μ M vs. imatinib 0.01 μ M alone $P<0.001$).

Saquinavir exerts a pro-apoptotic activity on NB cell lines. In cells cultured in the presence of 10% FCS, a significant saquinavir pro-apoptotic activity was observed only at concentrations of 20 μ M or higher (data not shown). In SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cells cultured in the presence of 1% FCS, saquinavir exhibited a significant concentration and time-dependent pro-apoptotic activity also at lower concentrations (10 and 15 μ M; $P<0.01$) (Fig. 3). We did not observe a significant difference in the percentage of apoptotic cells between the control experiments carried-out in the presence of 1% FCS and the control experiments carried-out in the presence of 10% FCS (data not shown), ruling out a possible pro-apoptotic effect of low concentrations of FCS. Imatinib at concentrations of 1 and 5 μ M caused a negligible

pro-apoptotic effect on the SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cell lines, whereas the association with imatinib significantly increased the pro-apoptotic activity of saquinavir (saquinavir 10 μ M in combination with imatinib 5 μ M vs. saquinavir 10 μ M: $P<0.05$ after 24 h; $P<0.001$ after 48 h; $P<0.001$ after 72 h).

Saquinavir inhibits nuclear translocation of NF- κ B in NB cells. Western blotting experiments demonstrated a significant reduction of nuclear p65 and p50 in PMA-stimulated NB cells after exposure to saquinavir 5 μ M. Fig. 4 shows a representative analysis in the SJ-N-KP cell line.

Discussion

The direct anti-cancer activity of some HIV PIs has been previously described (2-6). In the present study a significant dose-dependent anti-proliferative effect of saquinavir was observed in

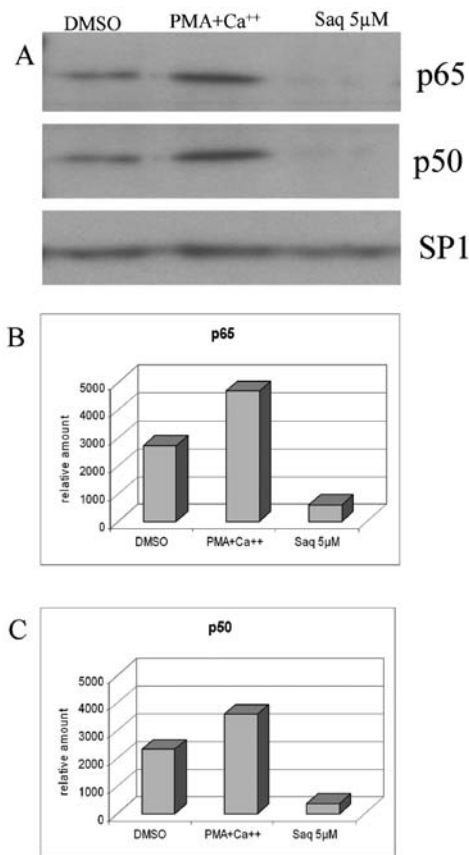


Figure 4. Saquinavir 5 μ M inhibits nuclear translocation of NF- κ B p50 and p65 in SJ-N-KP cell line. NB cells treated with or without 5 μ M saquinavir for 16 h were stimulated with PMA (phorbol 11-myristate-12-acetate) 10 ng/ml and calcium ionophore 500 ng/ml for 15 min. (A) Immunoblot of nuclear extracts of cells exposed to drug vehicle (DMSO), PMA plus calcium ionophore (PMA + Ca⁺⁺), PMA plus calcium ionophore and saquinavir 5 μ M (Saq, 5 μ M). Immunoreactive bands were reprobbed with anti SP-1. (B and C) Bar graphs showing p65 and p50 band intensity quantification (performed by Quantity One image analysis software; Bio-Rad, CA, USA).

NB cell lines. It is worth noting that the IC₅₀ determined in the present study was about 1 log lower than in the leukaemic K562 line (12).

Imatinib, a selective inhibitor of the tyrosine kinase activity of c-kit and PDGF-R, is active *in vitro* against NB cell lines (24-27). However, in spite of the pivotal role played by PDGF-R and c-kit in promoting cell proliferation and migration and in inhibiting apoptosis in NB cell lines (20-24), imatinib has shown little or no activity as a single agent in children with relapsed or refractory NB (28). The observation that imatinib is more effective in preventing the cell proliferation of CML lines when in combination with saquinavir (12) prompted us to investigate the effects of the association of saquinavir plus imatinib on the five NB cell lines, all expressing c-kit and PDGF-R. In the present study the association of saquinavir and imatinib showed an additive anti-proliferative activity at clinically attainable concentrations (33,34) of both compounds.

Saquinavir also exerted an anti-invasive activity on NB cells. This is in line with Sgadari *et al* (2) that PIs block Kaposi sarcoma cell invasion. Vitali *et al* (35) previously described an anti-migratory activity of imatinib in NB. Here, we demonstrated a significant increase of the anti-invasive activity of imatinib when associated with saquinavir at therapeutic

concentrations, suggesting an anti-metastatic effect that might have a significant impact on the outcome of NB patients.

In the presence of 10% FCS, saquinavir exerted a significant pro-apoptotic activity on NB cell lines at concentrations of 20 μ M or higher. However, in the cultures carried out in the presence of 1% FCS, saquinavir exhibited a significant pro-apoptotic activity at lower and clinically attainable concentrations, while low concentrations of FCS had not *per se* a pro-apoptotic activity as shown in control experiments. This might be explained by an anti-apoptotic activity of high concentrations of serum, as demonstrated in other experimental models (36). Imatinib alone had a negligible pro-apoptotic effect on NB cell lines, whereas the association with imatinib significantly increased the pro-apoptotic activity of saquinavir.

Altogether, our data suggest that, in NB cell lines, the anti-proliferative and anti-invasive activity of saquinavir, observed in the presence of 10% FCS, is prominent compared to its pro-apoptotic activity. Since some NB cell lines in the study were reported to be MYCN-unamplified/low-expressed (37) and some MYCN-amplified/overexpressed (IMR5) (38,39), the biological effects of saquinavir and of its association with imatinib on NB cell lines seem independent from MYCN amplification.

Pajonk *et al* (6) evidenced that saquinavir inhibits NF- κ B activation in prostate carcinoma cells. Here we show that low saquinavir concentrations inhibit NF- κ B p50 and p65 activation in NB cells. The ubiquitin-proteasome pathway plays a key role in the activation of NF- κ B and in the degradation of its inhibitor I κ -B. HIV PIs at therapeutic concentrations block proteasome activities (11) and thus may impair a large array of biological activities, such as cell-cycle progression, angiogenesis, and activation of transcriptional factors. The inhibitory effects of saquinavir on proteasome functions and NF- κ B activation may account, at least partially, for its antitumoral properties.

In conclusion, saquinavir is a drug utilized in the long-term treatment of HIV seropositive patients, with good oral bioavailability and a well known toxicity profile. Its anti-proliferative and anti-invasive effects on NB cell lines at therapeutic concentrations suggest a potential usefulness for the treatment of NB, particularly for remission maintenance. The additive activity of low concentrations of saquinavir and imatinib seems of particular interest. Targeted animal studies are warranted.

Acknowledgements

This study was partially supported by a grant from the Unione Genitori Italiani contro il tumore dei bambini (UGI). We thank Mr. Andrew Martin Garvey for editorial assistance.

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