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

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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-κB (Western immunoblotting for nuclear NF-κB 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-κB 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-κB 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 Abi 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).
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-Rβ. SJ-N-KP, IMR5, AF-8, SK-N-SH, SK-N-BE cells (5x10⁵) were incubated with an anti-PDGF-Rβ 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 FACS Canto (BD Pharmingen). The osteosarcoma cell line SJS-A1 (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.). Student’s t-tests were used to analyse the drug effects on cell proliferation, apoptosis and migration.

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-Rβ. Flow cytometry demonstrated in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines the
expression of membrane PDGF-Rβ at comparable levels (Fig. 1). The expression of c-kit in the same lines has been previously described by our group (22). PDGF-Rβ 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 µM with various concentrations of imatinib caused a significant increase of the imatinib antiproliferative activity (considering all cell lines taken together: saquinavir 5 µM plus imatinib from 0.001 to 10 µM 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 µM 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 µM also inhibited cell migration. The association of imatinib 0.01 µM with saquinavir 5 µM significantly increased the anti-invasive activity of the single compounds (saquinavir 5 µM

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

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
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<th>48 h</th>
<th></th>
<th>72 h</th>
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<tbody>
<tr>
<td></td>
<td>Saq</td>
<td>Im</td>
<td>Saq + Im</td>
<td>Saq</td>
<td>Im</td>
<td>Saq + Im</td>
</tr>
<tr>
<td>SJ-N-KP</td>
<td>6.96</td>
<td>2.01</td>
<td>0.02</td>
<td>3.17</td>
<td>2.70</td>
<td>0.27</td>
</tr>
<tr>
<td>IMR5</td>
<td>6.91</td>
<td>4.01</td>
<td>0.07</td>
<td>3.96</td>
<td>2.14</td>
<td>0.26</td>
</tr>
<tr>
<td>AF-8</td>
<td>6.56</td>
<td>4.54</td>
<td>0.63</td>
<td>3.72</td>
<td>1.07</td>
<td>0.08</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>6.54</td>
<td>1.79</td>
<td>0.10</td>
<td>3.33</td>
<td>0.63</td>
<td>0.02</td>
</tr>
<tr>
<td>SK-N-BE</td>
<td>4.78</td>
<td>2.63</td>
<td>0.41</td>
<td>4.73</td>
<td>1.27</td>
<td>0.18</td>
</tr>
</tbody>
</table>

IC50 (expressed in µM, calculated by non-linear regression analysis) of saquinavir (Saq), imatinib alone (Im) and imatinib in association with saquinavir 5 µM (Im + Saq) in SJ-N-KP, IMR5, AF-8, SK-N-SH and SK-N-BE cell lines.
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 ± SD of three independent experiments and are expressed as % of inhibition in treated cells. Statistical significance of the combination of saquinavir 5 µM 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.

<table>
<thead>
<tr>
<th></th>
<th>SJ-N-KP (%)</th>
<th>IMR5 (%)</th>
<th>AF-8 (%)</th>
<th>SK-N-SH (%)</th>
<th>SK-N-BE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µM Saq</td>
<td>39±8.5</td>
<td>48±4.9</td>
<td>35±12.0</td>
<td>33±7.1</td>
<td>33±9.2</td>
</tr>
<tr>
<td>10 µM Saq</td>
<td>46±9.9</td>
<td>59±6.4</td>
<td>55±0.2</td>
<td>46±0.5</td>
<td>55±11.3</td>
</tr>
<tr>
<td>20 µM Saq</td>
<td>67±4.9</td>
<td>72±15.5</td>
<td>71±7.1</td>
<td>69±12.7</td>
<td>75±8.5</td>
</tr>
<tr>
<td>0.01 µM Im</td>
<td>54±9.9</td>
<td>52±8.5</td>
<td>35±1.0</td>
<td>45±7.1</td>
<td>31±16</td>
</tr>
<tr>
<td>0.01 µM Im + 5 µM Saq</td>
<td>67±9.2</td>
<td>66±11.3</td>
<td>63±12.0</td>
<td>56±12.7</td>
<td>65±3.5</td>
</tr>
</tbody>
</table>

Evaluation of cell invasion through transwell plates with 8-µm 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 µM or saquinavir 5 µM plus imatinib 0.01 µM. Values are the mean ± standard deviation of three independent experiments. The association of imatinib 0.01 µM with saquinavir 5 µM significantly increased the anti-migratory activity of saquinavir 5 µM alone (P<0.0001). Saq, saquinavir; Im, imatinib.
plus imatinib 0.01 µM vs. saquinavir 5 µM alone P<0.001; saquinavir 5 µM plus imatinib 0.01 µM vs. imatinib 0.01 µM 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 µM 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 µM; 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 µM 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 µM in combination with imatinib 5 µM vs. saquinavir 10 µM: P<0.05 after 24 h, P<0.001 after 48 h; P<0.001 after 72 h).

**Saquinavir inhibits nuclear translocation of NF-κB 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 µM. 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
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 µM 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-κB activation in prostate carcinoma cells. Here we show that low saquinavir concentrations inhibit NF-κB p50 and p65 activation in NB cells. The ubiquitin-proteasome pathway plays a key role in the activation of NF-κB and in the degradation of its inhibitor IκB. 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-κB 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.

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References
