Chronic Myeloid Leukemia

The effects of saquinavir on imatinib-resistant chronic myelogenous leukemia cell lines

We evaluated the effect of the human immunodeficiency virus (HIV) protease inhibitor saquinavir on the imatinib-sensitive and imatinib-resistant chronic myelogenous leukemia cell lines. Saquinavir, which is also a proteasome blocker, showed dose- and time-related anti-proliferative activity, particularly on the imatinib-resistant lines and a pro-apoptotic effect. Association with imatinib caused a significant increase of activity.

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The introduction of imatinib to treat chronic myelogenous leukemia (CML) has significantly increased patients' life expectancy.[1,2] However, primary and acquired resistance to imatinib[3,4] indicates the need for new drugs targeting Bcr-Abl downstream pathways for patients with suboptimal response to imatinib in chronic phase and for patients with accelerated or blastic phase. In CML, the fusion protein Bcr-Abl activates the transcription factor NFκB, which is involved in tumorigenesis.[5] NFκB is activated by proteosomes, which also regulate the degradation of its inhibitor Iκ-B. Proteasome blockers are, therefore, optimal candidates for CML treatment. Bortezomib, a proteasome inhibitor used in multiple myeloma, induces apoptosis in Bcr/Abl-positive cell lines.[6] Several human immunodeficiency virus (HIV) protease inhibitors and, in particular, saquinavir, significantly inhibit[7] human proteasomes, NFκB activation and degradation of IκB.[8]

Here, we evaluated the activity of saquinavir on the imatinib-sensitive K562 and on the imatinib-resistant K562-R and KCL22-R CML cell lines (a kind gift from Junia Melo), established by growing parental cells in the presence of increasing concentrations of imatinib and cultured in basal conditions with 0.6 μM and 0.3 μM imatinib, respectively. Although the exact mechanism of resistance of these cell lines is unknown, we excluded the presence of point mutations or gene amplification.

Cell lines were cultured in RPMI 1640 plus 10% fetal calf serum (FCS) and 1% glutamine with increasing concentrations of saquinavir, imatinib alone or imatinib plus saquinavir 5 μM. After 24, 48 and 72 hours, viable cells were counted by trypan blue exclusion. Apoptosis was evaluated by annexin V binding (Apoptosis Detection Kits, R&D Systems), with a EPICS XL2 flow cytometer. All the experiments were carried out in triplicate. Concentrations producing 50% inhibition (IC_{50}) were calculated by the SPSS 11.5 program. As a control, we also evaluated apoptosis of purified cord blood (CB) $CD34^+$ cells (separated with MiniMACS, Miltenyi-Biotech) from three CB units, cultured in Iscove's modified Dulbecco's medium (IMDM) plus 10% FCS and exposed to saquinavir 10 and 20 μM.

Saquinavir inhibited proliferation and promoted apoptosis in cell lines in a dose- and time-related manner. Table 1 shows the IC_{50} of saquinavir, imatinib and imatinib plus saquinavir 5 μM. After 72 hours' exposure to saquinavir 10 and 20 μM the percentage (±SD) of apoptotic cells was 13.6±5.3% and 19.3±6.8% in K562 (control without saquinavir 12.8±4.2%), 28.4±7.6% and 70.2±12.1% in K562-R (control 12.7±3.6%), and 6.1±2.7% and 26.5±6.7% in KCL22-R (control 3.6±1.8%). Exposure to saquinavir 10 and 20 μM for 72 hours caused slight apoptosis in purified CB $CD34^+$ cells only at the higher concentration (14.9±7.4% and 21.1±13.1%, respectively, versus 14.6±5.4% in controls).

Table 1A. The 50% Inhibitory concentrations (IC_{50}) of saquinavir and imatinib mesylate in K562, K562-R and KCl22-R cell lines.

	K562	*K562-R*	*KCl22-R*
Saquinavir	43.90	6.0	9.6
Imatinib	0.19	1.13	15.19
Imatinib (+ saquinavir 5μM)	0.06	0.03	0.81

The cell lines were cultured in RPMI 1640 plus 10% FCS and 1% glutamine with or without saquinavir 1, 5, 10, 20, 30, 40 μM and with imatinib mesylate 0.001, 0.01, 0.1, 0.5, 1, 5, 10, 20 μM alone or in combination with saquinavir 5 μM.

Table 1B. Saquinavir inhibits peripheral blood CFU-GM growth also in CML blastic phase. Number of CFU-GM/10^5 low density mononuclear cells.

Saquinavir concentrations	*CML chronic phase at diagnosis*	*CML chronic phase during interferon treatment*	*CML blastic phase M*	*CML blastic phase F*	*CB controls (n=5) (mean±standard error)*
basal	17	10	34	192	56±12.3
10 μM	10 (41.2%)	6 (40%)	26 (24%)	180 (6%)	46±12 (20.4±8.2%)
20 μM	3 (82.4%)	4 (60%)	10 (71%)	139 (28%)	34±8.9 (41.6±4.9%)

CB: cord blood. The percentages of CFU-GM inhibition in comparison with control cultures, performed without saquinavir, are given in brackets.

Electrophoretic mobility shift assay (EMSA)[9] of the K562 and K562-R lines demonstrated higher NFκB transcriptional activity after stimulation in the imatinib-resistant line, inhibited by saquinavir (Figure 1).

Saquinavir activity on colony-forming units, granulocyte-monocyte (CFU-GM) from peripheral blood of four CML patients was also investigated: one 15-year old male in chronic phase at diagnosis, one 45-year old female in chronic phase during interferon treatment, one 60-year old male and one 40-year old female in imatinib-resistant blastic phase. CB CFU-GM (from five units) were utilized as a control. Saquinavir inhibited CFU-GM of CML patients in both chronic and blastic phases (Table 1B). Saquinavir 10 and 20 μM also inhibited control CB CFU-GM (20.4±SE8.2% and 41.6±4.9%, respectively).

In conclusion, saquinavir exerts a dose-related inhibitory and pro-apoptotic effect on CML lines. Our findings are consistent with the observation of Pajonk *et al.*,[10] who described a pro-apoptotic effect of saquinavir on the K562 line without, however, providing information about concentrations or time of exposure. Saquinavir acted on the imatinib-resistant lines at concentrations lower than those needed for the parental line and similar to those achievable and usually well tolerated for prolonged periods in HIV patients. The increased effect of

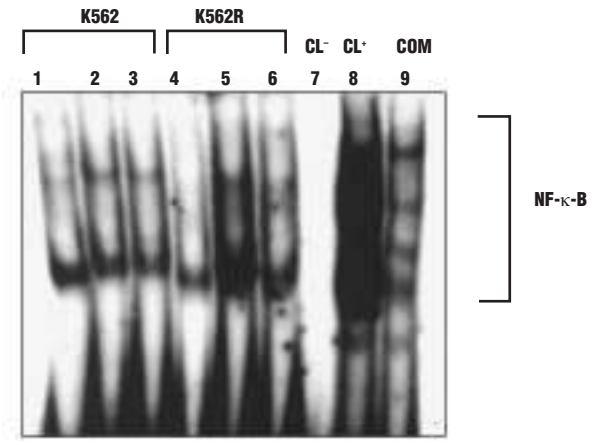

Figure 1. Electrophoretic mobility shift assay (EMSA) in K562 and K562-R cell lines treated or not with 20 μM saquinavir for 16h and stimulated with phorbol 11-myristate-12-acetate (PMA)10 ng/mL and calcium ionophore 500 ng/mL for 15 minutes.Lane 1) K562 control; 2) K562 + PMA + Ca ionophore: after stimulation the ability of NF-κ-B to bind its consensus sequence increases slightly; 3) K562 pre-exposed to Saquinavir 20 μM +PMA and Ca ionophore: preincubation with Saquinavir does not significantly affect NF-κ-B activity; 4) K562-R control; 5) K562-R + PMA + Ca ionophore: the ability of NFκB to bind its consensus sequence increases greatly; 6) K562-R pre-exposed to saquinavir 20 μM +PMA and Ca ionophore: preincubation with saquinavir significantly reduces the signal; 7) negative control (CL -); 8) Hela cells as positive control (CL +) ; 9) competitive (COM).

saquinavir on these resistant lines appears to be related to their higher NFκB transcriptional activity after stimulation. The association of imatinib with saquinavir 5 μM, a relatively low concentration, significantly increased the activity of imatinib on both sensitive and resistant lines. CFU-GM inhibition was not specific to Bcr-Abl positive cells, probably because saquinavir acts on the proteasome, a general cellular pathway. However, saquinavir had a stronger pro-apoptotic effect on CML cells than on normal $CD34^+$ cells. Moreover, hematologic toxicity has seldom been reported in saquinavir-treated HIV patients. Because of its downstream Bcr-Abl activity, saquinavir seems a good candidate for association with specific Bcr-Abl inhibitors such as imatinib. The substantial reduction of the IC_{50} of imatinib, when this drug is associated with saquinavir, supports this hypothesis. Saquinavir's prominent activity on resistant lines further suggests the potential usefulness of this drug in a combined therapeutic approach for CML.

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