HIV protease inhibitors are potent anti-angiogenic molecules and promote regression of Kaposi sarcoma

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Treatment with HIV-1 protease inhibitors (PI) is associated with a reduced incidence or regression of Kaposi sarcoma (KS). Here we show that systemic administration of the PIs indinavir or saquinavir to nude mice blocks the development and induces regression of angioproliferative KS-like lesions promoted by primary human KS cells, basic fibroblast growth factor (bFGF), or bFGF and vascular endothelial growth factor (VEGF) combined. These PIs also block bFGF or VEGF-induced angiogenesis in the chorioallantoic membrane assay with a potency similar to paclitaxel (Taxol). These effects are mediated by the inhibition of endothelial- and KS-cell invasion and of matrix metalloproteinase-2 proteolytic activation by PIs at concentrations present in plasma of treated individuals. As PIs also inhibit the *in vivo* growth and invasion of an angiogenic tumor-cell line, these data indicate that PIs are potent anti-angiogenic and anti-tumor molecules that might be used in treating non-HIV KS and in other HIV-associated tumors.

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Kaposi sarcoma (KS) is an angio-proliferative disease characterized by angiogenesis, endothelial spindle-cell growth (KS cells), inflammatory-cell infiltration and edema[1,2]. KS is associated with human herpesvirus 8 (HHV8) infection, and is particularly frequent and aggressive in HIV-1/HHV8 co-infected individuals (AIDS-KS)[1–3]. KS development is associated with HHV8 reactivation and spread to blood and tissues. Reactivation, in turn, is induced by Th-1 type cytokines (interferon-γ, interleukin-1β and tumor necrosis factor-α) that are increased in KS patients or in at-risk individuals[1,2,4]. Inflammatory cytokines (ICs) also activate vessels promoting the tissue extravasation of infected lympho-monocytes and spindle-cell progenitors[1,2]. In early-developing lesions, HHV8 load is low or undetectable whereas it is high in late-nodular lesions, which mostly show latent infection[1,2,5]. This finding suggests that HHV8 may have a key role in KS progression to a frank tumor as latently expressed viral genes may induce cell transformation[2], a feature observed in some late-nodular AIDS-KS lesions[6]. Increase of ICs can also induce production of angiogenic factors and other molecules that initiate lesion formation, as observed in HIV-1-infected individuals that develop KS or show KS progression after systemic administration of interferon-γ and interleukin-2 or tumor necrosis factor-α[7]. Other studies confirm that high HHV8 burden and expression of ICs and angiogenic factors are found in all forms of KS (refs. 1,2,7).

Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the most highly expressed angiogenic factors[8–10]. They are also increased in sera of KS patients or in at-risk individuals[11,12], and are expressed in early lesions by KS cells, endothelial cells and infiltrating cells in response to ICs (refs. 1,7,8,10,13,14). bFGF promotes in autocrine fashion the growth of KS cells and synergizes with VEGF to mediate the development of angioproliferative KS-like lesions induced by the inoculation of KS cells in nude mice[8,10,13,15–17]. Such KS-like lesions are composed of mouse cells and are induced by the factors produced by KS cells, and they are highly vascularized, closely resemble early KS and regress as KS regresses in humans[1,7]. Conversely, inoculation with bFGF in nude mice and its *in vivo* induction with ICs reproduces the vascular lesions promoted in mice by the inoculation of KS cells and those found in human KS (refs. 8,14). Finally, bFGF synergizes with the HIV-1 Tat protein released by infected cells and increases the frequency and aggressiveness of KS in HIV-1-infected individuals[8,18,19]. Thus, bFGF and VEGF are crucial to the development and progression of all forms of KS as well as to the growth of solid tumors[20].

Recent reports have described a reduced incidence[21] or the regression[22,23] of KS in HIV-1-infected patients treated with highly active antiretroviral therapy (HAART) that includes at least one HIV-protease inhibitor (PI) such as indinavir or saquinavir[24]. PIs have also been shown to directly affect cell metabolism, interfere with host or fungal proteases and block T-cell activation and dendritic-cell function[25,32], even though they were designed to selectively interfere with the catalytic site of HIV protease[24]. Given that proteases are also essential for angiogenic and inflammatory processes and for tumor growth, we reasoned that at least part of the lower incidence and regression of KS observed in PI-treated patients resulted

Table 1 Effects of PI on KS-like lesions induced by KS cells in nude mice

Treatment	Injection	No. mice with macroscopic lesions / Number of inoculated mice (%)	Mean external lesion size ($cm^2 \pm$ s.d.)
Saline	Medium	0/8 (0)	0.58 ± 0.13
Saline	KS cells	14/14 (100)	0.76 ± 0.08
Indinavir	KS cells	7/16 (43)	0.57 ± 0.06
Saquinavir	KS cells	4/16 (25)	0.56 ± 0.06

The number of mice developing lesions and the external lesion size at killing are shown. The decrease of lesion number and external size of lesions in PI-treated animals were statistically significant ($P < 0.001$ and $P < 0.01$, respectively).

from direct anti-KS and/or anti-angiogenic effect(s) of these drugs.

To test this hypothesis, we used *in vitro* and *in vivo* models of angiogenesis, KS-lesion formation and tumor growth that are devoid of any viral agents and T cells (that is, nude mice and chorioallantoic membrane (CAM) assays), and are established preclinical models to evaluate the efficacy of anti-KS and anti-angiogenic therapies[8,15,33,34]. Here we show that PIs have direct anti-angiogenic, anti-KS and anti-tumor activity at concentrations present in plasma of treated individuals.

PIs inhibit and promote regression of KS-like lesions

To study the effect of PIs on KS, we treated nude mice by intragastric gavage with indinavir, saquinavir or saline solution (as control) for two days and then injected the mice subcutaneously (s.c.) with different strains of KS cells or with their medium as negative control[5,8,10,14,15,34]. Treatment was continued for an additional five days. KS-cell inoculation promoted the development of visible angioproliferative KS-like lesions in 100% of untreated mice (Table 1). In contrast, in mice treated with PIs, lesion development was greatly reduced (43% and 25% for indinavir or saquinavir, respectively ($P < 0.001$)) (Table 1). In addition, the external lesional area in PI-treated mice was similar to the negative control ($P < 0.01$) (Table 1). Inoculation of different KS-cell strains gave similar results. Lesions were visibly florid and highly vascularized in untreated mice (Fig. 1*b*), but smaller, pail and regressive in PI-treated animals (Fig. 1*c* and *d*). Similarly, lesions in untreated mice had intense neovascularization, spindle-cell infiltration, edema and red blood-cell extravasation (Fig. 1*f*). PI-treated mice, however, showed a large necrotic area at the site of cell injection (Fig. 1*g* and *h*), involving up to 85% of the whole lesional area, and a marked reduction of both neo-formed vessels and spindle-cell infiltration, which were mostly confined at the periphery of the necrotic/regressing area (Fig. 1*g* and *h*). Similar results were observed with KS12 ($P = 0.05$) and KS8 ($P \leq 0.001$) cell strains. Thus, PIs can block the development of KS lesions in mice treated before KS-cell inoculation.

However, PIs also promoted KS regression without drug pre-treatment, as shown by starting treatment at the time of KS-cell inoculation. PI-treated mice showed lesion regression (mean external lesional area: 0.54 and 0.50 cm^2 for indinavir or saquinavir, respectively) as compared with untreated mice (1.02 cm^2) and negative controls (0.66 cm^2) ($P < 0.001$). Thus PIs can block development but also induce regression of KS. In addition, the histological features of PI-treated mice suggested that this was due to inhibition of angiogenesis.

PIs inhibit growth factor–induced angiogenic lesions

To verify whether PIs could directly inhibit angiogenesis, indinavir, saquinavir or saline was administered by intragastric gavage to nude mice for two days and treatment was continued upon s.c. injection with bFGF (1 μg) or its dilution buffer (negative control)[8,10,14]. Injection of bFGF induced the development of macroscopic angiogenic lesions in 71% of the untreated mice (Table 2 and Fig. 2*b*), as observed previously[8,10,14]. In contrast, PIs strongly inhibited angiogenic lesion formation (28% and 25% for indinavir and saquinavir, respectively ($P < 0.001$)) and either smaller lesions or no lesions were observed (Table 2 and Fig. 2*c* and *d*). This was accompanied by a strong reduction of angiogenesis and spindle-cell growth, which, in the case of total inhibition, were almost indistinguishable from the negative control. This was confirmed by staining for the endothelial-cell markers CD31 and FVIII-RA, which were reduced by up to 75% in PI-treated mice as compared with untreated controls ($P < 0.001$) (Table 2 and Fig. 2*e–h*).

As VEGF synergizes with bFGF in inducing angiogenesis and KS-lesion formation[10], mice were also inoculated with sub-optimal amounts of bFGF (0.1 μg) and VEGF (1 μg) combined[10] and treated with PIs. The combined growth factors induced lesion development in 83% of untreated mice; however, both indinavir or saquinavir reduced lesion forma-

Table 2 Effects of PI on bFGF- or VEGF-induced angiogenesis in mice or CAM

Mice

Treatment	Injection	No. mice with macroscopic lesions / No. inoculated mice (%)	Mean % of stained lesion area (± s.d.)	
			FVIII-RA	CD31
Saline	Buffer	0/22 (0)	0.20 (± 0.2)	0.06 (± 0.1)
Saline	bFGF	20/28 (71)	4.32 (± 2.0)	3.45 (± 1.7)
Indinavir	bFGF	8/28 (28)	1.32 (± 1.2)	1.00 (± 0.6)
Saquinavir	bFGF	7/28 (25)	1.13 (± 0.6)	0.83 (± 0.3)

Chorioallantoic membrane

Treatment (No. eggs)	Angiogenic factor (± s.d.)	Mean vessel number/mm^2
Saline (23)	Buffer	4.39 (± 1.12)
Saline (11)	bFGF	13.50 (± 3.03)
Indinavir (11)	bFGF	8.22 (± 2.54)
Saquinavir (13)	bFGF	6.08 (± 1.84)
Saline (8)	VEGF	13.51 (± 2.42)
Indinavir (9)	VEGF	7.70 (± 2.52)
Saquinavir (7)	VEGF	5.39 (± 0.67)
Paclitaxel (4)	bFGF	7.71 (± 1.63)

The reduction of angiogenic KS-like lesion formation and the decrease of FVIII or CD31 expression in residual lesions of PI-treated mice, or the inhibition of vessel formation by PIs in CAM were statistically significant ($P < 0.001$, $P < 0.001$ or $P < 0.05$, respectively). Administration of PIs or paclitaxel alone was not associated with toxicity and did not affect basal CAM vascularization (data not shown).

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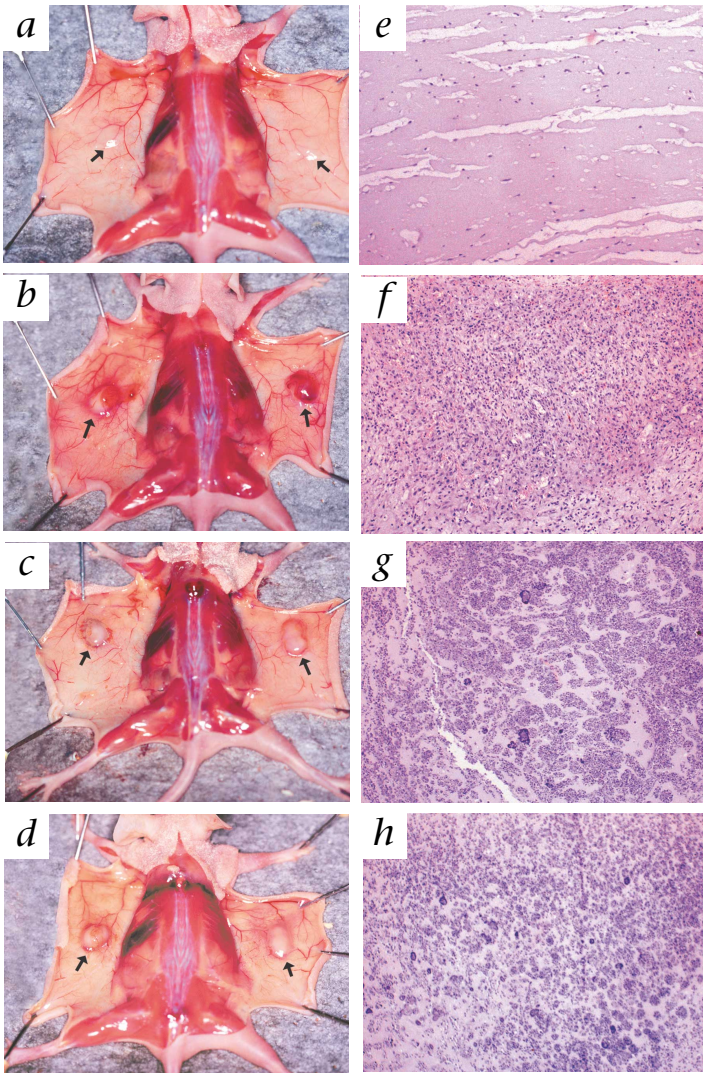

Fig. 1 PIs block the formation and promote necrosis of angiogenic KS-like lesions induced by the inoculation of KS cells in nude mice. ***a–d***, Representative examples of macroscopic lesions at the site of cell injection. Mice were injected on both sides with medium or KS cells and treated with saline or PI: *a*, medium injection and saline treatment; *b*, KS-cell injection and saline treatment; *c*, KS-cell injection and indinavir treatment; *d*, KS-cell injection and saquinavir treatment. Arrows indicate the sites of injection. ***e–h***, Histological features of the necrotic and regressing area of representative KS-like lesions induced by the inoculation of KS cells in nude mice treated or not with PIs (H&E staining; magnification, ×100). Central areas of sites of medium or KS-cell inoculation in mice treated with saline or PI are shown: *e*, medium inoculation and saline treatment; *f*, KS-cell inoculation and saline treatment; *g*, KS-cell inoculation and indinavir treatment; *h*, KS-cell inoculation and saquinavir treatment.

tion to 33% and 17% ($P < 0.05$) of treated mice, respectively. Thus, because PIs inhibit the angiogenesis and the KS-like lesion development induced by bFGF alone or in combination with VEGF, they must have direct anti-angiogenic effects.

PIs block angiogenesis at levels similar to paclitaxel

To confirm that PIs have direct anti-angiogenic effects, we used the CAM assay, an established *in vivo* assay used to measure angiogenesis[35]. As shown in Table 2, indinavir or saquinavir inhibited bFGF-induced angiogenesis to 42% and 19% of the untreated bFGF control, and to 36% and 11% of the untreated VEGF control, respectively ($P < 0.05$). Notably, the inhibition of bFGF-induced angiogenesis by both PIs was comparable with that of paclitaxel, a cytotoxic drug with both anti-tumor and anti-angiogenic activities, which is used in the therapy of KS and solid tumors[34,36] (Table 2). These data confirmed that PIs directly block angiogenesis *in vivo*.

PIs block the invasion but not the growth of HUVECs

Angiogenesis requires sequential steps that are induced by angiogenic factors. These include the degradation of the vessel basement membrane by matrix metalloproteinases (MMPs) with endothelial-cell migration into the perivascular space (invasion), and endothelial-cell proliferation[20,37,38]. To verify which of these steps was inhibited by PIs, we performed proliferation and invasion assays with primary human macrovascular (human umbilical-vein endothelial cells; HUVECs) and microvascular (human dermal microvascular endothelial cells; HDMVECs) endothelial cells with concentrations of PIs in the same range (0.1–10.0 μM) as those present in plasma of treated individuals[24].

PIs did not affect proliferation promoted by bFGF nor basal growth of both HUVECs (Fig. 3*a*) and HDMVECs (data not shown). Similarly, no effects were seen on cell survival (data not shown). In contrast, PIs totally blocked bFGF-induced invasion of HUVECs ($P < 0.001$) (Fig. 3*a*) and HDMVECs ($P \leq 0.05$) (data not shown), even at the lowest concentration (Fig. 3*a*). As cell invasion is essential for angiogenesis[20,37,38], these results explain the mechanism of angiogenic inhibition by PIs.

PIs block KS-cell invasion but not KS-cell growth

KS-cell cultures derived from different human KS lesions lose HHV8 at an early passage, contain no known virus, show the same pattern of marker expression of *in situ* KS spindle cells (that is, activated/transdifferentiated endothelial cells[1,7]), and produce high amounts of both bFGF and VEGF (refs. 8–10,13,15,17). To verify whether PIs had also direct effects on KS cells, we performed cell-growth and invasion experiments with both drugs (at 1 μM). Neither PI inhibited either KS-cell growth (Fig. 3*b*) or survival (data not shown); however, they both inhibited invasion of different KS-cell strains (Fig. 3*b*). Thus, PIs have inhibitory effects on invasion of both endothelial and KS cells.

PIs block MMP-2 activation

The invasion of endothelial cells is mediated by the proteolytic effect of active MMP-2, which degrades the vessel basement membrane allowing endothelial-cell migration[37,38]. MMP-2 is highly expressed by KS and endothelial cells *in vitro* and *in vivo* in response to bFGF (refs. 8, 19) and released as a proenzyme (latent 72-kD MMP-2), which is proteolytically activated to the 64/62-kD form through a complex mechanism involving other proteases[37]. Gel-activity assays[19] were therefore performed to verify the effect of indinavir on MMP-2 activity in endothelial cells. Indinavir had little or no effect on the synthesis of the latent 72-kD MMP-2; however, it dose-dependently blocked conversion of latent MMP-2 to its 64/62-kD active form (Fig. 3*c*). Similar results were observed with saquinavir (data not shown). Thus, PIs

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block the activation of proteases that are key for angiogenesis and tumor-cell invasion[37,38].

PIs block EA-hy 926 tumor growth and cell invasion

To determine whether PIs could inhibit the growth of a tumor model, we inoculated nude mice with the EA-hy 926 cell line. This cell line, which is derived from a hybrid between HUVECs and human lung adenocarcinoma cells[39], retains most of the endothelial-cell markers and is used as an angiogenic tumor model[40]. PIs were administered to nude mice starting two days before tumor-cell inoculation. Tumors arose in 83% of untreated mice but only in 33% and 25%, respectively, of mice treated with indinavir or saquinavir ($P < 0.05$) (Table 3). Similarly, the external tumor area was reduced in mice treated with both PIs ($P < 0.05$), reaching the size of the negative controls (Table 3). Residual tumors in treated animals showed a strong reduction of both tumor growth and angiogenesis as determined histologically and by staining with anti-FVIII-RA or anti-CD31 antibodies as compared with controls ($P < 0.001$) (Table 3). Inhibition of tumor growth was also observed in the absence of drug pre-treatment. In these animals, external tumor size was reduced by more than 50% as compared with untreated controls ($P < 0.001$). Finally, the invasion but not growth of EA-hy 926 cells (Table 3) was inhibited by both PIs (at 1 μM) ($P < 0.05$). Thus, PIs can inhibit *in vivo* growth of angiogenic tumors by directly blocking angiogenesis and tumor-cell invasion.

Discussion

The lower incidence and regression of KS observed in HIV-1-infected individuals treated with PIs (refs. 21–23) may result from a variety of drug effects. These may include one or more of the following: 1) the inhibition of HIV replication and production and release of the HIV-1 Tat protein, a KS progression factor[5,8,14,18,40,41]; 2) the reduction of the synthesis of ICs by HIV-1-infected or activated T cells[28,30,42], which triggers the production of angiogenic factors[1,2,5,7,10,13,14] and HHV8 reactivation[1,2,4]; 3) the direct inhibitory effects on HHV8 infection; 4) the improvement of protective immune responses against HHV8 (refs. 1,43,44); and 5) direct blocking effects on KS-lesion formation.

In relation to HHV8, although KS regression is often associated with a reduction of HHV8 load in peripheral blood mononuclear cells and tissues[1,22,23], no direct inhibitory effects of PIs have been detected by us (data not shown) or others[45] on HHV8-infected cells before or after virus reactivation with tetradecanoyl phorbol acetate (TPA). Previous data indicate an improvement of T-cell responses or natural killer–cell activity against HHV8-infected cells in patients with regressing KS responding to HAART (refs. 1,43). However, other studies indicate a lack of association between the increase of $CD4^+$ T-cell counts, HIV-1 suppression and KS regression upon HAART (refs. 46,47). In addition, the rate of KS regression with non PI-combination therapies, which are still efficacious in blocking HIV replication[48], is significantly lower as compared with PI HAART and similar to the low rate of spontaneous AIDS-KS regression in years preceding the use of PI-combination therapies[49]. By contrast, most studies indicate that a significant decrease of KS incidence and increased KS regression occurred only with PI HAART (refs. 21–23).

Thus, in addition to HIV suppression and immunoreconstitution, PIs must exert other activities on KS. This is also suggested by recent data indicating that PIs inhibit dendritic-cell function, antigen presentation and proteosome function[25,29], effects that reduce T-cell activation and IC production also in the absence of HIV (refs. 30,42). This may explain the reduction of HHV8 reactivation and virus load[22,23] and the lowered serum levels of bFGF (ref. 12) observed in PI-treated patients. However, this also suggests that the effect of PIs on KS may be largely dependent on direct and unknown effects of these drugs.

We have shown here that PIs have direct anti-angiogenic,

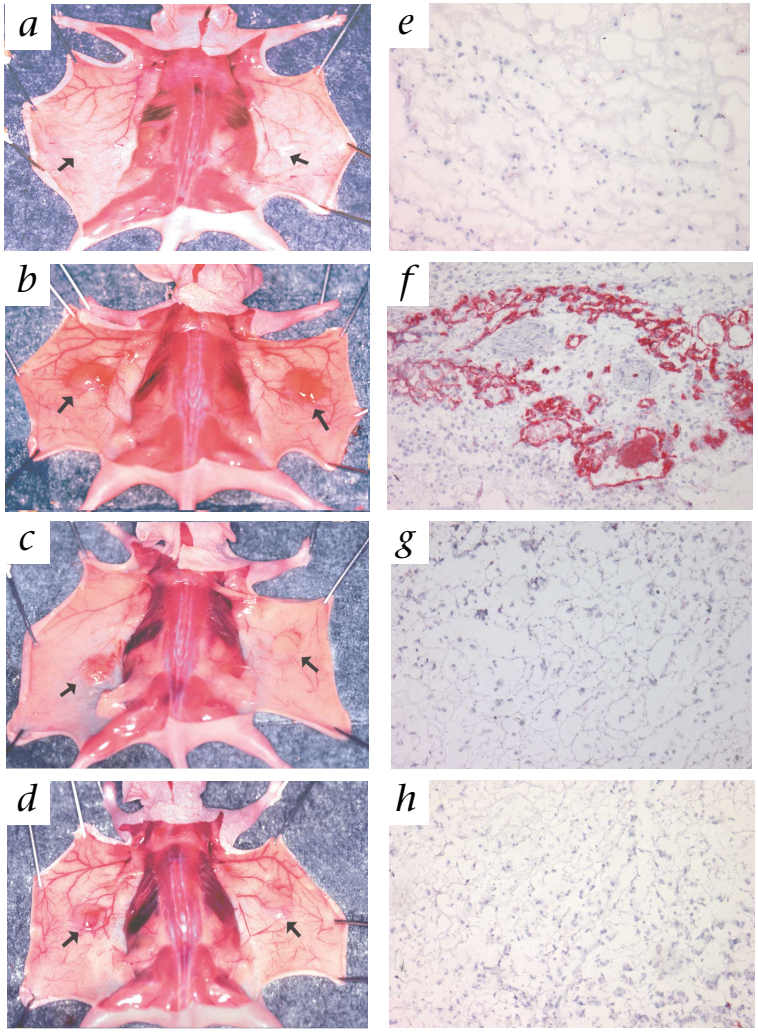

Fig. 2 PIs block the formation of bFGF-induced angiogenic lesions in nude mice. ***a–d***, Representative examples of macroscopic lesions at the site of bFGF injection. *a*, Mice injected on both sides with buffer and treated with saline. *b*, Mice injected on both sides with bFGF and treated with saline. *c*, Mice injected on both sides with bFGF and treated with indinavir. *d*, Mice injected on both sides with bFGF and treated with saquinavir, respectively. ***e–h***, Representative examples of CD31 expression at the injection sites of mice injected with bFGF or buffer and treated with saline, indinavir or saquinavir (magnification, ×100). ***e***, Site of buffer inoculation in a mouse treated with saline. *f*, Site of bFGF inoculation in a mouse treated with saline. *g*, Site of bFGF inoculation of in a mouse treated with indinavir. *h*, Site of bFGF inoculation of a mouse treated with saquinavir.

Table 3 Effects of PI on angiogenic tumors induced by EA-hy 926 cells

Mice

Injection	Treatment	No. mice with macroscopic lesions / No. inoculated mice (%)	External lesion size (cm^2 ± s.d.)	Mean % stained tissue area (± s.d.)	
				FVIII-RA	CD31
Medium	Saline	0/8 (0)	0.58 ± 0.15	0	0
EA-hy 926	Saline	10/12 (83.3)	0.71 ± 0.2	2.91 (± 1)	2.30 (± 1.7)
EA-hy 926	Indinavir	4/12 (33.3)	0.63 ± 0.17	1.01 (± 1.5)	0.70 (± 0.6)
EA-hy 926	Saquinavir	3/12 (25)	0.55 ± 0.14	0.71 (± 0.8)	0.08 (± 0.1)

Cell invasion

Stimulus	Treatment	Mean invaded cells/field (± variation range)
Buffer	Buffer	8.5 (± 0.5)
bFGF	Buffer	17 (± 4)
bFGF	Indinavir	13 (± 3)
bFGF	Saquinavir	9 (± 1)

The decrease of macroscopic angiogenic tumor lesion formation, external lesion size, and FVIII-RA or CD31 expression in PI-treated animals was statistically significant ($P < 0.05$, $P < 0.05$ and $P < 0.001$, respectively) as compared with saline-treated mice. PIs had no effects on EA-hy 926-cell proliferation (data not shown). The block of EA-hy 926-cell invasion (average and variation range of 2 independent experiments each in duplicate) by PIs was statistically significant ($P < 0.05$).

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anti-KS and anti-tumor effects. In particular, the *in vitro* and *in vivo* models of angiogenesis, KS-lesion formation and tumor growth used here contain neither HIV nor HHV8, and they are observed *in vivo* in nude mice or CAM assays and, therefore, in the absence of T cells. This combination of features supports a direct anti-angiogenic and anti-tumor effect of these drugs, which may contribute to the inhibition of KS seen in treated patients. In fact, by inhibiting endothelial-cell invasion, PIs block angiogenesis and tumor growth. Notably, PIs block the angiogenesis mediated by both bFGF and VEGF, which are the two key factors for KS-lesion formation. However, PIs did not block the production or release of bFGF or VEGF by KS cells (data not shown), as already suggested by the lack of effects on KS-cell growth, which requires bFGF in autocrine fashion[1,7,15].

bFGF and/or VEGF are also produced by most tumors to support their own angiogenesis[20]. Blocking endothelial-cell invasion is the basis of current anti-angiogenic and anti-tumor therapies[20,37,38]. However, PIs also directly inhibit KS- and tumor-cell invasion and therefore exert an additional effect. The inhibitory effect of PIs is similar to that observed with paclitaxel, a microtuble-binding drug with cytotoxic and anti-angiogenic activity used in the therapy of KS and solid tumors[34,36]. PIs also inhibited the growth of EA-hy 926 tumor cells in nude mice. Finally, PIs not only blocked the development of KS or tumors in mice treated with the drugs before cell inoculation, but they were also effective when given at the time of cell inoculation. This suggests that PIs can both prevent development and induce regression of KS or tumors, in agreement with both the lower incidence and the regression of KS seen with PI HAART.

Endothelial-cell invasion requires the degradation of the basement membrane by MMP-2 that is produced by endothelial cells in response to angiogenic factors[8,18,19,37]. MMP-2 is also produced by KS cells and highly expressed in human KS lesions[19], and it is required by tumor cells to invade tissues[37]. Blocking MMP-2 leads to inhibition of endothelial-cell invasion and, consequently, angiogenesis[37,38]. Similarly, treatment of endothelial cells with PIs was found to reduce the bFGF-induced activation of pro-MMP-2 to its active forms. Thus, although other proteases may be involved, inhibition of MMP-2 activity may be one of the mechanisms by which PIs inhibit cell invasion. However, no direct inhibitory effects of PIs were observed on recombinant activated MMP-2 (data not shown). In fact, MMP-2 cleaves after a glycine, whereas the HIV protease is an aspartyl protease, and MMP-2 has no sequence homology with the HIV protease catalytic site[26] targeted by PIs (data not shown). Thus, PIs may inhibit one or more steps leading to MMP-2 activation.

These results indicate that PIs are promising anti-angiogenic and anti-tumor compounds, and, in addition to AIDS patients with KS or at risk of KS, they should also be evaluated for the treatment of other tumors in HIV-infected individuals and for the therapy of KS in seronegative individuals. In particular, PIs may represent a pathogenetic therapy for all forms of KS given that—in addition to directly blocking angiogenesis—they also reduce T-cell activation and production of ICs (refs. 30,42). Consequently, they inhibit HHV8 reactivation and angiogenic factor production[1,2,7,10,13,18,22,23]. In addition, combination therapies containing at least one PI should be evaluated for the treatment of late-nodular KS—an aggressive cancer that is often refractory to chemotherapy alone, as suggested by the remission of AIDS-KS by associating PIs with paclitaxel[50]. By analogy, PIs should be investigated, alone or in combination with other drugs, for the therapy of other tumors in HIV-1-infected individuals.

Methods

Cell cultures. HUVECs and HDMVECs (BioWhittaker, Verviers, Belgium) (passages 3–7), primary AIDS-KS spindle-cell cultures (KS3, KS8 and KS12, passages 6–13) and the EA-hy 926 cell line, a HUVEC–adenocarcinoma immortalized cell hybrid, were derived and cultured as described[13,14,18,39-41].

Induction and treatment of lesions induced by tumor cells. The same indinavir or saquinavir formulation (Merck Sharp & Dohme, Haarlem, the Netherlands, and Roche, Hertfordshire, Great Britain, respectively) administered to AIDS patients was dissolved in 0.4 ml saline solution and administered by intragastric gavage to nude mice (5–6-wk-old female BALB/c nu/nu from Charles River, Calco, Italy). No systemic nor organ toxicity was ever observed in PI-treated animals (data not shown). Mice were treated with the same doses of PIs given to HIV-infected patients[24]

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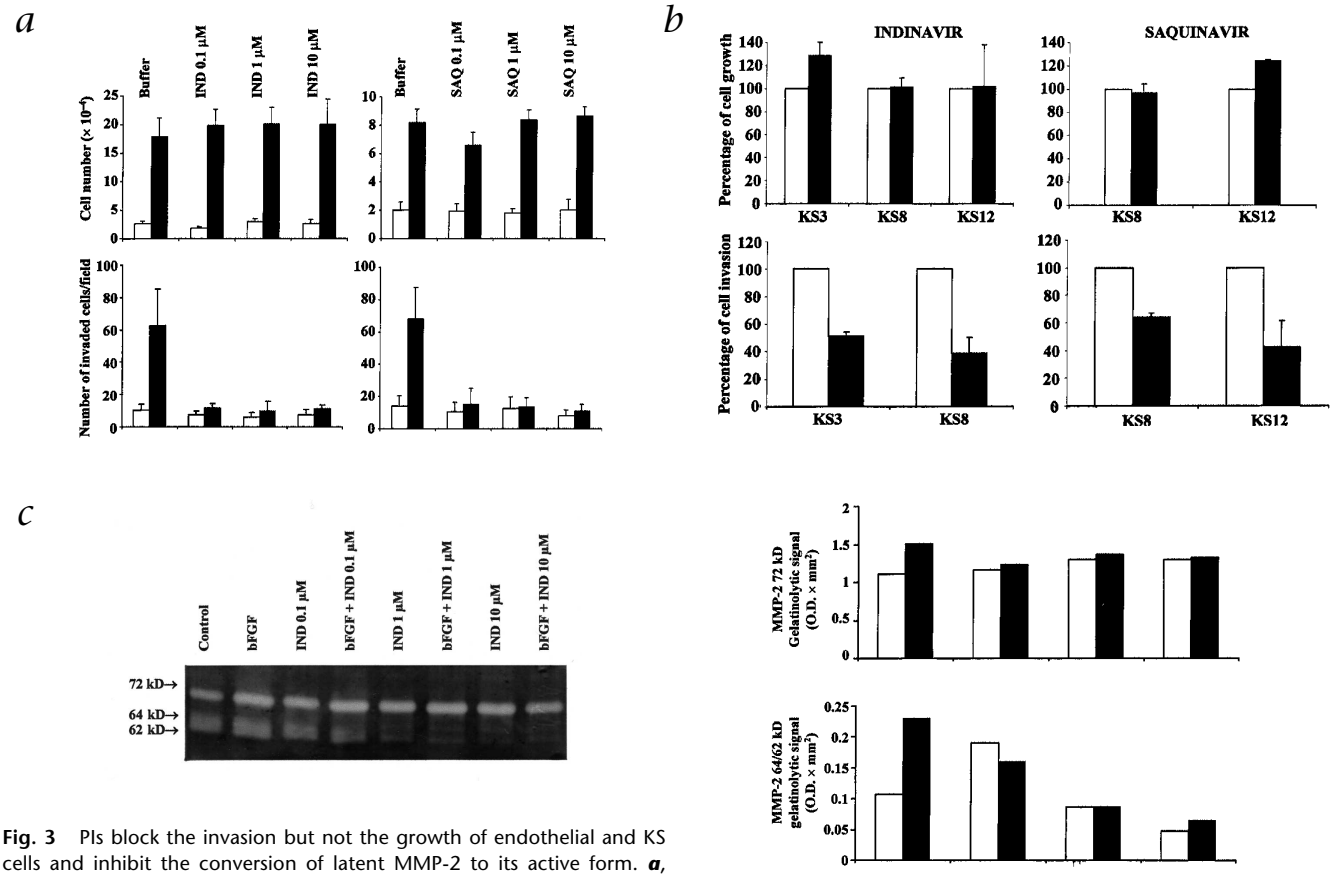

Fig. 3 PIs block the invasion but not the growth of endothelial and KS cells and inhibit the conversion of latent MMP-2 to its active form. ***a***, HUVEC growth (top) or invasion (bottom) assays. Data expressed as mean number (± s.d.) of cells grown or invaded in response to bFGF (■) or buffer (□) in the presence of PI or resuspension buffer, as indicated. ***b***, KS-cell growth (top) or invasion assays (bottom) (KS3, KS8 or KS12 cell strains) expressed as percentage of cells grown or invaded in absence or presence of PI (1 μM) (■) as compared with PI-buffer (□). The number of KS cells grown or invaded in the presence of buffer was given a 100% value in order to normalize for the different levels of basal growth or invasion of the different cell strains. The average and the variation range of independent experiments are shown. ***c***, Representative zymogram analysis of concentrated supernatants from HUVECs stimulated with bFGF (■) or its buffer (□) and cultured with indinavir. Top, arrows indicate the destained areas due to the gelatinolitic activity corresponding to the latent (72 kD) or the active (64 and 62 kD) MMP-2 forms. Middle and bottom panels show the densitometric quantification of the destained areas corresponding to the gelatinolitic activity of the latent 72-kD or the active 64/62-kD forms of MMP-2 released by the cells. Results expressed as optical densities of destained bands.

(70 mg/kg/day of indinavir or 36 mg/kg/die of saquinavir) or with saline solution (control mice) once a day for 7 d, starting 2 d prior to KS-cell injection (pre-treatment), or for a total of 5 d starting at the time of cell injection. In this case, indinavir or saquinavir (10 μM) were mixed with cells in Matrigel. Mice were inoculated s.c. into the lower back with different KS-cell strains (KS8 or KS12, 2–3 × 10^6 cells per mouse), EA-hy 926 cells (3 × 10^6 cells per mouse) or with the resuspension medium, all mixed with 0.2 ml (1:1) of growth factor–depleted Matrigel (BD Biosciences, Bedford, Massachusetts) as described[8]. Lesion size was evaluated daily by caliper measurement. 5 d after cell inoculation mice were killed and the sites of injection examined macroscopically and frozen in OCT for histochemistry or fixed in formalin for histological examination[8,15]. The central necrotic area present in KS-like lesions was quantified by capturing digital images (magnification, ×25) corresponding to the whole tissue section and by calculating the percentage of tissue showing cells with picnotic nuclei and undefined cell margins by using the KS300 (Carl Zeiss, Jena, Germany) image-analysis software. The care and use of mice were in accordance with the European Community and institutional guidelines.

Induction and treatment of lesions induced by growth factors. Nude mice were pre-treated with PIs or saline solution as described above, injected s.c. with human recombinant bFGF (1 μg) (Roche), or with bFGF and VEGF-A_{165} (R&D systems, Minneapolis, Minnesota) combined (0.1 and 1 μg, respectively) mixed with 0.2 ml Matrigel and PIs (1 μM) or saline solution, and treated for an additional 5 d before killing.

Immunohistochemistry. Frozen tissue sections were fixed with cold acetone and stained with rabbit anti-human FVIII-RA polyclonal antibodies (DAKO, Glostrup, Denmark; 1:2000 dilution) or anti-mouse CD31 rat monoclonal antibody (BD Biosciences; 1:1000 dilution) as described[14,34]. Digital images (magnification, ×200) were captured by a color CCD camera (Zeiss) and analyzed by acquiring 4–9 microscopic fields (about 0.15 mm^2 per field) corresponding to the whole histological sections. Staining was quantified by the KS300 (Zeiss) image analysis software and expressed as the percentage of positive area over the total tissue area.

CAM assay. CAM assays were performed with 1 mm^3 sterilized gelatin sponges (Gelfoam; Upjohn, Kalamazoo, Michigan) adsorbed with bFGF or VEGF (1 μg or 100 ng, respectively) in 5 μl of PBS, and with buffer, PI (10 μM) or paclitaxel (250 nM) (Bristol–Myers Squibb, Princeton, New Jersey) as described[35]. CAM were examined daily until day 12 under an Olympus SZX9 stereomicroscope. Images (1024 × 1024 pixels) were captured at a distance of 2 mm from the edge of the sponge using a cooled digital CCD ORCA camera (Hamamatsu Photonics Italia, Arese,

Italy). Vessel numbers were quantified with the ImageProPlus 4.0 imaging software (Media Cybernetics, Silver Spring, Maryland) in 3 randomly selected areas per egg (1 mm^3).

Cell-proliferation assay. HUVECs, HDMVECs or KS cells were seeded in triplicate or duplicate wells in gelatin-coated 12-well plates (1.5×10^4, 2×10^4 or 1×10^3 cells per well, respectively) in the presence of PI (0.1–10 μM) or dilution buffer (PBS). Assays were performed by the cell-counting method as described[15,18]. For all the *in vitro* studies, PIs as endotoxin-free pure powder (gift of Merck Sharp & Dohme and Roche) were resuspended in distilled water.

Cell-invasion assay. HUVECs, HDMVECs, KS cells or EA-hy 926 cells were cultured for 5–6 d with PI or buffer (control). Invasion assays were performed with the Boyden chamber as described[18] in the presence of PI or buffer. bFGF (20 or 50 ng/ml) was used as chemoattractant. The number of invaded cells was evaluated as described[18].

Gelatinolytic activity assay. HUVECs were cultured for 24 h in 10% FBS RPMI 1640 with PI (0.1–10 μM) or buffer with or without bFGF (100 ng/ml). Cells were incubated overnight in serum-free medium containing PIs and cell supernatants analyzed by gel zymography as described[19]. Densitometry of destained areas was quantified with an Imaging Densitometer GS-700 connected to a Macintosh Performa computer with Multi-Analyst software (Bio-Rad).

Statistical methods. Statistical significance of PI effects were determined by the standard test for proportions (macroscopic lesions), the generalized equation estimate (size of lesion), the one-way analysis of variance (ANOVA) and the Student–Neuman–Keuls test (CAM assays), and Student's *t*-test or log linear model (*in vitro* assays). Statistical analysis was performed using the STATA 7 software (STATA, College Station, Texas) or the STATISTICA software version 4.5 (StatSoft, Tulsa, Oklahoma).

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Competing interests statement

The authors declare that they have no competing financial interests.

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