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Human immunodeficiency virus protease inhibitors reduce the growth of human tumors *via* a proteasome-independent block of angiogenesis and matrix metalloproteinases

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Human immunodeficiency virus protease inhibitors (HIV-PIs), such as indinavir and saquinavir, have been shown to block angiogenesis and tumor cell invasion and to induce tumor cell apoptosis and growth arrest, respectively, both *in vitro* and *in vivo*. These findings have suggested that HIV-PIs or their analogues can be used as antitumor drugs. To this regard, indinavir and saquinavir were assessed for their ability to inhibit *in vivo* the growth of highly prevalent human tumors, such as lung, breast, colon and hepatic adenocarcinomas. We show here that both HIV-PIs significantly inhibited the growth of all adenocarcinomas tested in the mice model. This was not mediated by effects on proteasome-dependent cell growth arrest or on apoptosis but by the block of angiogenesis and matrix metalloproteinase activity. Accordingly, therapeutic steadystate concentrations of indinavir or saquinavir were highly effective in inhibiting invasion of tumor cells *in vitro*. In contrast, growth arrest was induced only by high concentrations of saquinavir that are not reached or are only transiently present in plasma of treated patients, likely through a proteasome-mediated mechanism. These data suggest that HIV-PIs or their analogues, characterized by a better biodistribution and lower toxicity, may represent a new class of antitumor drugs capable of targeting both matrix metalloproteinases and the proteasome for a most effective antitumor therapy.

The advent of the highly active antiretroviral therapy (HAART) has lead to a reduced incidence and/or regression of human immune deficiency virus (HIV)-associated tumors, particularly Kaposi's sarcoma (KS), non-Hodgkin's lymphomas, cervical cancer,[1–6] and other tumors.[7,8] Although HIV suppression and restoration of the immune response on HAART certainly play a key role in HIV-associated tumors,[9–12] several studies have indicated a lack of correlation of tumor incidence, regression and/or time-to-relapse with either HIV load reduction or CD4 T cell gain.[13–21] Furthermore, patients treated with HAART but failing in CD4 T cell recovery still show a significantly lower risk of non-Hodgkin's lymphomas or a lower rate of cervical intraepithelial neoplasia recurrence compared with CD4-matched patients treated with other antiretroviral (non-HAART) regimens.[17,22] These data have suggested that HAART exerts direct antitumor actions.[7,8,22]

Recent studies have shown that non-nucleoside reverse transcriptase inhibitors and HIV protease inhibitors (HIV-PIs), two classes of antiretroviral drugs included in HAART, exert several actions on cell targets and pathways that can affect tumor growth. In particular, non-nucleoside reverse transcriptase inhibitors inhibit tumor growth and aggressiveness by blocking endogenous reverse transcriptases involved in cell proliferation and differentiation.[23] On the other hand, HIV-PIs block angiogenesis and tumor cell invasion,[24,25] inhibit endothelial and tumor cell growth[26–28] and induce endoplasmic reticulum stress, autophagy and tumor cell apoptosis.[26,28–30] In addition, they modulate inflammation and T

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Abbreviations: bFGF: basic fibroblast growth factor; FBS: fetal bovine serum; HIV: Human immunodeficiency virus; HIV-PIs: HIV protease inhibitors; KS: Kaposi's sarcoma; MMP: matrix metalloproteinase; MMPI: MMP inhibitors
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cell-mediated cytotoxic responses.[28,31–38] These effects of HIV-PIs have been related to a few mechanisms of action that have been observed at different drug concentrations. In particular, at low concentrations, similar to the steady-state (C_{min}) drug plasma level present in HIV-infected patients treated with HAART, HIV-PIs inhibit activation of matrix metalloproteinase (MMP)-2 and production of MMP-7, which are both required for angiogenesis and/or tumor invasion and growth.[7,8,25] In contrast, at high concentrations, similar to or above the peak drug plasma level (C_{max}), HIV-PIs inhibit the proteasome, causing tumor cell growth arrest and apoptosis.[26–28,30,31,35,39] In addition, HIV-PIs perturb cellular signalling pathways, including the signal transducers and activators of transcription (STAT) 3 and the Akt pathways.[29,40–43] These are effects that can also be observed through proteasomal inhibition.[44] Because of these actions, HIV-PIs inhibit the growth of human tumor cell types in mice, including prostate and liver adenocarcinoma, squamous cell carcinoma, T-cell leukemia, KS, thymoma and breast and non-small cell lung carcinoma.[24,26,28,29,42,43,45] Moreover, they increase the effectiveness of radiotherapy (ritonavir and nelfinavir)[44,46–49] or chemotherapy (ritonavir and nelfinavir)[46,47] against several types of tumors by blocking Akt signaling and downregulating HIF-1α and VEGF expression, without, at least in some models, substantially affecting tumor growth.

The testing of different proteasome inhibitors in preclinical studies and the recent success of the proteasome inhibitor Bortezomib in Phases II and III trials against multiple myeloma, identify the proteasome as a promising tumor target.[50,51] On the other hand, the failure of large, controlled clinical trials for treatment of tumors with synthetic MMP inhibitors (MMPI)[52,53] has lead to renewed efforts for the identification of specific MMP, which can be considered as validated tumor targets.[54] In particular, several recent studies indicate that some MMPs can exert protective effects against tumors, thus acting as therapeutic "antitargets" rather then tumor targets.[54] Accordingly, third generation MMPI should be selective against validated MMP targets, which include MMP-1, MMP-2 and MMP-3, but should spare MMP validated antitargets (namely, MMP-3, MMP-8 and MMP-9).[54] Noteworthy, HIV-PIs indinavir and saquinavir have been shown to target MMP-2 and MMP-7 but not the antitarget MMP-9 that can be considered either target or antitarget depending on the tumor type.[25,54,55]

Thus, considering the capacity of HIV-PIs to target both the proteasome and validated MMP targets, we decided to explore both the effectiveness of HIV-PIs against human tumors with a high prevalence and incidence, and the role of MMPs *versus* proteasome inhibition in their antineoplastic activity.

Here, we report that therapeutic doses of indinavir and saquinavir inhibit the growth of human colon, breast, lung, and liver carcinomas in nude mice. We have demonstrated that these effects are due to a reduction of angiogenesis and MMP proteolytic activity but not to proteasome-mediated inhibition of cell proliferation or survival. Saquinavir, however, was highly effective in inhibiting tumor growth and survival in cultured tumor cells from the same tumors, but it required drug concentrations similar to or above the therapeutic peak drug concentrations present in plasma of treated patients, indicating that may not be effective on the proteasome *in vivo*. This suggests that HIV-PIs alone or combined with conventional cytotoxic therapy may be effective in treating aggressive tumors in HIV infected or uninfected individuals.

Material and Methods

Cell cultures

A549 (lung carcinoma), SW480 (colon carcinoma), MDA-MB-468 (breast carcinoma) and SK-HEP-1 (liver carcinoma) human tumor cell lines were obtained from American Type Culture Collection (Manassas, VA). SW480 and MDA-MB-468 cells were grown in DMEM medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (FBS) (Euroclone, Paignton, United Kingdom), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen); SK-HEP-1 cells were cultured in MEM with Earl's BSS (Invitrogen), 1% nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, antibiotics and 10% FBS; A549 cells were maintained in Ham's F12 medium (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 1.5 g/L sodium bicarbonate (Invitrogen) and antibiotics.

Induction and treatment of tumors in vivo

Nude mice (5–6 weeks old female Balb/c nu/nu from Charles River, Calco, Italy; 5 animals per group) were treated with the same indinavir (Merck Sharp & Dohme, Ldt., Haarlem, The Netherlands) or saquinavir (Roche, Hertfordshire, Great Britain) formulations and doses administered to patients with AIDS (indinavir, 70 mg/Kg/die; saquinavir, 36 mg/Kg/die)[56] or with saline solution. HIV-PIs were dissolved in 0.4 ml of saline solution and administered by intragastric gavage, starting 2 days (A549 cell injected mice) or 3 days (MDA-MB-468, SK-HEP-1 and SW480 cell injected mice) before tumor cell inoculation. Mice received 400-Rad total body irradiation and 24 hr later were inoculated subcutaneously into the right and left lower back, with A549 cells (4×10^6 cells/site), MDA-MB-468 cells (5×10^6 cells/site), SW480 cells or SK-HEP-1 cells (both at 3×10^6 cells/site), as described.[57] Animals were observed twice a week and size of tumors developing at the injection site were evaluated daily by caliper measurement (longest perpendicular length and width). Animals were sacrificed 68, 94, 38 and 85 days after A549, MDA-MB-468, SW480 and SK-HEP-1 cell injection, respectively. The care and use of mice were in accordance with the European Community guidelines.

Histological and immunohistochemical evaluation of tumors

At sacrifice, tumors were fixed in 10% neutral buffered formalin (Sigma) and blocked in paraffin or frozen in OCT compound (Tissue Tek, Sakura Finetek Europe, The

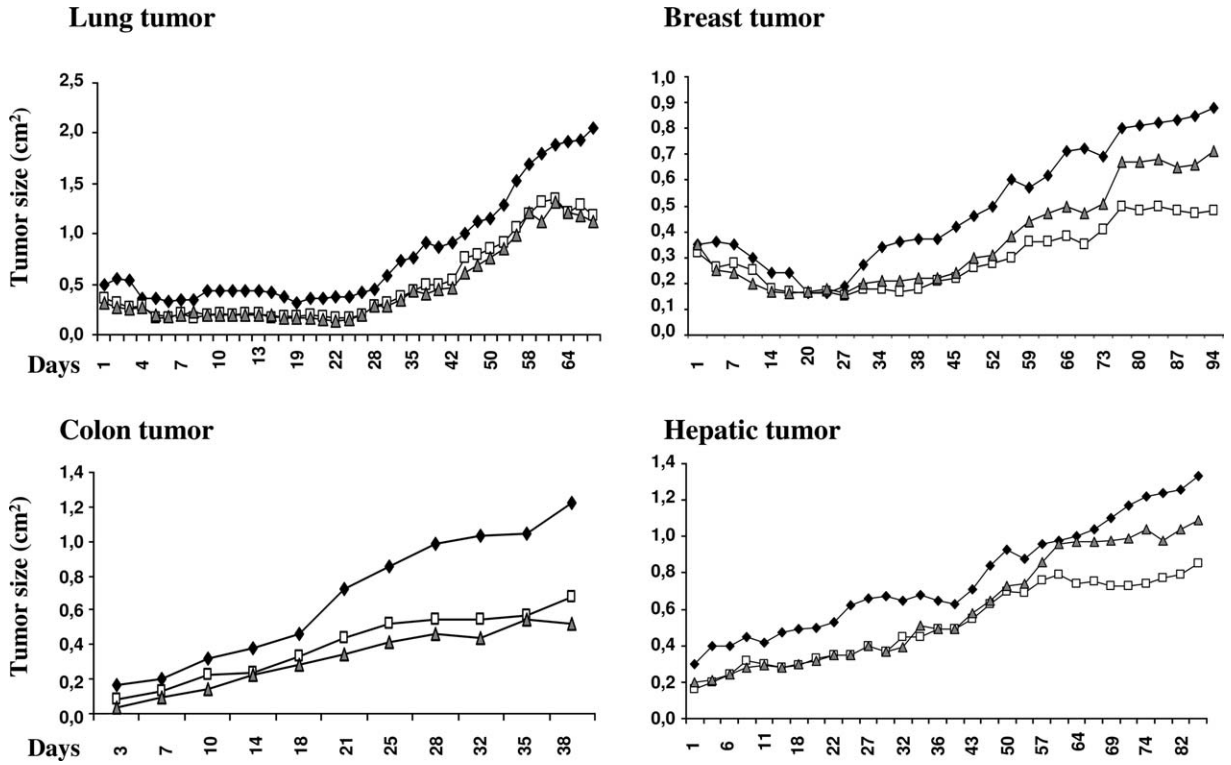

Figure 1. Indinavir or saquinavir inhibit the growth of all tumors induced by the inoculation of human lung, breast, colon or hepatic carcinoma cells in nude mice. The mean of external tumor area measured in untreated (black symbols), indinavir- (white symbols) or saquinavir- (gray symbols) treated mice injected subcutaneously with the indicated tumor cells was plotted as a function of time from tumor cell injection. The treatment with indinavir or saquinavir significantly reduced the growth of xenograft tumors compared with saline-treated mice ($p < 0.01$). In breast and hepatic tumors, indinavir resulted significantly more effective compared with saquinavir in inhibiting tumor growth ($p < 0.05$).

Netherlands).[25,57] For histological examination, paraffin-embedded tumors were sectioned (4 μM) and stained with hematoxylin and eosin.

For immunohistochemical analysis, frozen tissue sections of representative tumors (6 tumors/group) were fixed with cold acetone and stained with a rat anti-mouse CD31 monoclonal Ab (1:1000 dilution; BD Biosciences, Bedford, MA), mouse anti-human Ki67 monoclonal Ab (1:50 dilution; Dako, Glostrup; Denmark) or mouse anti-human p21 monoclonal Ab (1:25 dilution; Dako) as described.[25,57,58] Digital images (200× magnification) of the whole histologic sections (about 0.15 mm^2/field) were captured by a color CCD camera (Carl Zeiss, Jena, Germany). CD31, Ki67 or p21 staining were quantified by using the KS300 image analysis software (Zeiss) and expressed as the percentage of positive area over the total tissue area. Tumor apoptosis was evaluated by the TdT-mediated dUTP nick end labeling *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instruction.

Analysis of MMP activity in tumors

In situ zymography was performed as described previously.[59] Briefly, acetone-fixed tumor cryostat sections (8-μM thick) were air dried for 10 min and covered with a mixture of DQ-gelatin (1 mg/mL) containing DAPI (1.0 μg/mL; Enz-Chek; Molecular Probes, Eugene, OR). After agar gelling at 4°C, slides were incubated for 1 hr at RT. Fluorescence of FITC and DAPI was detected using a Axioskop 2 plus microscope (Zeiss) using AxioVision 3.06 software (Zeiss) under 20× original magnification. To ascertain the enzymatic nature of the appearance of fluorescence and its specific localization patterns, the sections were fixed with 4% paraformaldehyde for 10 min as a negative control. For combined localization of gelatinolytic activity and MMP-2 protein in the same section, immunohistochemical analysis was performed by using the ARK (Animal Research Kit) Peroxidase system (Dako), with a mouse anti-human MMP-2 monoclonal antibody (1:25 dilution; Calbiochem, Darmstadt, Germany), as previously described,[25,57,58] followed by *in situ* zymography.

Cell invasion assay

Tumor cells were cultured for 5 days in the presence of indinavir or saquinavir (a kind gift of Merck Sharp and Dohme and Roche) (0.1–10 μM) or drug resuspension buffer. Tumor cells were then harvested, resuspended in serum-free medium containing 0.1% BSA and seeded in

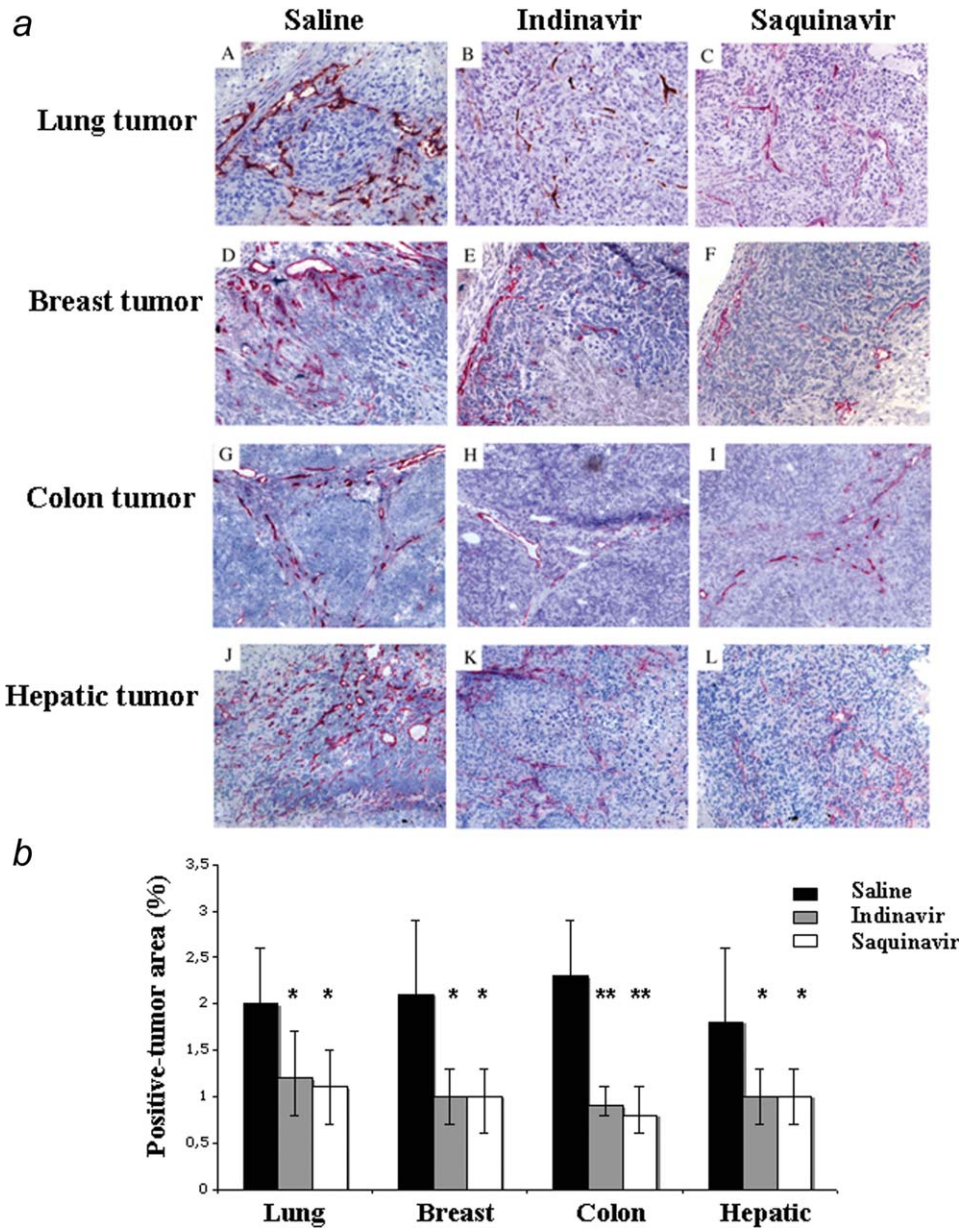

Figure 2. Drug-mediated inhibition of tumor angiogenesis in nude mice injected with tumor cells. Representative microphotographs of tumor cross-sections stained for CD31 expression (magnification, ×100) from mice bearing lung (panels *a*, *b* and *c*), breast (panels *d*, *e*, *f*), colon (panels *g*, *h* and *i*) or hepatic (panels *j*, *k* and *l*) human tumor xenografts, respectively, and treated daily with saline solution (left panels) or HIV-therapeutic doses of indinavir (middle panels) or saquinavir (right panels), respectively, are shown. Tumor-associated angiogenesis was evaluated by immunohistochemical analysis of tumor cross-sections using the endothelial cell marker CD31. The decrease of CD31 expression (mean with 95% confidence interval) was statistically significant (*$p < 0.05$; **$p < 0.001$) in all tumors from HIV-PI-treated animals compared with controls (*b*).

duplicate in the presence of indinavir, saquinavir or buffer (1.5×10^5 cells/chamber for A549 cell line or 2.5×10^5 cells/chamber for MDA-MB-468, SW480 and SK-HEP-1 cell lines) in the upper compartment of Boyden chambers separated from the lower compartment containing basic fibroblast growth factor (bFGF; 50 ng/mL) (Roche Diagnostic, Mannheim, Germany) by polycarbonate filters (12-μ pore filters for A549 cells or with 8-μ pore filters for MDA-MB-468, SW480 and SK-HEP-1 cells; Nucleopore; Whatman, Clifton, NJ) coated with matrigel (25 μg/50 μL) (BD Biosciences). After 6 hr, noninvaded cells present on the upper surface of the filters were mechanically removed, whereas cells that had migrated to the lower surface were fixed in ethanol and stained with toluidine blue (Sigma). The number of invaded cells/field was evaluated and expressed as described previously.[25,60]

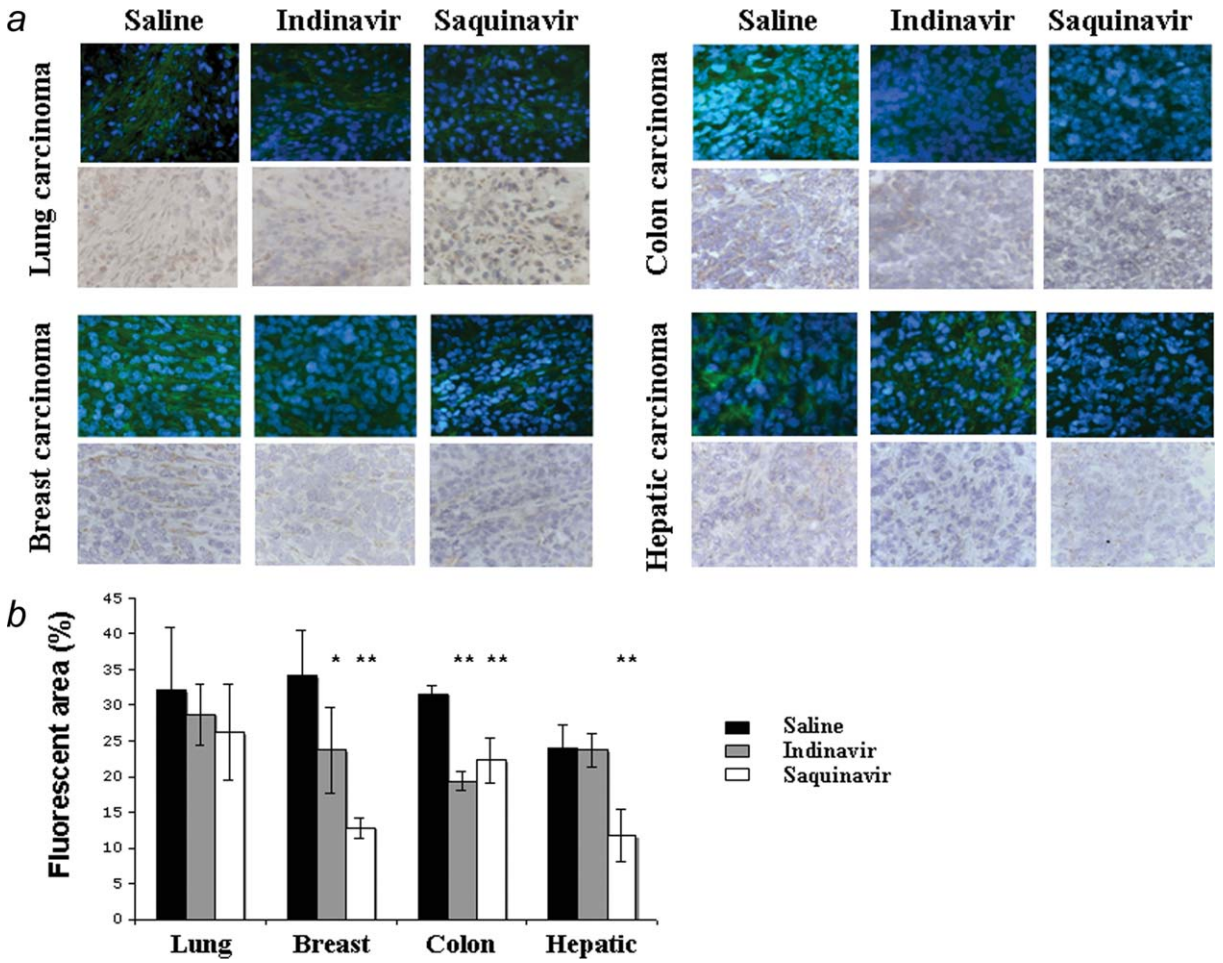

Figure 3. Indinavir and saquinavir decrease MMP activity *in vivo*. (*a*) The *in situ* zymography (upper panels) and the correspondent MMP-2 staining (lower panels) in lung, breast, colon and hepatic carcinoma tissues, respectively. Green fluorescence in tumor section marks the areas of MMP-2 and/or MMP-9 activity (see Material and Methods). Cell nuclei are stained in blue color. Immunohistochemical MMP-2 staining has been performed on the same section analyzed with *in situ* zymography. The inhibition of gelatinolytic activity induced by indinavir and saquinavir is superimposable with the decrease of MMP-2 staining in breast and colon carcinoma tissues (*a*). MMP activity was evaluated *in situ* zymography analysis of tumor cross-sections using DQ-gelatin as substrate. Quantification of the fluorescent areas in the tumor tissues was performed using the KS300 image analysis software (Zeiss). The decrease of fluorescent area in breast, colon and hepatic tumors from HIV-PI-treated animals (mean with 95% confidence interval) is statistically significant (*$p < 0.05$; **$p < 0.001$) (*b*).

Cell proliferation assay

Tumor cells were seeded in triplicate in 12-well plates (8×10^4 cells/well for A549, SW480 and SK-HEP-1 cell lines and 1×10^5 cells/well for the MDA-MB-468 cell line) and cultured in the presence of 0.1, 1, 10 and 25 μM indinavir or saquinavir or drug resuspension buffer for 5 days. The drugs were added to the cell culture every other day. Cell proliferation was determined by the cell counting method as described previously.[25,60]

IκBα expression

Tumor cells were grown in the presence of indinavir or saquinavir (1 and 25 μM) or drug resuspension buffer for 4 days or in the presence of epoxomicin (10 μM) for 6 hr. TNFα (10 ng/mL) was then added to the medium for 20 min, and cells were lysed in a buffer containing 50 nM TrisHCl, pH 7.5, 150 nM NaCl, 1% Triton X-100, 2.5 mM EGTA, 2.5 mM EDTA, 1.5 mM $MgCl_2$, 1 mM Na orthovanadate, 5 mM NaF, 5 mM *N*-ethyl-maleimide and a protease inhibitor mixture (Sigma, St. Louis, MO). Equal amounts of total proteins were separated by SDS-PAGE followed by immunoblotting with a purified rabbit polyclonal serum against IκBα (Santa Cruz, CA), incubation with a horseradish peroxidase-conjugated secondary antibodies (Amersham, Pharmacia Biotech), and detection by the ECL system (Amersham Pharmacia Biotech).

The p21 immunocytochemical analysis

Cells were grown on chamber slides for 4 days in the presence of indinavir or saquinavir (1 and 25 μM), drug

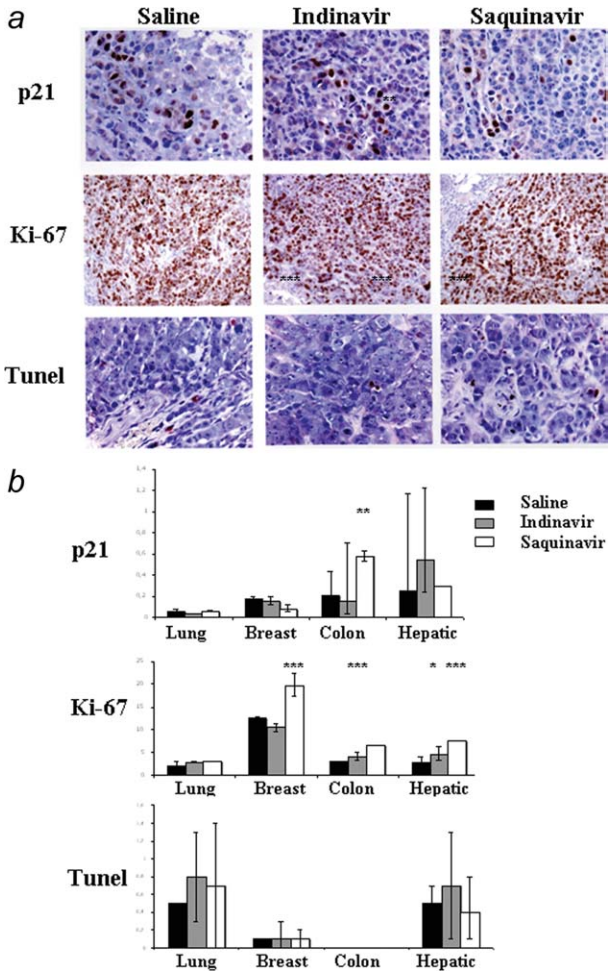

Figure 4. Indinavir and saquinavir effects on p21, Ki67 and cell apoptosis *in vivo*. (*a*) Representative microphotographs of breast carcinoma xenograft cross-sections analyzed for p21, Ki67 expression and the TUNEL assay, respectively (magnification, '400). Left panels: saline; middle panels: indinavir; right panels: saquinavir. (*b*) Quantification of the expression of p21, Ki67 and apoptosis in tumor tissues are expressed as geometric mean with 95% confidence interval, respectively (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, respectively).

resuspension buffer or epoxomicin (10 μM) (Biomol, PA). The cells were then fixed in cold acetone and stained with a monoclonal Ab against p21 (1:25 dilution; Dako). After washing with PBS, the slides were incubated with a biotinylated anti-mouse Ab (Vector Laboratories, Burlingame, CA), and p21 was detected by the ABC method (Vector Laboratories) followed by PBS washing and incubation with 3,39-diaminobenzidine substrate (Dako). Cells were counterstained with hematoxylin, visualized using the Axioskop 2 plus microscope (Zeiss), and images were captured and processed using the Axiocam camera (Zeiss) under 40× original magnification. The p21-positive cells were quantified as the percentage of total cells by using the KS 300 software (version 3.2).

Statistical methods

Regression model for correlated data (Generalized Estimating Equations for repeated measures) was applied to detect differences among treatment groups (Indinavir, Saquinavir, Saline); when data were not normally distributed, geometric mean was used.

All statistical tests were carried out at two sided with a 5% significance level. Statistical analyses and data processing were performed using SAS software (SAS Institute, Cary, NC).

Results

Indinavir and saquinavir reduce the growth of highly aggressive human tumors in nude mice

Our previous work indicated that either indinavir or saquinavir inhibit KS-tumor growth and angiogenesis in nude mice.[25] Others showed that indinavir has a similar effect on human liver cancer cells.[24]

To determine the effectiveness of HIV-PIs in inhibiting the growth of aggressive and prevalent human tumors, nude mice were inoculated subcutaneously with human cell lines from lung carcinoma (A549 cells), breast carcinoma (MDA-MB-468 cells), colon carcinoma (SW480 cells) and hepatic carcinoma (SK-Hep-1 cells), respectively. These are experimental models of tumor outgrowth that are widely used for testing the effectiveness of antiangiogenic and antitumor drugs.[57] Animals were treated by intragastric gavage for 2 to 3 days with doses of indinavir or saquinavir comparable with those used in HIV-infected patients or with saline solution as control and then injected subcutaneously with the human tumor cells.[25] Treatment with HIV-PI or saline was continued daily until sacrifice. The growth of all tumors was significantly inhibited by either indinavir or saquinavir (Fig. 1). All tumors, in fact, remained significantly smaller in treated animals compared with saline-treated mice throughout the entire treatment period ($p < 0.01$), and at sacrifice, the mean external size of tumor xenografts from treated mice was up to 2.3-fold smaller compared with controls (Fig. 1). The comparison of the two different treatments indicated that indinavir was significantly more effective than saquinavir at inhibiting breast and hepatic tumor growth ($p < 0.05$) (Fig. 1).

Finally, in established angiogenic tumor models,[25] indinavir and saquinavir were capable of inhibiting colon tumor xenograft development (induced by the same cell line and injection modalities) also when administered after tumor appearance (data not shown). These findings indicate that HIV-PI can promote tumor regression also when administered after tumor formation.

Indinavir and saquinavir block tumor growth by inhibiting angiogenesis and MMP activity

To investigate the effects of indinavir and saquinavir on the angiogenesis and MMPs and on the proteasome, tumor

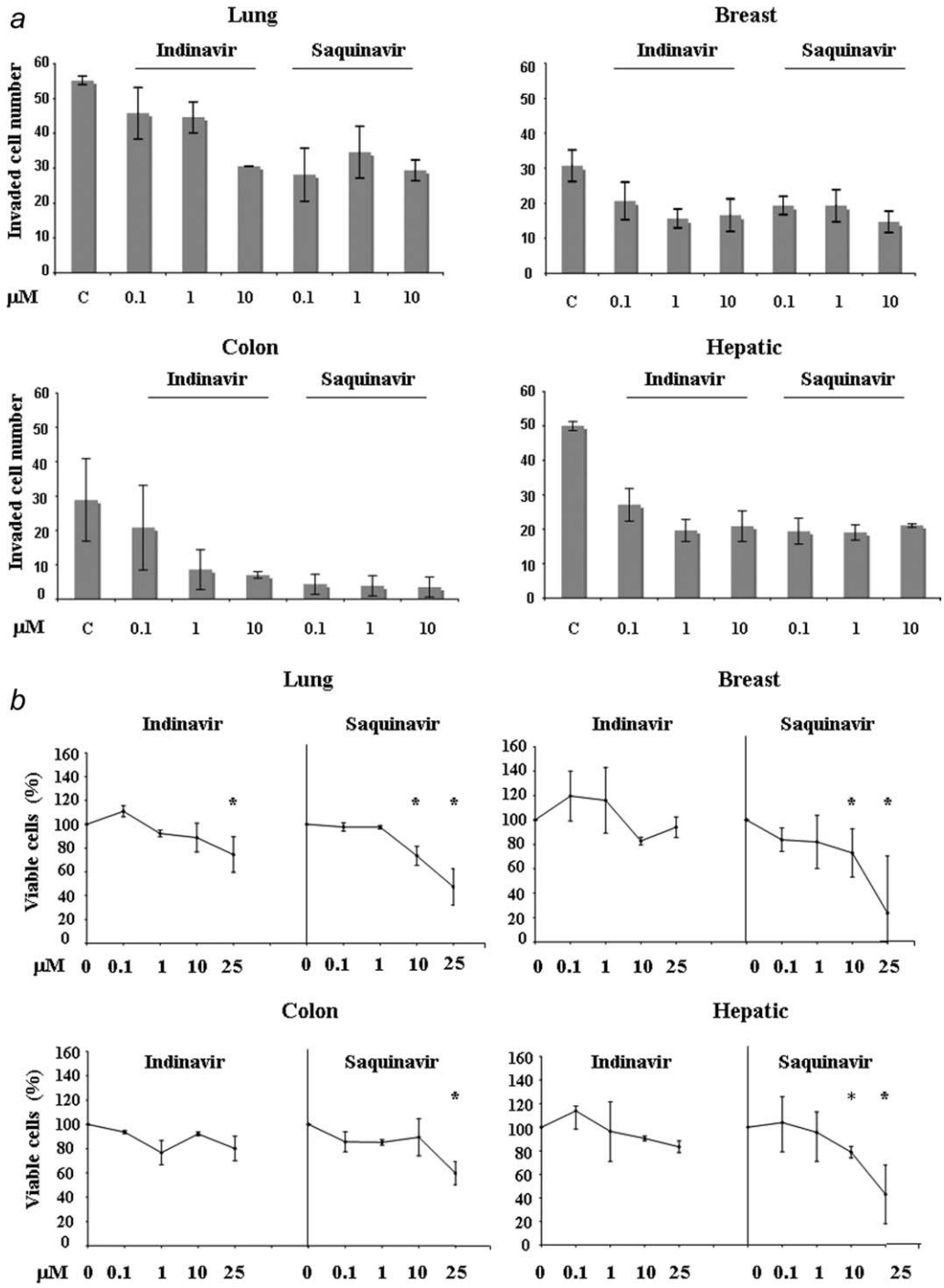

Figure 5. Inhibition of tumor cell invasion and cell growth/viability by indinavir and/or saquinavir. Data are expressed as the mean number (with 95% confidence interval) of invading cells in response to bFGF for cells treated with indinavir, saquinavir and HIV-PI-resuspension buffer (C). The decrease of bFGF-induced cell invasion by indinavir or saquinavir was statistically significant at all drug concentrations in all tumor cell lines ($p < 0.0001$) (*a*). Tumor cells cultured in the presence or absence of indinavir or saquinavir and viable cells were counted after 5 days (*b*). Data are expressed as the mean number of cells grown (with 95% confidence interval) in the presence of indinavir or saquinavir compared with control cultures (*$p < 0.02$).

xenografts were assessed for microvascular density through CD31 expression,[25] gelatinase activity by *in situ* zymography[59] and expression of cell proliferation, apoptosis and cell cycle markers, including Ki67 and p21, respectively.

Treatment with either saquinavir or indinavir significantly inhibited angiogenesis in all tumors, reducing microvascular density by 40 to 65% ($p < 0.05$; $p < 0.001$), respectively (Fig. 2*a*,*b*).

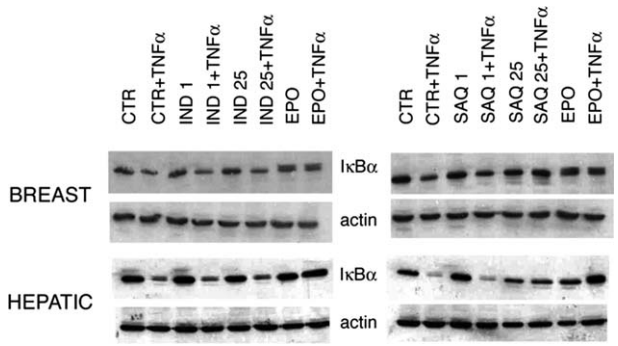

Figure 6. High concentrations of saquinavir, but not indinavir, block IκBα degradation. Tumor cells were cultured in the presence or absence of indinavir (IND) or saquinavir (SAQ) (1 or 25 μM), or epoxomicin (10 μM) (EPO) and analyzed for IκBα expression. Saquinavir (25 mM) blocked TNFα-induced IκBα degradation in breast and hepatic tumor cells but not in lung and colon tumor cells (data not shown).

Furthermore, the *in situ* zymographic analyses indicated that indinavir and saquinavir significantly reduced MMP activity in breast, colon and hepatic tumors (Fig. 3*a*,*b*). Immunohistochemical staining (Fig. 3*a*, lower panel) performed in parallel on the same slides analyzed for the gelatinolytic activity demonstrated that a reduction of MMP activity by indinavir and saquinavir was mostly associated with a lower expression of MMP-2, although an effect on MMP-9 activity could not be excluded by these assays (Fig. 3*b*).

In addition, we did not observe a significant difference either of the expression of p21 or of cell apoptosis in tumors from treated mice, with the exception of the colon tissues from mice treated with saquinavir (Fig. 4*a*,*b*). However, an increase of Ki67 expression was detected in most tumor tissues following either indinavir or saquinavir treatment (Fig. 4*b*). Altogether these data suggest that indinavir and saquinavir do not block cell cycle progression, proliferation or apoptosis but rather they reduce the tumor mass *in vivo* mostly through the inhibition of angiogenesis and MMP-2 activity.

Effects of indinavir and saquinavir on cultured tumor cells

HIV-PIs have been previously shown to promote tumor cell growth arrest and/or apoptosis *via* the functional impairment of the cellular proteasome.[26,28,30,31] Thus, to assess the effects of indinavir and saquinavir on cell invasion and cell growth/viability, tumor cells were cultured in the presence or absence of drugs at concentrations ranging from the steadystate to the peak drug levels present in plasma from treated patients (0.1 and 10 μM, respectively)[61–63] or higher (25 μM).

The results indicated that both indinavir and saquinavir decreased or completely blocked cell invasion in response to bFGF[25] in all tumor cell lines at all therapeutic drug concentrations ($p < 0.0001$) (Fig. 5*a*). No effects were observed on the noninduced cell motility by either drug (data not shown). In contrast, cell growth/viability was significantly inhibited only at peak drug concentrations which are comparable with those observed in plasma. Statistically significant inhibition was evident in all cell lines treated with saquinavir and, for indinavir, only in lung carcinoma cells treated with drug concentrations above the HIV therapeutic peak level (Fig. 5*b*) ($p < 0.02$). Tumor cells were then cultured in the presence of low (1 μM) or high (25 μM) drug concentrations or in the presence of the proteasome inhibitor epoxomicin[64] and analyzed for IκBα and p21 expression, which are known target of cellular proteasome proteolytic activity.[65] To induce proteasome-mediated IκBα degradation, cells were exposed to TNFα.[65] High concentrations of saquinavir, but not indinavir, blocked TNFα-induced degradation of IκBα in both breast and hepatic tumor cells to levels similar to epoxomicin (Fig. 6). In addition, high concentrations of saquinavir induced a significant p21 nuclear accumulation in lung, breast and hepatic carcinoma cells ($p < 0.001$) (Fig. 7*a*,*b*). By contrast, indinavir induced a statistically significant p21 accumulation only in hepatic tumor cells treated with a high drug concentration (25 μM), which is above the HIV therapeutic drug peak level (Fig. 7*a*,*b*). Altogether these results indicated that high concentrations of saquinavir, but not indinavir, are able to reduce the degradation of IκBα induced by TNFα and to promote a consistent accumulation of p21, thus acting on the cellular proteasome activity.

Discussion

Our present data show that HIV-PIs are effective in inhibiting the *in vivo* growth of aggressive tumors that have a high incidence rate in humans. This has been proved in animal models that are free of viruses and T cells and which detect direct antitumor effects of HIV-PIs that are independent of drug-mediated HIV suppression and immune reconstitution. We have examined the effectiveness of two different HIV-PIs, indinavir and saquinavir, against lung, breast, colon and hepatic adenocarcinoma.

In accordance with previous data obtained by us and others,[24,25] we have observed that the growth of all tumors was significantly inhibited *in vivo* by both indinavir and saquinavir. The tumors, in fact, remained significantly smaller in treated animals compared with control mice throughout the entire treatment period.

Previous studies have shown that HIV-PIs can affect several steps and host responses involved in tumor initiation, development and/or outgrowth (reviewed in Monini *et al.*[7]). These include angiogenesis, tumor cell invasion, tumor cell survival and/or proliferation, inflammation and cytotoxic T lymphocyte responses. Most of these pathways are inhibited or modulated by HIV-PIs through actions on two main targets: MMPs and the proteasome (reviewed in Monini *et al.*[7]). MMPs and the proteasome are also the targets of novel "pathogenetically oriented" antitumor therapies that are currently under clinical evaluation.

Herein, we have demonstrated that therapeutic doses of both HIV-PIs inhibit these tumors by blocking angiogenesis and tumor invasion mostly through targeting of MMP

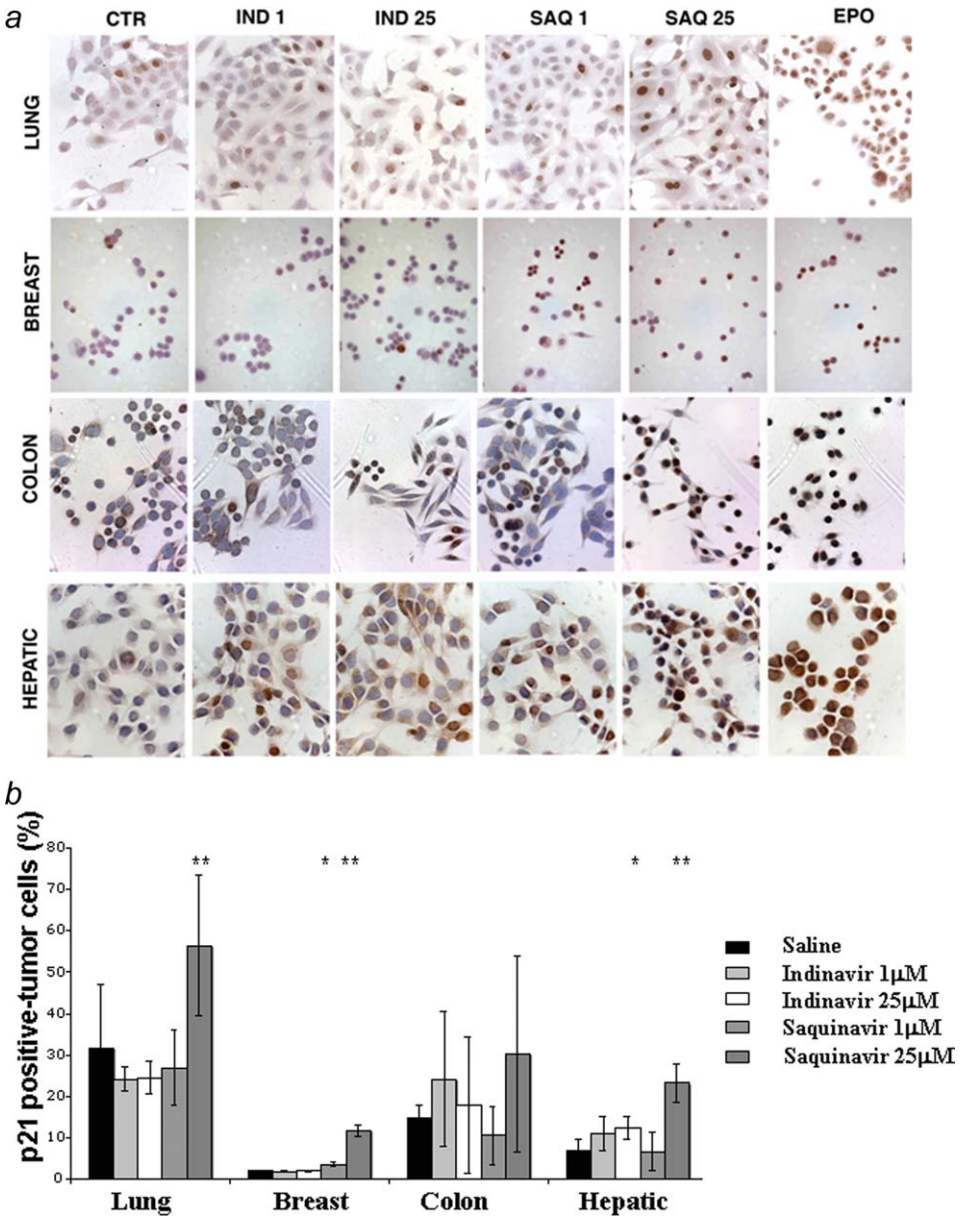

Figure 7. High concentrations of saquinavir, but not indinavir, block p21 degradation. The human lung, breast, colon and hepatic carcinoma cells treated with different doses of indinavir and saquinavir (1 and 25 μM) or resuspension buffer for 4 days were then analyzed for p21 expression by immunohistochemical analysis. A consistent accumulation of p21 was observed in the nucleus of cells treated with high doses of saquinavir or with epoxomicin (*a*). The amount of the tumor cells positive for p21 expression was quantified using the KS300 image analysis software (Zeiss). p21 expression was significantly increased in lung, breast and hepatic tumor cells treated with saquinavir and in the hepatic tumor cells treated with 25mM indinavir (*$p < 0.05$; **$p < 0.001$) (*b*).

activity and cell motility. In fact, a significant reduction of both the tumor mass and the microvascular density was observed in all types of tumor xenografts from HIV-PI-treated mice compared with untreated animals. By contrast, we found an increase of tumor cell Ki67 labeling *in vivo* that does not correlate with the reduction of the intratumoral microvessel density. Similar results have been previously observed in others studies on breast carcinomas and non-small-cell lung carcinomas[66–69] and suggest that tumor cell proliferation and capillary growth and/or density might be regulated by different mechanisms. Furthermore, our *in situ* zymographic analyses have demonstrated that therapeutic concentrations of both indinavir and saquinavir induce a significant reduction of MMP activity in our tumor models. Consistently with this, we have also observed that both HIV-PIs, at all therapeutic drug concentrations, efficiently block tumor cell invasion, which is strongly influenced by MMPs.[70] By contrast, in the *in vitro* experiments, tumor cell growth

was affected only by peak drug concentrations of saquinavir (Fig. 5*b*). These findings indicate that therapeutic concentrations of HIV-PIs may block tumor growth principally by decreasing MMP-dependent tumor cell invasion rather than acting on the cell cycle and are consistent with previous reports indicating that cell migration and DNA synthesis do not necessarily occur simultaneously [71,72] Cell migration and proliferation are required for wound healing, embryonic development and angiogenesis. However, during these processes, cells can either migrate in the absence of proliferation or proliferate without migrating, and this has been explained through the existence of signaling pathways that distinguish between these two responses.[71–74] Signaling molecules, such as the Shc family of adaptor proteins, may be crucial for DNA synthesis or cell migration depending whether their activation is induced by growth factors or integrin receptors ligation, respectively.[75]

Our ongoing experiments indicate that the decrease of MMP activity by HIV-PIs might not be due to a direct effect of the drugs on MMP-2, but rather an effect on αvβ3, the integrin receptor involved in the process of MMP-2 activation (unpublished observations and Bjorklund *et al.*[76]), thus contributing to the reduction of tumor invasion and angiogenesis.

Previous studies have shown that tumor cell growth arrest and/or apoptosis caused by high concentrations of ritonavir and saquinavir in lymphoma, KS and prostate cancer cell lines are associated with the accumulation of IκBα and p21 due to proteasome inhibition.[26,28,30] Consistent with this, our *in vitro* experiments have demonstrated that although saquinavir upregulated p21, rescued TNFα-induced degradation of IκBα and inhibited the growth and/or viability of cultured tumor cells due to effects on the proteasome, this required high drug concentrations that most likely were not reached for prolonged time periods in mice treated with HIV-PI therapeutic doses. This is further suggested by the lack of Ki67 downregulation, p21 upregulation and induction of tumor cell apoptosis in tumor xenografts from treated animals, and it is in agreement with other data indicating that ritonavir can block glioma growth *in vitro* but not *in vivo* due to the very low drug concentration achieved across the blood-brain barrier.[77]

In conclusion, this study provides evidence that HIV-PIs may be considered promising new tools to treat cancer particularly because of their antineoplastic potential associated also with the absence of complications due to prolonged treatment, as previously observed in other preclinical models and in HIV-1 negative KS patients treated with indinavir.[25,78] Angiogenesis, cell proliferation and apoptosis represent important mechanisms involved in malignant growth, and inhibition of tumor growth by low concentrations of indinavir and saquinavir was most likely mediated through a blockade of tumor angiogenesis and cell invasion, without significantly affecting cell cycle, cell apoptosis and/or proteasome activity. Clearly, the therapeutic potential of a combined approach using an antiangiogenic agent with conventional cytotoxic therapy might greatly improve the antitumor efficacy. To this goal, we have recently started a Phase II clinical trial in HIV-1 negative advanced KS patients, which are treated with indinavir in combination with a debulking chemotherapy.

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References

1. Cattelan AM, Calabro ML, Aversa SM, Zanchetta M, Meneghetti F, De RA, Chieco-Bianchi L. Regression of AIDS-related Kaposi's sarcoma following antiretroviral therapy with protease inhibitors: biological correlates of clinical outcome. *Eur J Cancer* 1999;35: 1809–15.
2. Heard I, Schmitz V, Costagliola D, Orth G, Kazatchkine MD. Early regression of cervical lesions in HIV-seropositive women receiving highly active antiretroviral therapy1. *AIDS* 1998;12:1459–64.
3. Hocqueloux L, Agbalika F, Oksenhendler E, Molina JM. Long-term remission of an AIDS-related primary effusion lymphoma with antiviral therapy 1. *AIDS* 2001;15: 280–2.
4. International Collaboration on HIV and Cancer. Highly active antiretroviral therapy and incidence of cancer in human immunodeficiency virus-infected adults. *J Natl Cancer Inst* 2000;92:1823–30.
5. Lebbe C, Blum L, Pellet C, Blanchard G, Verola O, Morel P, Danne O, Calvo F. Clinical and biological impact of antiretroviral therapy with protease inhibitors on HIV-related Kaposi's sarcoma. *AIDS* 1998;12:F45–F49.
6. Robinson WR, Freeman D. Improved outcome of cervical neoplasia in HIV-infected women in the era of highly active antiretroviral therapy 1. *AIDS Patient Care STDS* 2002;16:61–5.
7. Monini P, Sgadari C, Toschi E, Barillari G, Ensoli B. Antitumour effects of antiretroviral therapy. *Nat Rev Cancer* 2004;4:861–75.
8. Sgadari C, Monini P, Barillari G, Ensoli B. Use of HIV protease inhibitors to block Kaposi's sarcoma and tumour growth. *Lancet Oncol* 2003;4:537–47.
9. Bower M, Powles T, Nelson M, Mandalia S, Gazzard B, Stebbing J. Highly active antiretroviral therapy and human immunodeficiency virus-associated primary cerebral lymphoma. *J Natl Cancer Inst* 2006;98:1088–91.
10. Grabar S, Abraham B, Mahamat A, Del GP, Rosenthal E, Costagliola D. Differential impact of combination antiretroviral therapy in preventing Kaposi's sarcoma with and without visceral involvement. *J Clin Oncol* 2006;24:3408–14.
11. Stebbing J, Gazzard B, Mandalia S, Teague A, Waterston A, Marvin V, Nelson M, Bower M. Antiretroviral treatment regimens and immune parameters in the prevention of systemic AIDS-related non-Hodgkin's lymphoma. *J Clin Oncol* 2004; 22:2177–83.
12. Stebbing J, Portsmouth S, Nelson M, Mandalia S, Kandil H, Alexander N,

Davies L, Brock C, Bower M, Gazzard B. The efficacy of ritonavir in the prevention of AIDS-related Kaposi's sarcoma. *Int J Cancer* 2004;108:631–3.
13. Benfield TL, Kirk O, Elbrond B, Pedersen C. Complete histological regression of Kaposi's sarcoma following treatment with protease inhibitors despite persistence of HHV-8 in lesions. *Scand J Infect Dis* 1998; 30:613–5.
14. Carrio M, Mazo A, Lopez-Iglesias C, Estivill X, Fillat C. Retrovirus-mediated transfer of the herpes simplex virus thymidine kinase and connexin26 genes in pancreatic cells results in variable efficiency on the bystander killing: implications for gene therapy. *Int J Cancer* 2001;94:81–8.
15. Gill J, Bourboulia D, Wilkinson J, Hayes P, Cope A, Marcelin AG, Calvez V, Gotch F, Boshoff C, Gazzard B. Prospective study of the effects of antiretroviral therapy on Kaposi sarcoma—associated herpes virus infection in patients with and without Kaposi sarcoma. *J Acquir Immune Defic Syndr* 2002;31:384–90.
16. Heard I, Tassie JM, Kazatchkine MD, Orth G. Highly active antiretroviral therapy enhances regression of cervical intraepithelial neoplasia in HIV-seropositive women. *AIDS* 2002;16: 1799–802.
17. Kirk O, Pedersen C, Cozzi-Lepri A, Antunes F, Miller V, Gatell JM, Katlama C, Lazzarin A, Skinhoj P, Barton SE. Non-Hodgkin lymphoma in HIV-infected patients in the era of highly active antiretroviral therapy. *Blood* 2001;98: 3406–12.
18. Martinez V, Caumes E, Gambotti L, Ittah H, Morini JP, Deleuze J, Gorin I, Katlama C, Bricaire F, Dupin N. Remission from Kaposi's sarcoma on HAART is associated with suppression of HIV replication and is independent of protease inhibitor therapy. *Br J Cancer* 2006;94:1000–6.
19. Nunez M, Machuca A, Soriano V, Podzamczer D, Gonzalez-Lahoz J. Clearance of human herpesvirus type 8 viraemia in HIV-1-positive patients with Kaposi's sarcoma treated with liposomal doxorubicin. Caelyx/KS Spanish Study Group 1. *AIDS* 2000;14:913–9.
20. Torre-Cisneros J, Pozo F, Serrano R, Vidal E, Rivero A, Tenorio A. Patterns of lymphotropic herpesvirus viraemia in HIV-infected patients with Kaposi's sarcoma treated with highly active antiretroviral therapy and liposomal daunorubicin. *AIDS* 2000;14:2215–7.
21. Uberti-Foppa C, Ferrari D, Lodini S, Reina S, Ameglio F, Grasso MA, Gallotta G, Ferrari A, Taccagni G, Lazzarin A, Lillo FB. Long-term effect of highly active antiretroviral therapy on cervical lesions in HIV-positive women. *AIDS* 2003;17: 2136–8.
22. Monini P, Toschi E, Sgadari C, et al. The use of HAART for biological tumour therapy. *J HIV Ther* 2006;11:53–6.
23. Sciamanna I, Landriscina M, Pittoggi C, Quirino M, Mearelli C, Beraldi R, Mattei E, Serafino A, Cassano A, Sinibaldi-Vallebona P, Garaci E, Barone C, et al. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. *Oncogene* 2005;24:3923–31.
24. Esposito V, Palescandolo E, Spugnini EP, Montesarchio V, De LA, Cardillo I, Cortese G, Baldi A, Chirianni A. Evaluation of antitumoral properties of the protease inhibitor indinavir in a murine model of hepatocarcinoma. *Clin Cancer Res* 2006;12:2634–9.
25. Sgadari C, Barillari G, Toschi E, Carlei D, Bacigalupo I, Baccarini S, Palladino C, Leone P, Bugarini R, Malavasi L, Cafaro A, Falchi M, et al. HIV protease inhibitors are potent anti-angiogenic molecules and promote regression of Kaposi sarcoma. *Nat Med* 2002;8:225–32.
26. Gaedicke S, Firat-Geier E, Constantiniu O, Lucchiari-Hartz M, Freudenberg M, Galanos C, Niedermann G. Antitumor effect of the human immunodeficiency virus protease inhibitor ritonavir: induction of tumor-cell apoptosis associated with perturbation of proteasomal proteolysis. *Cancer Res* 2002;62:6901–8.
27. Jiang W, Mikochik PJ, Ra JH, Lei H, Flaherty KT, Winkler JD, Spitz FR. HIV protease inhibitor nelfinavir inhibits growth of human melanoma cells by induction of cell cycle arrest. *Cancer Res* 2007;67:1221–7.
28. Pati S, Pelser CB, Dufraine J, Bryant JL, Reitz MS, Jr., Weichold FF. Antitumorigenic effects of HIV protease inhibitor ritonavir: inhibition of Kaposi sarcoma. *Blood* 2002;99:3771–9.
29. Gills JJ, Lopiccolo J, Tsurutani J, Shoemaker RH, Best CJ, bu-Asab MS, Borojerdi J, Warfel NA, Gardner ER, Danish M, Hollander MC, Kawabata S, et al. Nelfinavir, a lead HIV protease inhibitor, is a broad-spectrum, anticancer agent that induces endoplasmic reticulum stress, autophagy, and apoptosis in vitro and in vivo. *Clin Cancer Res* 2007;13: 5183–94.
30. Pajonk F, Himmelsbach J, Riess K, Sommer A, McBride WH. The human immunodeficiency virus (HIV)-1 protease inhibitor saquinavir inhibits proteasome function and causes apoptosis and radiosensitization in non-HIV-associated human cancer cells. *Cancer Res* 2002;62: 5230–5.
31. Andre P, Groettrup M, Klenerman P, de GR, Booth BL, Jr., Cerundolo V, Bonneville M, Jotereau F, Zinkernagel RM, Lotteau V. An inhibitor of HIV-1 protease modulates proteasome activity, antigen presentation, and T cell responses. *Proc Natl Acad Sci USA* 1998;95:13120–4.
32. Chavan S, Kodoth S, Pahwa R, Pahwa S. The HIV protease inhibitor Indinavir inhibits cell-cycle progression in vitro in lymphocytes of HIV-infected and uninfected individuals. *Blood* 2001;98: 383–9.
33. Gruber A, Wheat JC, Kuhen KL, Looney DJ, Wong-Staal F. Differential effects of HIV-1 protease inhibitors on dendritic cell immunophenotype and function. *J Biol Chem* 2001;276:47840–3.
34. Lu W, Andrieu JM. HIV protease inhibitors restore impaired T-cell proliferative response in vivo and in vitro: a viral-suppression-independent mechanism. *Blood* 2000;96:250–8.
35. Schmidtke G, Holzhutter HG, Bogyo M, Kairies N, Groll M, de GR, Emch S, Groettrup M. How an inhibitor of the HIV-I protease modulates proteasome activity. *J Biol Chem* 1999;274:35734–40.
36. Sloand EM, Kumar PN, Kim S, Chaudhuri A, Weichold FF, Young NS. Human immunodeficiency virus type 1 protease inhibitor modulates activation of peripheral blood CD4(+) T cells and decreases their susceptibility to apoptosis in vitro and in vivo. *Blood* 1999;94:1021–7.
37. Tovo PA. Highly active antiretroviral therapy inhibits cytokine production in HIV-uninfected subjects. *AIDS* 2000;14: 743–4.
38. Weichold FF, Bryant JL, Pati S, Barabitskaya O, Gallo RC, Reitz MS Jr. HIV-1 protease inhibitor ritonavir modulates susceptibility to apoptosis of uninfected T cells. *J Hum Virol* 1999;2: 261–9.
39. Piccinini M, Rinaudo MT, Chiapello N, Ricotti E, Baldovino S, Mostert M, Tovo PA. The human 26S proteasome is a target of antiretroviral agents. *AIDS* 2002;16: 693–700.
40. Gupta AK, Cerniglia GJ, Mick R, McKenna WG, Muschel RJ. HIV protease inhibitors block Akt signaling and radiosensitize tumor cells both in vitro and in vivo. *Cancer Res* 2005;65:8256–65.
41. Ikezoe T, Saito T, Bandobashi K, Yang Y, Koeffler HP, Taguchi H. HIV-1 protease inhibitor induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2. *Mol Cancer Ther* 2004;3:473–9.
42. Srirangam A, Mitra R, Wang M, Gorski JC, Badve S, Baldridge L, Hamilton J, Kishimoto H, Hawes J, Li L, Orschell CM, Srour EF, et al. Effects of HIV protease

inhibitor ritonavir on Akt-regulated cell proliferation in breast cancer. *Clin Cancer Res* 2006;12:1883–96.
43. Yang Y, Ikezoe T, Takeuchi T, Adachi Y, Ohtsuki Y, Takeuchi S, Koeffler HP, Taguchi H. HIV-1 protease inhibitor induces growth arrest and apoptosis of human prostate cancer LNCaP cells in vitro and in vivo in conjunction with blockade of androgen receptor STAT3 and AKT signaling. *Cancer Sci* 2005;96: 425–33.
44. Gupta AK, Li B, Cerniglia GJ, Ahmed MS, Hahn SM, Maity A. The HIV protease inhibitor nelfinavir downregulates Akt phosphorylation by inhibiting proteasomal activity and inducing the unfolded protein response. *Neoplasia* 2007;9:271–8.
45. Dewan MZ, Uchihara JN, Terashima K, Honda M, Sata T, Ito M, Fujii N, Uozumi K, Tsukasaki K, Tomonaga M, Kubuki Y, Okayama A, et al. Efficient intervention of growth and infiltration of primary adult T-cell leukemia cells by an HIV protease inhibitor, ritonavir. *Blood* 2006;107:716–24.
46. Ikezoe T, Hisatake Y, Takeuchi T, Ohtsuki Y, Yang Y, Said JW, Taguchi H, Koeffler HP. HIV-1 protease inhibitor, ritonavir: a potent inhibitor of CYP3A4, enhanced the anticancer effects of docetaxel in androgen-independent prostate cancer cells in vitro and in vivo. *Cancer Res* 2004;64:7426–31.
47. Jiang Z, Pore N, Cerniglia GJ, Mick R, Georgescu MM, Bernhard EJ, Hahn SM, Gupta AK, Maity A. Phosphatase and tensin homologue deficiency in glioblastoma confers resistance to radiation and temozolomide that is reversed by the protease inhibitor nelfinavir. *Cancer Res* 2007;67:4467–73.
48. Maggiorella L, Wen B, Frascogna V, Opolon P, Bourhis J, Deutsch E. Combined radiation sensitizing and anti-angiogenic effects of ionizing radiation and the protease inhibitor ritonavir in a head and neck carcinoma model. *Anticancer Res* 2005;25:4357–62.
49. Pore N, Gupta AK, Cerniglia GJ, Jiang Z, Bernhard EJ, Evans SM, Koch CJ, Hahn SM, Maity A. Nelfinavir down-regulates hypoxia-inducible factor 1alpha and VEGF expression and increases tumor oxygenation: implications for radiotherapy. *Cancer Res* 2006;66:9252–9.
50. Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* 2004; 4:349–60.
51. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clin Cancer Res* 2008;14: 1649–57.
52. Clamp AR, Jayson GC. The clinical potential of antiangiogenic fragments of extracellular matrix proteins. *Br J Cancer* 2005;93:967–72.
53. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; 295:2387–92.
54. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 2006;6:227–39.
55. Ensoli B. European Patent application, No. RM2001 A000210; Publication No.WO02/ 087583.
56. Deeks SG, Smith M, Holodniy M, Kahn JO. HIV-1 protease inhibitors. A review for clinicians. *JAMA* 1997;277:145–53.
57. Angiolillo AL, Sgadari C, Sheikh N, Reaman GH, Tosato G. Regression of experimental human leukemias and solid tumors induced by Epstein-Barr virus-immortalized B cells. *Leuk Lymphoma* 1995;19:267–76.
58. Sgadari C, Toschi E, Palladino C, Barillari G, Carlei D, Cereseto A, Ciccolella C, Yarchoan R, Monini P, Sturzl M, Ensoli B. Mechanism of paclitaxel activity in Kaposi's sarcoma. *J Immunol* 2000;165:509–17.
59. Frederiks WM, Mook OR. Metabolic mapping of proteinase activity with emphasis on in situ zymography of gelatinases: review and protocols 1. *J Histochem Cytochem* 2004;52:711–22.
60. Barillari G, Sgadari C, Fiorelli V, Samaniego F, Colombini S, Manzari V, Modesti A, Nair BC, Cafaro A, Sturzl M, Ensoli B. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. *Blood* 1999; 94:663–72.
61. Perry CM, Noble S. Saquinavir soft-gel capsule formulation. A review of its use in patients with HIV infection. *Drugs* 1998;55: 461–86.
62. Sadler BM, Gillotin C, Lou Y, Stein DS. In vivo effect of alpha(1)-acid glycoprotein on pharmacokinetics of amprenavir, a human immunodeficiency virus protease inhibitor. *Antimicrob Agents Chemother* 2001;45: 852–6.
63. Yeh KC, Deutsch PJ, Haddix H, Hesney M, Hoagland V, Ju WD, Justice SJ, Osborne B, Sterrett AT, Stone JA, Woolf E, Waldman S. Single-dose pharmacokinetics of indinavir and the effect of food. *Antimicrob Agents Chemother* 1998;42: 332–8.
64. Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Natl Acad Sci U S A* 1999;96:10403–8.
65. Krappmann D, Wulczyn FG, Scheidereit C. Different mechanisms control signal-induced degradation and basal turnover of the NF-kappaB inhibitor IkappaB alpha in vivo. *EMBO J* 1996;15:6716–26.
66. Horak ER, Leek R, Klenk N, LeJeune S, Smith K, Stuart N, Greenall M, Stepniewska K, Harris AL. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 1992;340:1120–4.
67. Tsoli E, Zacharatos P, Siou-Plakida D, Peros J, Evangelou K, Zavras AI, Yannoukakos D, Konstantopoulou I, Asimacopoulos PJ, Kittas C, Gorgoulis VG. Growth index is independent of microvessel density in non-small-cell lung carcinomas. *Hum Pathol* 2002;33:812–8.
68. Vartanian RK, Weidner N. Correlation of intratumoral endothelial cell proliferation with microvessel density (tumor angiogenesis) and tumor cell proliferation in breast carcinoma. *Am J Pathol* 1994;144: 1188–94.
69. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;84:1875–87.
70. Fingleton B. Matrix metalloproteinases: roles in cancer and metastasis. *Front Biosci* 2006;11:479–91.
71. Henderson DJ, Copp AJ. Role of the extracellular matrix in neural crest cell migration. *J Anat* 1997;191(Pt 4): 507–15.
72. Martin P. Wound healing—aiming for perfect skin regeneration. *Science* 1997;276: 75–81.
73. Perris R. The extracellular matrix in neural crest-cell migration. *Trends Neurosci* 1997; 20:23–31.
74. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386:671–4.
75. Collins LR, Ricketts WA, Yeh L, Cheresh D. Bifurcation of cell migratory and proliferative signaling by the adaptor protein Shc. *J Cell Biol* 1999;147:1561–8.
76. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* 2005;1755: 37–69.
77. Laurent N, de Bouard S, Guillamo JS, Christov C, Zini R, Jouault H, Andre P, Lotteau V, Peschanski M. Effects of the proteasome inhibitor ritonavir on glioma growth in vitro and in vivo. *Mol Cancer Ther* 2004;3:129–36.
78. Monini P, Sgadari C, Grosso MG, Bellino S, Di Biagio A, Toschi E, Bacigalupo I, Sabbatucci M, Cencioni G, Salvi E, Leone P, Ensoli B. Clinical course of classic Kaposi's sarcoma in HIV-negative patients treated with the HIV protease inhibitor indinavir. *AIDS* 2009;23:534–8.