Ritonavir or saquinavir impairs the invasion of cervical intraepithelial neoplasia cells via a reduction of MMP expression and activity

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Objective and design: Treatment of human immunodeficiency virus (HIV)-infected women with the highly active antiretroviral therapy (HAART) has reduced the onset of uterine cervical intraepithelial neoplasia (CIN), and halted its progression to cervical carcinoma. We and others demonstrated that the HIV protease inhibitors (HIV-PIs) used in HAART can exert direct antitumour activities also in HIV-free preclinical or clinical models. As uterine cervical carcinoma is a leading cause of death in women independently of HIV infection, herein we assessed the impact of therapeutic concentrations of HIV-PIs including indinavir (IDV), saquinavir (SQV) or ritonavir (RTV) on cells obtained from CIN or cervical carcinoma lesions of HIV-negative women.

Methods: HIV-PI effects were evaluated by cell invasion, growth or toxicity assays, and by RNA, protein or zymogram analyses.

Results: Both SQV and RTV inhibited CIN cell invasion, and this was paralleled by a reduced expression and proteolytic activity of the matrix metalloproteinase (MMP)-2 and 9 in treated cells. SQV and RTV also reduced CIN cell growth rate, but did not affect the invasion or growth of cells derived from highly progressed cervical carcinoma.

Conclusion: As MMP-2 and MMP-9 have a key role in CIN evolution into cervical carcinoma, these results support the use of SQV or RTV for the block of CIN clinical progression in either HIV-infected or uninfected patients.

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Introduction

Uterine cervical carcinoma is the second most common cancer among women worldwide [1].

The risk of cervical carcinoma development is highly increased by the persistent infection of cervical epithelial cells with oncogenic human papilloma viruses (HPVs), including HPV16, HPV18 and HPV31 [1]. Infection occurs through micro-wounds of the uterine cervix allowing HPV entry into the proliferating stem cells of the epithelial basal layer [1]. Infectious viral particles are then produced in the supra-basal regions of cervical epithelium by the keratinocytes differentiated from the infected basal stem cells [1]. This initially results in low-grade cervical intraepithelial neoplasia (CIN1), a dysplastic lesion which

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can either disappear spontaneously or persist, possibly evolving into high-grade CIN (CIN2-3) and cervical carcinoma [1].

Cervical intraepithelial neoplasia clinical progression occurs through the proliferation of HPV-infected, poorly differentiated cervical cells which invade the basement membrane at the stromal/epithelial junction of the lesions [1]. In the meantime, new capillaries are formed from pre-existing blood vessels (angiogenesis) in the growing lesion, significantly contributing to CIN clinical progression and, later, to cervical carcinoma growth [2].

Whereas cervical cell proliferation is believed to depend on the inactivation of cellular growth-suppressive factors by the HPV E6 and E7 proteins [1], CIN or cervical carcinoma cell invasion is mainly mediated by the matrix metalloproteinase (MMP), a family of basement membrane-degrading enzymes [3]. Among them, MMP-2 and MMP-9 are expressed in CIN and cervical carcinoma lesions at levels paralleling the evolution of low-grade CIN into high-grade CIN and cervical carcinoma [3–8].

The frequency of CIN development and its clinical aggressiveness are particularly dramatic in HIV/HPV doubly infected women [9]. However, in these patients, highly active antiretroviral therapy (HAART) has significantly reduced the risk of developing CIN [10], has caused CIN regression [11,12] and halted CIN progression to invasive cervical carcinoma [13,14], or has inhibited CIN recurrence after surgical excision [15].

Noteworthy, HAART can exert similar effects against other AIDS-associated tumours, including Kaposi's sarcoma and non-Hodgkin lymphoma (reviewed in [16–20]).

Suppression of HIV replication and restoration of the immune response upon HAART certainly play a key role in counteracting the development and/or progression of AIDS-related cancers [16–20]. However, clinical evidence indicates a lack of correlation between tumour incidence, regression, time to relapse and HIV load reduction, or $CD4^+$ T cells gain [16–20]. Furthermore, HAART-treated CIN, Kaposi's sarcoma or lymphoma patients failing in $CD4^+$ T-cell recovery still show a significantly lower risk of tumour recurrence, as compared to CD4-matched patients treated with antiviral regimens other than HAART [16–20].

In this regard, the HIV protease inhibitors (HIV-PIs), a class of antiviral drugs included in HAART, exert antitumour and antiangiogenic activities also in experimental models devoid of HIV-1 or immune cells [16–20].

In particular, the HIV-PI indinavir (IDV), saquinavir (SQV) or ritonavir (RTV) directly impairs MMP activity, thus efficiently inhibiting tumour cell invasion and angiogenesis [21–25]. Furthermore, IDV, SQV, or RTV compromises the function of the cellular proteasome, a cytosolyc complex of enzymes regulating the turnover of intracellular proteins [24,26–29]. In doing so, SQV and RTV augment the levels of growth-suppressive proteins in tumour cells, thus leading to tumour cell growth arrest and/or apoptosis [24,26,27].

In agreement with these findings, results from a phase II clinical trial in classical, non-AIDS-related Kaposi's sarcoma indicate that, as for AIDS-associated Kaposi's sarcoma, IDV can promote the regression of early-stage lesions, and the long-lasting stabilization of late-stage lesions [30]. Interestingly, responses occur only in patients with high IDV plasma levels, indicating a 'therapeutic' drug threshold, and again therapeutic effects are associated with a decrease in MMP activity [30].

Starting from these findings, herein we have evaluated IDV, SQV and RTV effects on the invasion and growth of human primary CIN cells or human cell lines derived from progressed cervical carcinoma.

Methods

Reagents

Indinavir and SQV (endotoxin-free pure powder) were a gift of Merck Sharp & Dome (Haarlem, the Netherlands) and Roche (Hertfordshire, UK), respectively. The HIV-PIs were suspended in distilled water, and further diluted in saline solution [21]. RTV was obtained from the National Institutes of Health Repository (Bethesda, Maryland, USA), and diluted and handled according to the provider's instructions. Human recombinant epidermal growth factor (EGF) was purchased from Becton and Dickinson (Franklin Lakes, New Jersey, USA). Anti-p53 monoclonal antibodies and fluorescin-isothiocyanate-labelled polyclonal antibodies directed against human EGF receptor were from Santa Cruz Biotechnologies (Santa Cruz, California, USA). Anti-β-actin monoclonal antibodies, staurosporine, and the chemicals employed for protein extraction were from Sigma (St. Louis, Missouri, USA). Growth media and their supplements were purchased from Invitrogen Life Technologies (Milan, Italy).

Two clones of $HPV31^+$ primary keratinocytes derived from a low-grade CIN of an HIV-negative woman (CIN612–7E and CIN612–9E cells) were obtained, characterized and grown as previously described [31,32]. Normal human epidermal keratinocytes (NHEKs; Lonza, Verviers, Belgium), and the cervical carcinoma-derived SiHa and CaSki cell lines (American Type Culture Collection, Manassas, Virginia, USA) were cultured according to the providers' instructions.

Cell invasion, growth and toxicity assays

Cells were cultured for 96 h in the absence or presence of 10 μmol/l IDV, SQV or RTV [21,25]. HIV-PI effect on cell invasion and growth was assessed by the Boyden chamber and the cell counting methods, as described earlier [21,25].

In the invasion assays, human recombinant EGF (20 ng/ml) was employed as the chemo-attractant, since fluorescence microscopy revealed that NHEK, CIN or cervical carcinoma cells express EGF receptor (data not shown).

Cervical intraepithelial neoplasia cell sensitivity to HIV-PI was detected with a XTT-based in-vitro toxicology assay kit (TOX2, Sigma), which measures the capability that mitochondrial dehydrogenase, active in living cells, has to reduce XTT tetrazolium ring, yielding an orange, water-soluble formazan derivative [33]. The absorbance (A) of the resulting orange solution was measured spectrophotometrically: a decrease in viable cells relative to control cells resulted in a decrease in the amount of formazan formed, indicating the degree of cellular toxicity caused by the drugs [33]. The A values of formazan were calculated as a percentage of the control untreated wells as follows: survival rate = 100% X (A drug treated − A blank/A control − A blank). Each concentration of drugs was measured in triplicate wells on the same plate in three independent experiments. The cytotoxic drug staurosporine was employed as the positive control [34].

Real-time polymerase chain reaction

Total RNA was extracted from the cells and used to synthesize cDNA, which was then amplified using oligonucleotide primers derived from the MMP-2 or MMP-9 cDNA sequence (Hs_MMP2_1_SG and Hs_MMP9_1_SG Quantitect Primer Assays, Qiagen Inc., Valencia, CA, USA). The reaction was normalized by amplifying samples for glyceraldehyde-3-phosphate dehydrogenase as house-keeping gene (Hs_GADPH_2_SG Quantitect Primer Assay, Qiagen). Real-time polymerase chain reaction (RT-PCR) was performed by employing the QuantiFast SYBR Green PCR kit (Qiagen), and the ABI PRISM 7700 thermocycler (Applied Biosystems, Monza, Italy). Data were analysed by employing the ABI sequence detection system software (Applied Biosystems).

Zymograms

Total proteins from conditioned media of HIV-PI-treated or control cells were run onto a nonreducing SDS-PAGE co-polymerized with gelatin. Gels were then incubated overnight at 37°C to allow enzyme degradation of the gelatin. After staining with blue Coomassie, the decrease in staining of each band of protease activity was quantified by densitometry [21].

Western blot analysis

Cells were lysed in radio-immunoprecipitation assay buffer containing aprotinin and leupeptin. Proteins from each sample were separated onto SDS-PAGE. After protein transferring, filters were probed with the primary antibody, incubated with the specific secondary horse-radish peroxidase-conjugated antibody (Santa Cruz), and developed with the use of the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Intensity of the bands was quantified by employing the GS300 calibrated densitometer connected to a computer with the Quantity One software (Bio-Rad, Segrate, Italy).

Statistical analysis

All experiments were repeated at least three to four times. Means ± SD were calculated for each group. The nonparametric method of Kruskal–Wallis one-way analysis of variance (which is especially indicated for small sample size) was used to compare the data between each treatment group with the control. Statistical analysis was carried out at two-sided with a 0.05 significance level, using SAS software (SAS Institute, Cary, North Carolina, USA).

Results

Therapeutic concentrations of SQV or RTV inhibit the invasion of human primary CIN cells, and down-regulate MMP-2 and MMP-9 expression by these cells

We have previously shown that HIV-PIs efficiently inhibit the invasion of a variety of human tumour cells *in vitro* [21,25]. Thus, initial experiments assessed IDV, SQV or RTV effect on CIN cell invasion. The three HIV-PIs were employed at concentrations corresponding to the drugs peak (10 μmol/l) levels detectable in plasma of treated, HIV-infected individuals [35], or uninfected patients experiencing the complete remission or regression of early-stage Kaposi's sarcoma with low or no toxicity [30]. NHEKs from neonatal foreskin were employed as controls, as these cells have the same embryonic origin of CIN cells [31]. EGF, a growth factor which has a major role in the progression of uterine cervical tumours [36], was used as the chemoattractant.

Results indicated that differently from NHEKs, CIN cells efficiently invaded the basement membrane in response to EGF (Fig. 1a). In this context, SQV or RTV inhibited EGF-promoted CIN612–7E cell invasion by −82% ($P = 0.03$) and −81% ($P = 0.02$), respectively, whereas IDV effect was less evident (Fig. 1a). Similar results were obtained with the CIN612–9E cells (Fig. 1a).

Our previous work indicated that the inhibition of tumour cell invasion by HIV-PIs parallels the functional

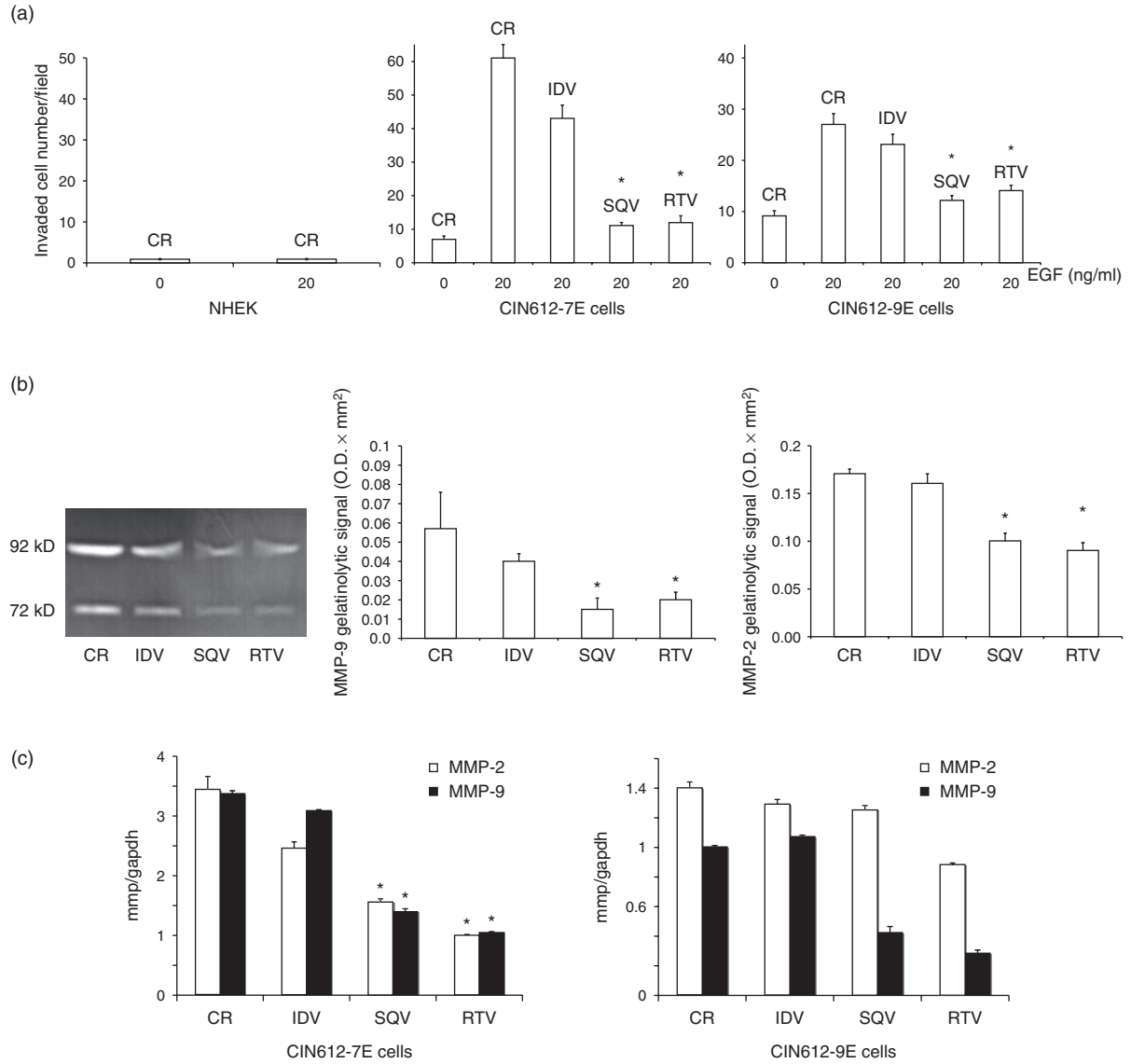

Fig. 1. IDV, SQV or RTV effect on human primary CIN cell invasion, and MMP activity or expression. In (a) NHEK (left panel), CIN612-7E cells (central panel) or CIN612-9E cells (right panel), were cultured for 96 h in the absence (control, CR) or presence of 10 μmol/l IDV, SQV or RTV [21,25], and then stimulated to invade a reconstituted basement membrane in response to 20 ng/ml of human recombinant EGF. The EGF suspension buffer (phosphate-buffered saline solution – 0.1% bovine serum albumin, here indicated as EGF 0 ng/ml) was employed as control. Results are from three to four experiments, and they are expressed as mean number (+SD) of invaded cells. (b) In the left panel is a representative zymogram analysis of supernatants from CIN612-7E cells treated as described above. Shown are the de-stained areas due to the gelatinolytic activity corresponding to MMP-9 (92 kD) or MMP-2 (72 kD) released by CIN612-7E cells. Quantification of MMP-9 or MMP-2 gelatinolytic activity is shown in the central and right panels, respectively. Results are expressed as optical densities (OD)/mm^2 of de-stained bands. Bars represent the mean (± SD) from three experiments. Similar data were obtained with the CIN612-9E cells. In particular, the OD/mm^2 of de-stained areas corresponding to MMP-2 activity was 0.40 in control cells, 0.31 in IDV-treated cells, 0.27 in SQV-treated cells, and 0.26 in RTV-treated cells. The OD/mm^2 of de-stained areas corresponding to MMP-9 activity was 0.64 in both control and IDV-treated cells, 0.12 in SQV-treated cells, and or 0.21 in RTV-treated cells. In (c) is the RT-PCR analysis of MMP-2 (white bars) or MMP-9 (black bars) RNA levels in CIN612-7E cells (left panel) or CIN612-9E cells (right panel). Cells were cultured in the absence (control, CR) or presence of 10 μmol/l IDV, SQV or RTV. Results refer to the relative mmp-2 or mmp-9 gene expression, normalized to the GAPDH housekeeping gene. Bars represent the mean (± SD) from three to four experiments. CIN, cervical intraepithelial neoplasia; EGF, epidermal growth factor; IDV, indinavir; MMP, matrix metalloproteinase; NHEK, normal human epidermal keratinocyte; RTV, ritonavir; SQV, saquinavir.

impairment of basement membrane-degrading enzymes belonging to the MMP family [21,25]. To determine whether this occurs also with CIN-derived cells, we tested IDV, SQV or RTV effect on MMP-2 and MMP-9, two MMPs which are expressed in CIN or cervical carcinoma lesions *in vivo* [3–6].

As first, we measured MMP-2 and MMP-9 proteolytic activity released by the CIN cells [21]. Consistent with the results obtained with the Boyden chambers, in CIN cells EGF induced MMP-9 activity, and increased that of MMP-2 (data not shown). Therefore, we evaluated IDV, SQV or RTV effect on MMP-2 and/or MMP-9 activity present in supernatants from EGF-treated CIN cells.

Results indicated that SQV or RTV reduced by −74 and 65% ($P=0.04$), respectively, MMP-9 proteolytic activity released by CIN612–7E cells (Fig. 1b). Similarly, SQV diminished MMP-2 proteolytic activity by −41%, and RTV by −47% (Fig. 1b). In contrast, IDV had little effect on the proteolytic activity of either MMP-9 or MMP-2 (Fig. 1b).

Again, either SQV or RTV impaired MMP-2 and MMP-9 proteolytic activity released by CIN612–9E cells, whereas IDV had minimal inhibitory effects (see legend to Fig. 1b).

Previous studies indicated that IDV or SQV down-regulates MMP levels in primary astrocytes or preadipocytes [37,38]. Aimed at investigating the molecular mechanism(s) for HIV-PI inhibitory effect on CIN cell invasion and MMP gelatinolytic activity, we evaluated whether IDV, SQV, or RTV could modify mmp-2 and/or mmp-9 gene expression in CIN cells.

Noteworthy, when CIN612-7E cells were incubated with 10 μM SQV or RTV the RNA levels of mmp-9 were reduced by −58% ($P=0.01$) and −69% ($P=0.02$), respectively (Fig. 1c). Moreover, SQV decreased mmp-2 expression by −55% ($P=0.01$), and RTV by −71% ($P=0.04$). In CIN612-9E cells, both SQV and RTV effectively inhibited mmp-9 expression, whereas their effect on mmp-2 RNA levels was not impressive (Fig. 1c). Finally, consistent with the results from the invasion and gel activity assays, IDV did not significantly inhibit MMP gene expression by either CIN612-7E or CIN612-9E cells (Fig. 1c).

Therapeutic concentrations of HIV-PIs do not affect the invasive phenotype of cervical carcinoma-derived human cell lines

Additional experiments assayed the impact of IDV, SQV or RTV on the invasion of cervical carcinoma cell lines which are widely employed as preclinical model of highly progressed human cervical carcinoma [39–44]. In particular, we utilized SiHa cells, a cell line obtained from an invasive cervical carcinoma [43], and CaSki cells, which are derived from a cervical carcinoma metastasis [40].

In agreement with previous findings [39,41,42,44], results from RT-PCR analyses indicated that CaSki cells express both mmp-2 and mmp-9 genes, whereas SiHa cells express only mmp-2 (Fig. 2). Accordingly, gel activity assays showed that SiHa cell supernatants display MMP-2, but not MMP-9 proteolytic activity, whereas CaSki cells release both MMP-2 and MMP-9 (Fig. 2).

As shown in Fig. 2a, neither IDV nor RTV impaired SiHa cell invasion, whereas SQV promoted a 1.32-fold increase of SiHa cell invasion, which was not statistically significant ($P=0.27$). In addition, none of the three drugs significantly affected mmp-2 gene expression nor MMP-2 proteolytic activity in these cells (Fig. 2b and c).

As for SiHa cells, IDV, SQV or RTV did not inhibit CaSki cell invasion, nor did they reduce MMP-2 expression and activity (Fig. 2 a–c). However, as compared to untreated cells, SQV diminished by −44% ($P=0.02$), and RTV by −23% ($P=0.04$), mmp-9 gene expression in CaSki cells (Fig. 2b). Again, SQV or RTV reduced by −71 and −61% ($P=0.04$), respectively, MMP-9 proteolytic activity in CaSki cell supernatants (Fig. 2c). Treatment of CaSki cells with IDV reduced mmp-9 expression by −26% (Fig. 2b) and MMP-9 proteolytic activity by −39% (Fig. 2c), although these effects were not statistically significant ($P=0.27$ and 0.12, respectively).

IDV, SQV or RTV have no or limited effect on CIN or cervical carcinoma cell growth

As HIV-PIs can promote cell growth arrest and/or death [24,26,27], the impact of IDV, SQV or RTV on the growth of the human primary CIN cells or cervical carcinoma-derived cell lines was also evaluated. Results indicated that SQV and RTV were cytostatic for both CIN612-7E and CIN612-9E cells, whereas IDV had only minimal effects (Fig. 3a). Specifically, as compared to control CIN cells, either SQV or RTV reduced the number of CIN cells collected after 5 days of drug treatment; however, neither SQV nor RTV decreased CIN cell number below that of cell seeding (Fig. 3a).

In contrast with CIN cells, the growth rate of SiHa or CaSki cells was not affected by any of the drugs (Fig. 3a).

Toxicity assays confirmed that IDV did not compromise CIN cell viability, whereas SQV or RTV caused little CIN cell death (Fig. 3b), as compared to the pro-apoptotic anticancer drug staurosporine [34].

Previous results indicated that exposure of cells transfected with HPV DNA to high doses (0.1–1 mmol/l) of IDV or RTV reduced cellular proteasome capability of quenching the p53 growth-suppressive, pro-apoptotic

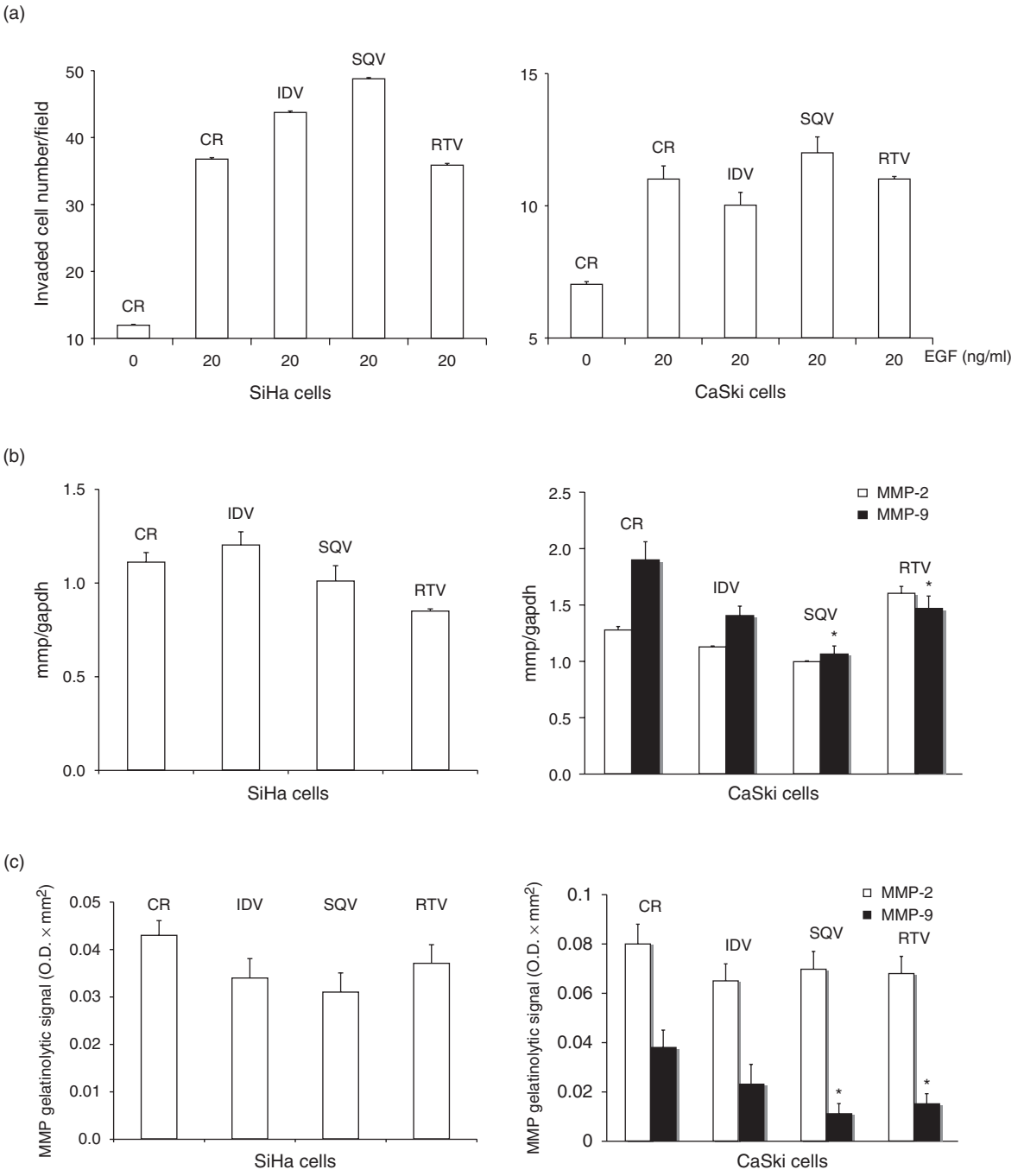

Fig. 2. IDV, SQV or RTV effect on the invasive phenotype of CC-derived human cells. In (a) SiHa cells (left panel) or CaSki cells (right panel), were cultured in the absence (control, CR) or presence of 10 μmol/l IDV, SQV or RTV, and then stimulated to invade in response to EGF or its suspension buffer (here indicated as EGF 0 ng/ml), which was employed as control. Results are from three experiments, and they are expressed as mean number (+ SD) of invaded cells. In (b) is the RT-PCR analysis of MMP-2 (white bars) or MMP-9 (black bars) RNA levels in SiHa cells (left panel) or CaSki cells (right panel). At variance with CaSki cells, SiHa cells expressed only the mmp-2 gene. Both SiHa and CaSki cells were cultured in the absence (control, CR) or presence of 10 μmol/l IDV, SQV or RTV. Results refer to the relative mmp-2 or mmp-9 gene expression, normalized to the GAPDH housekeeping gene. Bars represent the mean (± SD) from three experiments. In (c) is the densitometric quantification of the de-stained areas corresponding to the gelatinolytic activity of MMP-2 (white bars) or MMP-9 (black bars) released by SiHa (left panel) or CaSki (right panel) cells. Consistent with the results from RT-PCR analyses, no MMP-9 activity was detected in conditioned media from SiHa cells. For both cell lines, results are expressed as described in the legend to Fig. 1c, and they represent the mean (± SD) from three experiments. CC, cervical carcinoma; EGF, epidermal growth factor; IDV, indinavir; MMP, matrix metalloproteinase; RTV, ritonavir; SQV, saquinavir.

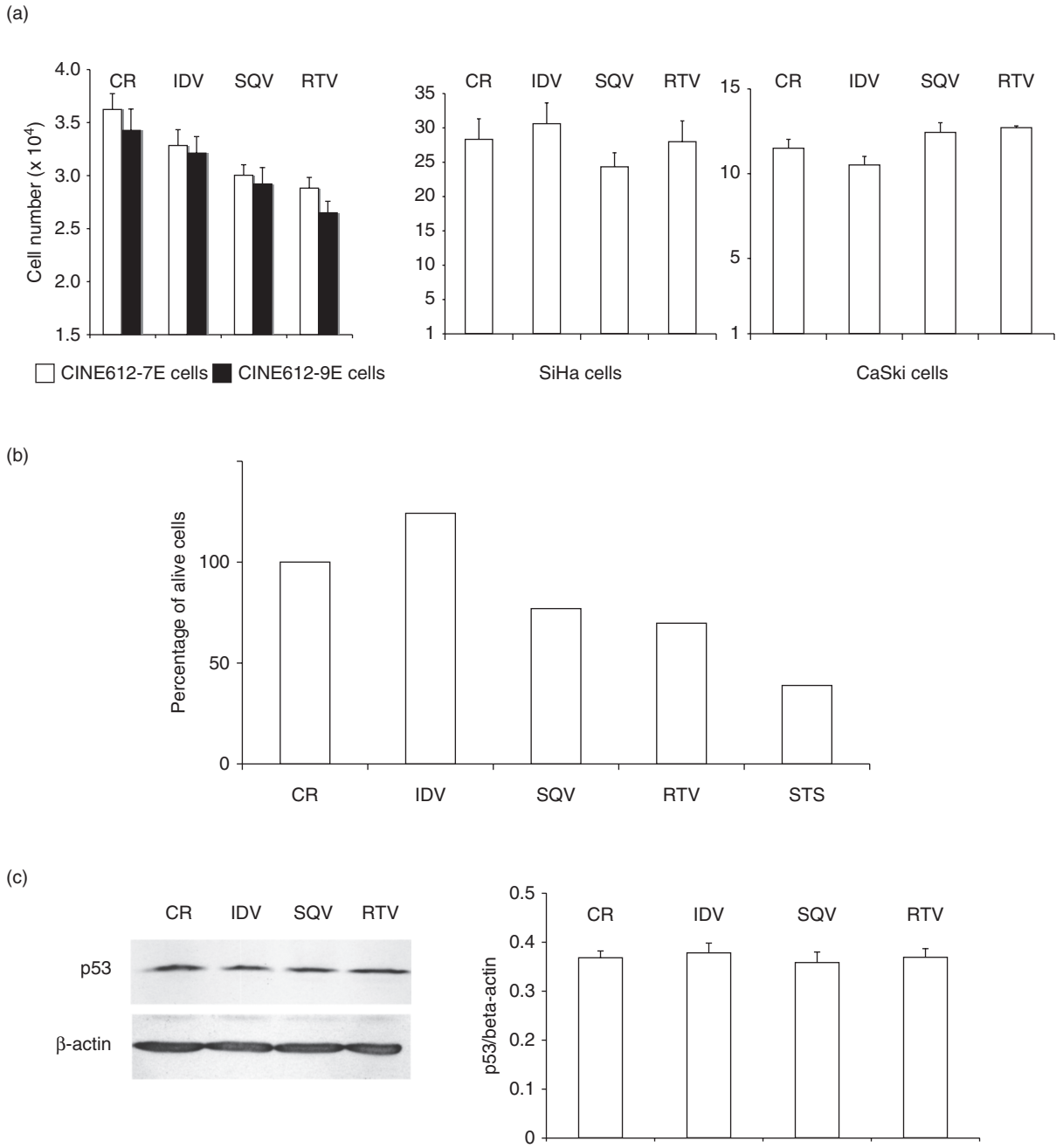

Fig. 3. IDV, SQV or RTV effect on the growth rate of CIN or CC-derived cells. In (a) CIN612-7E (left panel, white bars) or CIN612-9E (left panel, black bars) cells were seeded at 1.5×10^4 cells/ well. Because of their high growth rate, SiHa cells (central panel) or CaSki cells (right panel) were plated at 0.5×10^4 cells/ well. Shown is the number of cells collected after 5 days of culture in the absence (control cells, CR) or presence of 10 μmol/l IDV, SQV or RTV. Data represent the mean (±SD) from three to four experiments. In (b) CIN612-7E sensitivity to HIV-PI was evaluated by a XTT-based in-vitro toxicity assay, as described in the Materials and methods section. Data are from three experiments, and they are expressed as the percentage of live cells measured after 48 h of culture in the presence of IDV, SQV, RTV, or the anticancer drug staurosporine (STS), which was employed as the positive control of toxicity [34]. Cells grown in the absence of HIV-PIs were the negative control (CR). In (c) CIN612-7E cells were cultured in the absence (control, CR) or presence of 10 μmol/l IDV, SQV or RTV. Cells were lysed, and equal amounts of total proteins were electrophoresed and analysed by Western blotting using a monoclonal antibody directed against p53. Blots were re-probed with anti-β-actin monoclonal antibody to verify equal loading of protein in each lane. On the left are representative Western blots of p53 or β-actin. On the right is the quantitative (densitometric) analysis of p53 protein levels (normalized to β-actin) in control or HIV-PI-treated CIN cells. Bars represent the mean (±SD) from three experiments. CIN, cervical intraepithelial neoplasia; IDV, indinavir; RTV, ritonavir; SQV, saquinavir.

protein: this leaded to a stable p53 increase in the treated cells, with minimal cell death [40]. Based on these findings, we evaluated whether the reduction of CIN cell growth rate promoted by 10 μmol/l SQV or RTV was accompanied by increased p53 protein levels. As shown in Fig. 3c, when employed at these low (therapeutic) doses, none of the HIV-PIs modified the levels of p53 protein in CIN cells. This finding indicated that SQV and RTV inhibit CIN cell invasion at doses not impairing cellular proteasome function.

Discussion

In the effort of improving the efficacy of antitumour therapies, many current medical strategies are aimed at designing novel inhibitors of the molecular pathways leading to cancer progression. In this context, we are focused on investigating the antitumour effect of HIV-PI, a class of drugs which has reduced the incidence and/or promoted the regression of AIDS-associated cancers, independently of its anti-HIV or immune-reconstituting activities [16–20].

In this regard, we and others have shown that HIV-PI including IDV, SQV or RTV can directly target molecules with a key role in tumour progression, such as the MMP or the cellular proteasome, with antitumour and antiangiogenic effects [21–28,40].

These properties render IDV, SQV or RTV attractive candidates for novel therapies directed against CIN and/or cervical carcinoma in both HIV-positive and negative patients. In fact, preclinical data indicated that classical MMP inhibitors greatly reduce CIN progression in HPV-transgenic mice [45], whereas classical proteasome inhibitors sensitize CIN cells to apoptosis-inducing agents *in vitro* [46]. However, clinical trials have shown that classical MMP or proteasome inhibitors are often toxic and/or ineffective, especially against solid tumours (reviewed in [47,48]).

In this context, treatment of HIV-infected women with HIV-PI-based therapies has inhibited the risk of CIN progression to invasive cervical carcinoma, or CIN recurrence after surgical excision, with little or no side effects [10,11].

Starting from these findings, herein we evaluated whether IDV, SQV or RTV concentrations corresponding to their peak levels in plasma of treated patients [30,35] possess any direct activity against cells obtained from CIN or cervical carcinoma lesions of HIV-negative women.

Results indicated that at the doses used, SQV or RTV strongly reduces CIN cell invasion, and that this is paralleled by a down-regulation of the expression and proteolytic activity of MMP-9 and, to a lesser extent, MMP-2. These findings confirm previous work showing that HIV-PIs reduce MMP levels both *in vitro* [37,38], and in plasma from treated patients [30].

Concerning a possible mechanism for HIV-PI inhibitory effect on MMP expression by CIN cells, it has to be highlighted that induction of MMP expression is preceded by the phosphorylation of signalling molecules, including p38MAPK or protein kinase B, resulting in the binding of a variety of transcription factors to the promoter region of mmp genes [49]. In this context, results from previous work indicated that HIV-PIs inhibit p38MAPK and/or protein kinase B phosphorylation [24,50,51]. Future work will evaluate whether this occurs in CIN cells.

Differently from CIN cells, however, neither SQV or RTV inhibited the invasion of the cervical carcinoma-derived SiHa or CaSki cells, and they had no effect on MMP-2 expression by these cells. In contrast, both drugs reduced MMP-9 expression and proteolytic activity in CaSki cells, although this was not sufficient to halt cellular invasion. Possible explanations for this could be that CaSki cells are highly transformed cells which are derived from an undifferentiated cervical carcinoma, and are known to express other pro-invasive enzymes in addition to MMP [52,53].

Nevertheless, the results obtained with the primary CIN cells and the CaSki cell line indicate that HIV-PIs are particularly effective at inhibiting MMP-9, and this has important clinical implications. Among other basement membrane-degrading enzymes, in fact, MMP-9 levels increase both in tumour tissues and in plasma as low-grade CIN progresses to high-grade CIN [7,8], and then to cervical carcinoma [5], and its activity is directly related to CIN or cervical carcinoma recurrence after surgical excision [3]. In this context, we found that primary cells derived from low-grade CIN express MMP-2 but not MMP-9, which is induced by EGF. This finding is particularly intriguing, considering the role of EGF in the progression of low-grade CIN to high-grade CIN [36], and SQV or RTV effect on EGF-induced MMP-9.

In addition to their anti-invasive properties, either SQV or RTV also reduces the growth rate of CIN cells, but not that of cervical carcinoma-derived cell lines, without modifying the levels of the proteasome-targeted, growth-suppressive p53 protein. These data are consistent with the fact that SQV or RTV therapeutic concentrations are much lower than those required to impair p53 quenching by the cellular proteasome [40], and they are in agreement with our recent finding that HIV-PIs exert direct antitumour activities in a proteasome-independent fashion [25]. Thus, the modest inhibition of CIN cell growth promoted by SQV or RTV could depend, at least in part, on the reduction of MMP expression and activity caused in the CIN cells by these HIV-PIs. In fact, MMPs

are known to affect tumour cell growth by modulating both cell–cell and cell–matrix interactions [48].

Altogether, the results described herein suggest that SQV or RTV may be effective against CIN, whereas they could lack clinical efficacy in progressed cervical carcinoma. This, and the presence or absence of HIV-PIs in HAART, could partially explain the contrasting conclusions from clinical-epidemiological studies indicating on one side a diminished risk of CIN onset or progression, and on the other side an unmodified incidence of uterine cervical carcinoma in the HAART era [9–15,54–57].

In conclusion, here we have shown in-vitro data supporting SQV or RTV effectiveness against CIN independently of their action on HIV. In addition to provide further information on the mechanisms for HIV-PI antitumour activity, and to identify markers possibly predictive of tumour response to therapy, the results described herein, together with those from our previous and recent work [21,25,30], further support the use of HIV-PIs as efficient antagonists of cancer progression, to be used alone or in combination with conventional cytotoxic drugs. Altogether, these data also recommend continuing to include HIV-PIs in HAART.

At present time surgical excision represents the only therapy preventing CIN progression to cervical carcinoma. However, surgical intervention can have severe side effects (haemorrhages, infection, and/or cervical stenosis), without avoiding CIN recurrence risk [1]. This is alarming, considering that about 30% of the CIN patients persistently infected with oncogenic HPV is estimated to progress to cervical carcinoma within 2 years [1].

Differently from surgery, a pharmacological treatment may provide a widespread accessible, low-cost tool to prevent CIN progression/recurrence, while minimizing the impact on the quality of life of the patients. In this regard, it has to be highlighted that CIN evolution into cervical carcinoma is paralleled by an 'angiogenic switch' in the lesion [2]. Thus, HIV-PIs could be very effective against CIN clinical progression also because of their direct antiangiogenic effect [21,25].

Indeed, the use of SQV or RTV for CIN patients is advantageous as these drugs have been used since years on a wide spectrum of individuals, with no safety concerns, and that a large body of data exists on their pharmacokinetic and tissue distribution. Therefore, these HIV-PIs could be easily adopted in the clinical practice of CIN patients.

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G.B. designed the experiments, analysed results, and wrote the manuscript. A.I. and I.B. performed cell invasion assays, and RNA, protein or zymogram analyses. C.P. and P.L. carried on the cell growth and toxicity assays. S.B. performed the statistical analysis of the results. P.M. and B.E. participated to experiment design and manuscript writing. This work was supported by grants from the Italian Ministry of Health (Progetti Ricerca Finalizzata Oncologia) to B.E. and G.B. We thank Professor L.A. Laimins (Northwestern University, Chicago, Illinois, USA) for providing the CIN612-7E and CIN612-9E cells, and M. Falchi and G. Fornari-Luswergh (National AIDS Center) for helping in figures preparation and for editorial assistance.

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Conflicts of interest

Authors declare that no conflict of interest exists.

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