Evaluation of Antitumoral Properties of the Protease Inhibitor Indinavir in a Murine Model of Hepatocarcinoma

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Abstract

Purpose: Accumulating evidences show a higher incidence of hepatic neoplasm in HIV/hepatitis C virus (HCV)–coinfected individuals compared with HCV-monoinfected patients. Treatment with HIV-1 protease inhibitors inhibited cancer-promoted angiogenesis in HIV-infected patients affected by Kaposi sarcoma. We aimed to evaluate the antineoplastic potential activities of the protease inhibitor indinavir (Crixivan) in *in vitro* and *in vivo* hepatocarcinoma models.

Experimental Design: We analyzed effects of indinavir on cell growth and invasiveness in Huh7 and SK-HEP-1 hepatocarcinoma cell lines and on *in vivo* tumor growth of the same cells in nude mice. Morphologic and molecular analyses on explanted tumors were carried out to evaluate vascularization and apoptosis.

Results: We observed a reduced ability to invade an *in vitro* extracellular matrix for both cell lines treated with indinavir compared with controls ($P = 0{,}001$). Moreover, indinavir treatment was able to inhibit matrix metalloproteinase-2 proteolytic activation, whereas there was no effect on cell proliferation. The drug was also able to delay *in vivo* tumor growth. The inhibition of tumor growth was statistically significant from days 6 to 21 ($P = 0.004$ and $P = 0.003$, respectively). Moreover, the drug showed antiangiogenic and proapoptotic actions, as revealed by vessel count and apoptotic index by terminal deoxynucleotide transferase–mediated nick end labeling in explanted tumors. Finally, treatment with indinavir did not block the production of vascular endothelial growth factor in the tumors.

Conclusion: Indinavir could be helpful to prevent the development of hepatocarcinomas in HIV/HCV–coinfected individuals. In view of the current trend to substitute protease inhibitors with other antiretroviral agents, this information may have clinical implications.

Despite the introduction of new molecules in multidrugs protocols, chemotherapy treatment of many solid neoplasms still remains unsatisfactory (1–3). Numerous studies have shown that cytokines and growth factors play a key role in tumor growth, angiogenesis, inhibition of host defences, and metastatic cascade (4, 5). Among the others, basic fibroblast growth factor and vascular endothelial growth factor (VEGF) are highly expressed in many human and canine tumors (6–9), where they are responsible for neoangiogenesis. Moreover, VEGF and basic fibroblast growth factor seem to play a pivotal role in the development and progression of Kaposi sarcoma and other solid neoplasms (10, 11). Several preclinical studies in rodents have suggested a possible successful use of different molecules with antiangiogenic properties (12, 13).

HIV protease inhibitors are antiretroviral drugs able to block the active site of HIV aspartyl protease, preventing production of infectious viral particles. Their use in combination with nucleoside inhibitors of HIV reverse transcriptase (highly active antiretroviral therapies) has led to a better clinical outcome in AIDS patients (14). Recent publications suggest that protease inhibitors used in the therapy of HIV-1 patients could have antineoplastic properties through a mechanism of inhibition of the growth of tumor-induced blood vessels, as suggested by the regression of Kaposi sarcomas in HIV-1 human herpes virus 8–coinfected patients (15–17).

To evaluate the clinical potentials of the protease inhibitor indinavir, we did *in vitro* studies in a hepatocarcinoma model. The rationale for this choice lays in the high incidence of hepatic neoplasm in HIV-affected individuals. Hepatitis C virus (HCV) and HIV frequently coexist due to shared routes

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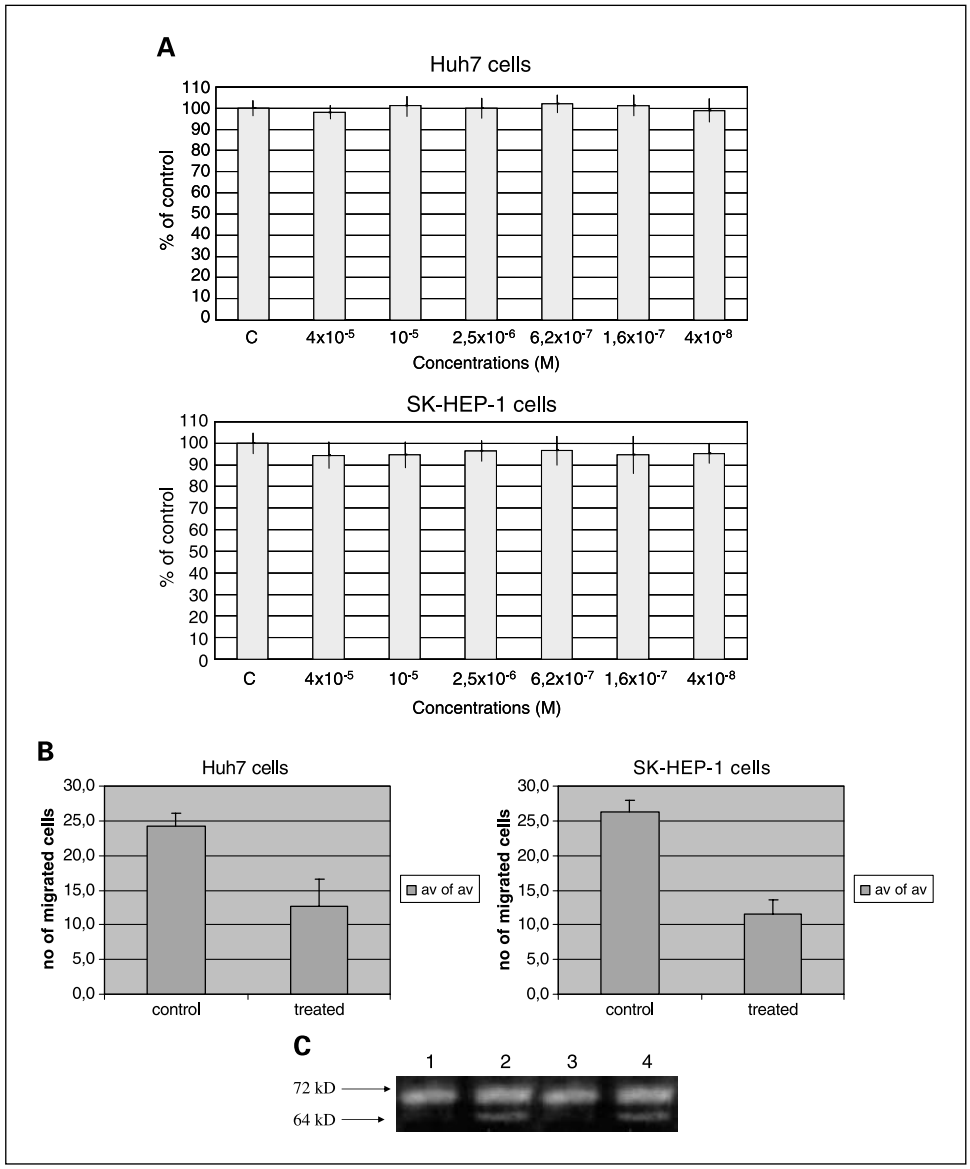

Fig. 1. Indinavir inhibits cell invasion and MMP-2 activation but not cell proliferation of the Huh7 and SK-HEP-1 hepatocarcinoma cells in vitro. *A, in vitro* proliferation assay. Proliferation rate was expressed as the amount of orange formazan dye spectrophotometrically determined at 492 nm. *B, in vitro* chemoinvasion assay. Cell invasivity was expressed as the number of invaded (migrated) cells on the lower surface of the coated membrane. *C, in vitro* gelatinolytic assay. Concentrated supernatants from Huh7 and SK-HEP-1 cells stimulated with bFGF and cultured with indinavir. *Lanes 2* and *4,* Huh7 and SK-HEP-1 cells stimulated with bFGF; *lanes 1* and *3,* Huh7 and SK-HEP-1 cells stimulated with bFGF and cultured with indinavir.

of transmission. The prevalence of HCV infection among HIV-positive patients averages about 35% in the United States and Europe, but in clinical populations where there is a great prevalence of i.v. drug use as a risk factor for acquiring HIV, this value may be as high as 80% to 90% (18). HIV/HCV–coinfected patients with ongoing HIV viremia have a faster rate of HCV-related liver fibrosis progression and a more rapid progression to liver failure or hepatocellular carcinoma than HCV-monoinfected persons. In the past, the effect of HCV on overall morbidity and mortality of coinfected patients was minimal, due to the poor prognosis of HIV. However, since the introduction of highly active antiretroviral therapies, HCV has become a significant pathogen in this population. HIV clearly exacerbates HCV infection and accelerates progression to cirrhosis, end-stage liver disease, and hepatocellular carcinoma (19–21).

HCV coinfection dramatically promotes the development of cirrhosis and hepatic carcinoma that has a more aggressive clinical course compared with monoinfected patients (22). Thus, preventive strategies should be implemented in the management of HIV/HCV–coinfected patients.

Materials and Methods

Cell cultures. Huh7 cell line is an hepatoma cell line established from an hepatocellular carcinoma (23). The cells were cultured in DMEM plus 10% fetal bovine serum in a 5% CO_2 atmosphere. SK-HEP-1 cell line is an hepatocarcinoma cell line derived from the ascites of an hepatic carcinoma. The cells were cultured in EMEM, supplemented as suggested by the American Type Culture Collection (Rockville, MD).

Chemicals. For all the *in vitro* and *in vivo* experiments, indinavir as endotoxin-free pure powder (Crixivan, kindly provided by Merck Sharp and Dohme Italia, Rome, Italy) was resuspended in distilled water at the concentrations indicated.

Proliferation assay. Huh7 and SK-HEP-1 cells were seeded in a 96-well plate (5×10^3 per well) 12 hours before the experiment. Then, cells were incubated for 24 and 48 hours with indinavir at concentrations ranging from 40 μmol/L to 40 nmol/L. Cell growth in untreated cells was compared with cell growth in cells treated with indinavir using

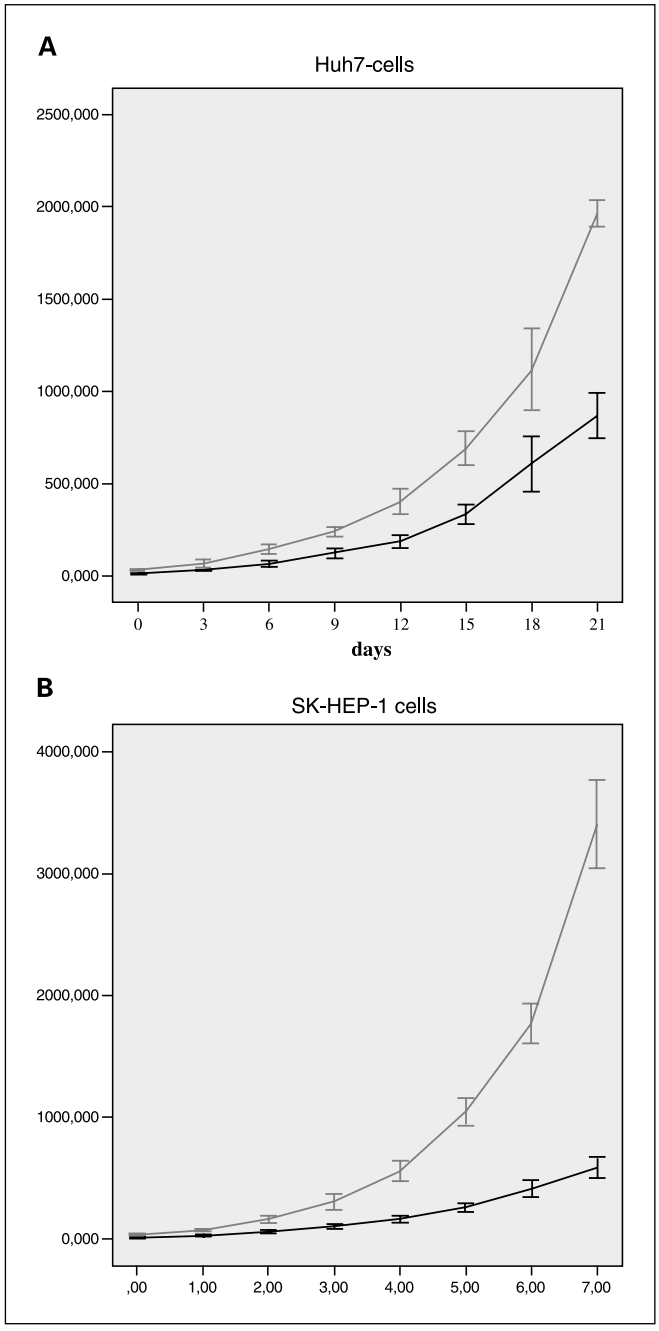

Fig. 2. Indinavir inhibits the growth of the Huh7 and SK-HEP-1 hepatocarcinoma cells *in vivo* in nude mice. *A, in vivo* growth rates of the Huh7 cells in mice treated with indinavir (*black line*) or with saline solution (*gray line*). The inhibition of tumor growth was statistically significant from days 6 to 21. *Points,* average; *bars,* SD. *B, in vivo* growth rates of the SK-HEP-1 cells in mice treated with Indinavir (*black line*) or with saline solution (*gray line*). The inhibition of tumor growth was statistically significant from days 6 to 21. *Points,* average; *bars,* SD.

the Cell Proliferation kit II (Roche Molecular Biochemicals, Indianapolis, IN), following manufacturer's instructions. This method employs a colorimetric procedure based upon the tetrazolium salt sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate, which is transformed in a formazan dye only in metabolically active cells. The formazan dye was measured after 4 hours at a 492 nm wavelength. Values ± SD are the average of three experiments done in quadruplicate.

In vitro *invasion assay*. The chemoinvasion assay was done as previously described (24) with some minor modifications. Briefly, Huh7 and SK-HEP-1 cells were cultured for 5 days with indinavir or PBS buffer. Invasion assays were done, setting the cells in inserts incorporating 6.4-mm-diameter membranes (8-μm pore size; Becton Dickinson, Franklin Lakes, NJ). Each membrane was coated with 10 μg of Matrigel basement membrane matrix (Becton Dickinson) and adsorbed with PBS buffer or Crixivan (10 μmol/L). Invading cells on the lower surface of the membrane were counted by optical microscopy. Ten fields per each membrane were counted. Each experiment was done at least five times. Spearman's rank correlation or Fisher's exact test was used to assess relationship between ordinal data. Two-tailed $P \leq 0.05$ was considered significant. SPSS software (version 10.00; SPSS, Chicago, IL) was used for statistical analysis.

***Gelatinolytic activity assay*.** Huh7 and SK-HEP-1 cells were cultured for 24 hours with indinavir or buffer and stimulated with basic fibroblast growth factor at 100 ng/mL (R&D Systems, Minneapolis, MN). Cells were incubated overnight in serum-free medium containing indinavir, and cell supernatants were analyzed by gel zymography as described (17).

In vivo *analysis of tumor growth inhibition*. Huh7 and SK-HEP-1 cells (5×10^6) were injected s.c. into the back (between scapulae) of male CD1 *nu/nu* mice (Charles River, Calco, Italy) in a final volume of 200 μL. Ten mice for each experimental point were employed. Tumor volume was monitored by caliper measurement. Mice were treated with Crixivan administered by intragastric gavage at the dose of 70 mg/kg/d, or with saline solution (control mice), for 3 weeks. Treatment started the day of cell inoculation. One week after the last indinavir administration, animals were sacrificed, and tumors were removed and processed for RNA and protein extraction and histologic and immunohistochemical analyses. In a second trial, treatment with crixivan or buffer started when tumor measurement was possible, and the administration was continued for 3 weeks from first tumor evaluation. Descriptive statistical analysis was used to monitor tumor volume modifications (expressed as median value and 95% confidential interval of tumor volumes). All procedures involving animals and their care were conducted in conformity with the institutional guidelines, in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare No. 8, G.U., July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996). Sheffe test and two-way ANOVA analysis were used to compare tumor growth (tumor volume) of different groups (multiple comparisons). Two-tailed $P \leq 0.05$ was considered significant.

***Western blotting*.** Cell lysates were prepared by treating tissues with ice-cold lysis buffer [20 mmol/L Tris/HCl (pH 8), 10% NP40, 10% glycerol, 137 mmol/L NaCl, 10 mmol/L EDTA (pH 8), and Complete; Roche Applied Science, Mannheim, Germany] for 20 minutes on ice followed by centrifugation at 4°C for 15 minutes to sediment particulate materials. The protein concentration was measured using Bradford Protein Assay (Bio-Rad Laboratories; Hercules, CA). Proteins (35 μg) were separated on 10% SDS-PAGE gels and then transferred on polyvinylidene difluoride membrane. Membranes were incubated with VEGF monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 or with anti-actin antibody (Santa Cruz Biotechnology), diluted 1:1,000, to normalize the sample loading. Horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) were used at 1:3,000 dilution. Antibodies reactions were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia, Uppsala, Sweden). All experiments were done in duplicate.

***Histology and immunohistochemistry*.** Briefly, sections from each specimen were cut at 3 to 5 μm, mounted on glass, and dried overnight at 37°C. All sections then were deparaffinized in xylene, rehydrated through a graded alcohol series, and washed in PBS. This buffer was used for all subsequent washes and for dilution of the antibodies.

Light microscopic examination was done after staining with H&E and hematoxylin/Van Gieson. For immunohistochemistry, tissue sections were heated twice in a microwave oven for 5 minutes each at 700 W in citrate buffer (pH 6) and then processed with the standard streptavidin-biotin-immunoperoxidase method (DAKO Universal kit, DAKO Corp., Carpinteria, CA). Rabbit anti-human FVIII-RA polyclonal antibody and anti-CD31 monoclonal antibody from DAKO were used at a 1:100 dilution. These two antibodies cross-reacted with endothelial cells from mouse vessels (data not shown). All the primary antibodies were incubated for 1 hour at room temperature. Diaminobenzidine was used as the final chromogen and hematoxylin as the nuclear counterstain. Negative controls for each tissue section were done leaving out the primary antibody. Positive controls included in each experiment consisted of tissue previously shown to express the antigen of interest. Two observers (V.E. and A.B.) evaluated the staining pattern of the two proteins separately and scored the protein expression in each specimen by scanning the entire section and estimating the number of vessels visible for high-power field 10×20. The level of concordance, expressed as the percentage of agreement between the observers, was 92%. In the remaining specimens, the score was obtained after collegial revision and agreement. Spearman's rank correlation or Fisher's exact test were used to assess relationship between ordinal data. Two-tailed $P \leq 0.05$ was considered significant.

Terminal deoxynucleotide transferase–mediated nick end labeling assay. Terminal deoxynucleotide transferase–mediated nick end labeling reaction was done using the peroxidase-based Apoptag kit (Oncor, Gaithersburg, MD). Terminal deoxynucleotide transferase–mediated nick end labeling–positive cells were detected with diaminobenzidine and H_2O_2 according to the supplier's instructions. The experiment was repeated on different sections for each specimen (two to four). One hundred random fields ($\times 250$) per section were analyzed (12.5 mm^2). The same statistical analyses used for the immunohistochemical quantification were applied.

Results

***Indinavir inhibits cell invasion and matrix metalloproteinase-2 activation but not cell proliferation of the Huh7 and SK-HEP-1 hepatocarcinoma cells* in vitro.** To verify whether indinavir had a direct effect on Huh7 and SK-HEP-1 cells, we did cell growth and invasion experiments. Indinavir, used at concentrations ranging from 40 μmol/L to 40 nmol/L, did not substantially affect cell proliferation of Huh7 and SK-HEP-1 cells at 48 hours (Fig. 1A). On the other hand, we were able to show a reduced ability to invade an *in vitro* constituted extracellular matrix for both cell lines treated with indinavir compared with the untreated cells ($P = 0.001$; Fig. 1B). Matrix metalloproteinase-2 (MMP-2) is highly expressed in hepatocarcinoma cells (25). It is released as a proenzyme (latent 72-kDa MMP-2) and is proteolitically activated to the 64/62 kDa by a complex mechanism involving several proteases, when cells are stimulated with basic fibroblast growth factor (26).

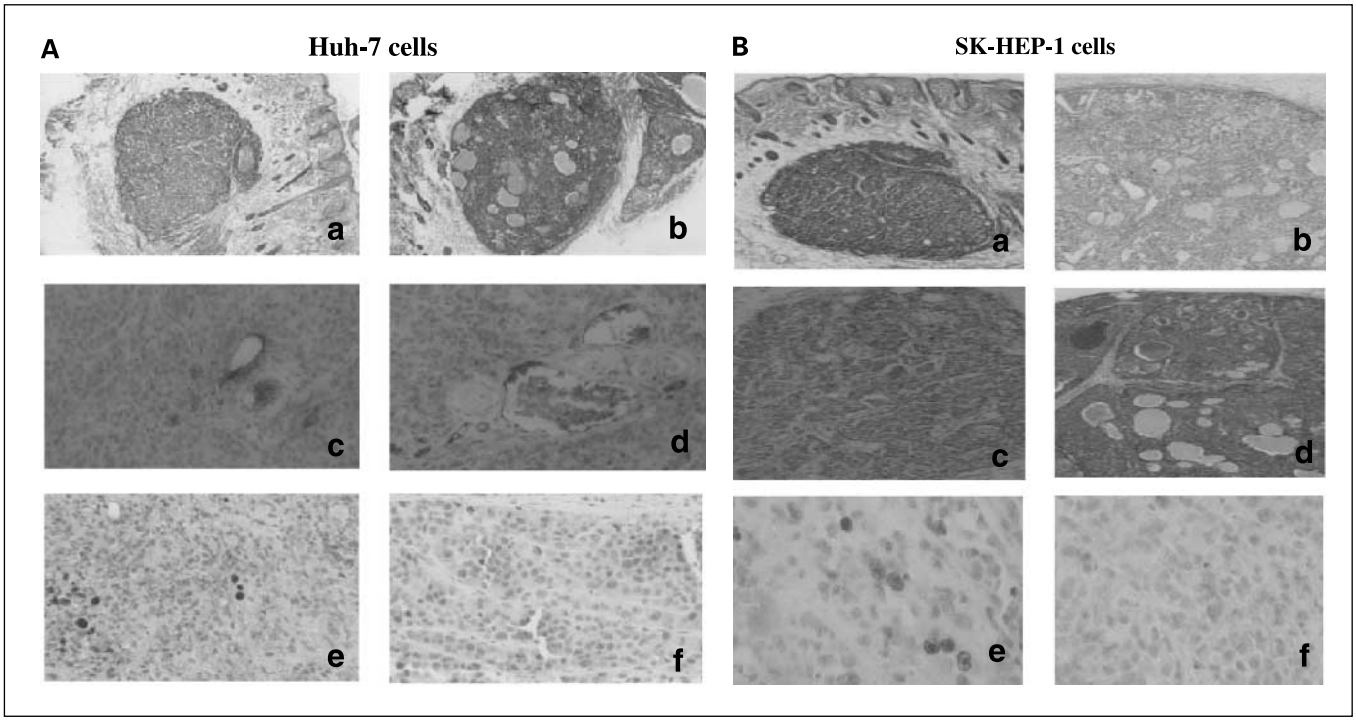

Fig. 3. Indinavir promotes regression of hepatocarcinoma growth in vivo by its antiangiogenic action in Huh7 cells and in SK-HEP-1 cells. *A,* morphologic and immunohistochemical analysis in Huh7 cells. *A,* H&E staining of the xenografts showed small, pail, and regressive tumors in protease inhibitor – treated animals. Original magnification, ×20. *B,* H&E staining of the xenografts revealed lesions visibly florid and highly vascularized in untreated mice. Original magnification, ×20. *C,* immunohistochemistry for factor VIII showed few vessels in the tumors of treated animals (ABC). Original magnification, ×40. *D,* immunohistochemistry for factor VIII revealed numerous vessels in the tumors of untreated animals (ABC). Original magnification, ×40. *E,* terminal deoxynucleotide transferase – mediated nick end labeling assay showed several apoptotic cells in the tumors of treated animals. Original magnification, ×40. *F,* terminal deoxynucleotide transferase – mediated nick end labeling assay revealed very few apoptotic cells in the tumors of untreated animals. Original magnification, ×40. *B,* Morphologic and immunohistochemical analysis in SK-HEP-1 cells. *A,* H&E staining of the xenografts showed small and regressive tumors in protease inhibitor – treated animals. Original magnification, ×20. *B,* H&E staining of the xenografts revealed lesions florid and highly vascularized in untreated mice. Original magnification, ×20. *C,* immunohistochemistry for factor VIII showed few vessels in the tumors of treated animals (ABC). Original magnification, ×40. *D,* immunohistochemistry for factor VIII revealed numerous vessels in the tumors of untreated animals (ABC). Original magnification, ×40. *E,* terminal deoxynucleotide transferase – mediated nick end labeling assay showed several apoptotic cells in the tumors of treated animals. Original magnification, ×40. *F,* terminal deoxynucleotide transferase – mediated nick end labeling assay revealed very few apoptotic cells in the tumors of untreated animals. Original magnification, ×40.

Table 1. Number of vessels and apoptotic cells in experimental tumors derived from HCC cell lines

	No. vessels	Apoptotic index
Huh7 cell tumors untreated	6 ± 2	1 ± 0.5
Huh7 cell tumors treated with indinavir	2 ± 1	7 ± 2
SK-HEP-1 cell tumors untreated	7 ± 2	1 ± 0.5
SK-HEP-1 cell tumors treated with indinavir	3 ± 1	9 ± 2

Gel activity assay showed that indinavir blocked the conversion of latent MMP-2 to its 62/64-kDa active form (Fig. 1C). Thus, indinavir has direct inhibitory effects on invasion but not on proliferation of Huh7 and SK-HEP-1 cells *in vitro*.

Indinavir inhibits the growth of hepatocarcinoma cells* in vivo *in nude mice. To study the effect of indinavir on hepatocarcinoma cells *in vivo*, Huh7 and SK-HEP-1 cells (5×10^6) were injected s.c. into the back (between scapulae) of nude mice. Then, the animals were treated by intragastric gavage with indinavir at the dose of 70 mg/kg/d or saline solution (as a control) for 3 weeks, and tumor volumes were measured three times per week. After a week from the end of indinavir administration, animals were sacrificed, and tumors were removed and processed. The dosage used were similar to that used for HIV patients treatment and confirmed in a previous *in vivo* trial (25). In this experimental setting, tumors originated in the treated mice grew significantly slower than controls (Fig. 2). The inhibition of tumor growth was statistically significant from days 6 to 21 ($P = 0.004$ and $P = 0.003$, respectively) for both cell lines. We then did a second trial, where the treatment with indinavir or buffer started when tumor measurement was possible, and the administration was continued for 3 weeks from first tumor evaluation. In this case, the drug was not effective in the inhibition of tumor growth (data not shown). Therefore, indinavir was able as single agent therapy to delay the growth of s.c. implanted hepatocarcinoma xenografts in nude mice compared with placebo. Of interest, the therapy failed to prevent further growth of already established tumor xenografts from both cell lines.

Indinavir promotes regression of hepatocarcinoma growth* in vivo *by its antiangiogenic and proapoptotic action. Histopathologic and immunohistochemical analyses of the explanted hepatocarcinoma xenografts revealed that indinavir was effective at blocking tumor growth essentially by inhibition of tumor angiogenesis and tumor cell invasion, with no or little effects on tumor cell proliferation. Exemplificative stainings are depicted in Fig. 3A and B. Microscopic examination by H&E staining of the xenografts, using both cell lines, revealed lesions visibly florid and highly vascularized in untreated mice but smaller, pail, and regressive in protease inhibitor–treated animals. Consistently, by hematoxylin-Van Gieson staining, a greatly reduced number of vessels were visible in protease inhibitor–treated mice compared with control animals. This was confirmed by immunohistochemical staining of lesions with endothelial markers, such as CD31 and FVIII-RA, that gave similar results. Counting of the number of vessels per high-power field resulted in 2 ± 1 in treated animals and 6 ± 2 in control animals ($P = 0.003$), when using Huh7 cells, and in 3 ± 1 in treated animals and 7 ± 2 in control animals ($P = 0.003$), when using SK-HEP-1 cells. Finally, analysis of cell survival by terminal deoxynucleotide transferase–mediated nick end labeling technique showed that the number of apoptotic cells was significantly different in the two group of tumors. The apoptotic rate was of 7 ± 2 in the treated animals and 1 ± 0.5 in the control group ($P = 0.002$), when using Huh7 cells, whereas it was 9 ± 2 in the treated animals and 1 ± 0.5 in the control group ($P = 0.001$), when using SK-HEP-1 cells. Table 1 summarizes the data about vessels number and apoptotic rate.

Finally, we investigated the status of VEGF, a proangiogenic molecule, directly on tumor tissues. Western blot analyses of the tissue lysates from tumor xenografts revealed that VEGF protein expression did not diminish in tumors treated with indinavir, compared with the controls, when using both Huh7 and SK-HEP-1 cells (Fig. 4).

Discussion

It has been recently described that several widely used protease inhibitors can affect important cellular and tissue processes mostly through inhibition of cell invasion and MMPs. This observation has been supported by experimental and clinical data describing suppressive effect of protease inhibitor on Kaposi sarcoma tumor growth (15–17).

We have analyzed the antineoplastic effects of one protease inhibitor, indinavir, on the hepatocarcinoma cell lines Huh7 and SK-HEP-1 both *in vitro* and *in vivo*. Treatments *in vitro* with indinavir were able to partially inhibit the cell invasion but did not affect the cell growth. Moreover, the drug inhibited the MMP-2 proteolytic activation as well. Systemic administration of indinavir to nude mice by intragastric gavage was able to partially block the development and induce regression of the tumor growth. Moreover, morphologic and molecular analyses on the explanted tumors showed that this effect on tumor growth was mostly due to antiangiogenic and proapoptotic actions of the protease inhibitor on tumor cells. These data are in agreement with several observations conducted on different tumor cell lines (27–29). Interestingly, tumors from animals treated with indinavir did not show a significant down-regulation of VEGF protein levels when compared with tumors from controls. These data are consistent with previous observations on Kaposi sarcoma cells (17). Therefore, indinavir exerts its antiangiogenic action mostly by inhibiting MMP-2

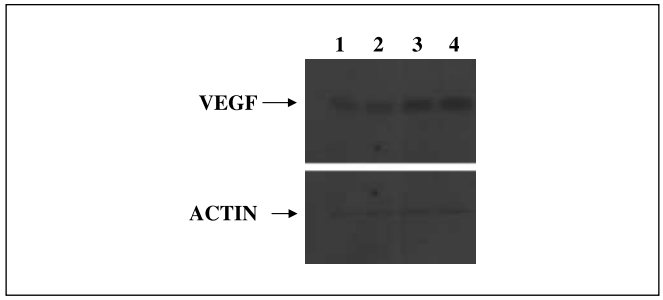

Fig. 4. Indinavir does not exert its antiangiogenic action in Huh7 and SK-HEP-1 cells by down-modulating VEGF. Western blot analysis of VEGF in protein extracts of tumors produced by Huh7 cells in control and treated animals (*lanes 1* and *2*) and of tumors produced by SK-HEP-1 cell in control and treated animals (*lanes 3* and *4*). Expression of β-actin is shown as a control of loading.

proteolytic activation in tumor cells, leading to inhibition of cell invasion and angiogenesis (26).

These findings suggest that protease inhibitors, such as indinavir, could be exploited for the therapy of other tumors beside Kaposi sarcoma occurring both in HIV-infected and noninfected individuals. To note, an increased risk for HCC has been independently associated with HIV infection in a study among patients with parenteral risk exposure (21, 22). Moreover, in patients with HIV infection, liver disease stage is often significantly more advanced, and liver tumor has a significantly higher prevalence of multifocal and infiltrating lesions. Finally, in a recent study, the proportion of HIV-positive patients with extranodal and extrahepatic metastases from HCC has been reported to be much higher with respect to HIV negatives (22).

Actual therapeutic strategies for hepatic carcinoma treatment do not achieve satisfying results. In fact, when surgery is precluded, local nonsurgical therapies are applied. Percutaneous treatments (radiofrequency thermal ablation, ethanol injection, and chemoembolization) provide good results in HIV-negative patients (5-year survival, 40-50%), but frequently, the high degree of malignancy of this tumor in HIV-positive patients makes them not eligible for this kind of therapy due to the size and number of lesions, their large size, and the frequent metastatic spread (30). Systemic treatments with chemotherapy agents, such as doxorubicin, gemcitabine, oxaliplatin, cisplatin, and fluorouracil, combined in different therapeutic regiments lead only to a low rate of partial responses and/or stable disease, with overall response rate ≤50% and 25% of the 1-year survival (31).

The shorter interval between the estimated date of first HCV exposure and first diagnosis of hepatocarcinoma in HIV-coinfected patients should be also highlighted. Hepatocarcinogenesis could be a more rapid process in HIV/HCV–coinfected patients; therefore, an increasing number of cases is expected within the next few years in this population, also considering the frequent young age of coinfected patients and the improved survival due to highly active antiretroviral therapies therapeutic regiment (21, 22). Based on these observations, it could be speculated that chronic treatment of HIV/HCV patients with indinavir gives an advantage in terms of tumor prevention and eventual reduced progression. This suggests the possibility to associate indinavir with other antitumoral treatments to improve the outcome of this kind of patients and to recommend the use of this drug in highly active antiretroviral therapies association regimens for HIV patients coinfected with HCV. Previous studies have already outlined that protease inhibitors can enhance the anticancer effects of some chemotherapeutics, such as docetaxel, in the treatment of prostate cancer (29). In view of the current trend to substitute protease inhibitors with other antiretroviral agents, this information has important implications (32). Therefore, further epidemiologic and prospective studies are urgently required to confirm this hypothesis.

Acknowledgments

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