Antitumor Effect of the Human Immunodeficiency Virus Protease Inhibitor Ritonavir: Induction of Tumor-Cell Apoptosis Associated with Perturbation of Proteasomal Proteolysis

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ABSTRACT

Ritonavir is an HIV protease inhibitor used in the therapy of HIV infection. Ritonavir has also been shown to inhibit the chymotrypsin-like activity of isolated 20S proteasomes. Here, we demonstrate that ritonavir, like classical proteasome inhibitors, has antitumoral activities. In vitro, ritonavir strongly reduced the rate of proliferation of several tumor cell lines and induced their apoptosis. Nontransformed cell lines and terminally differentiated bone-marrow macrophages were comparatively resistant to the apoptosis-inducing effect. In vivo, ritonavir, administered p.o. for a week at doses of 6–8.8 mg/mouse/day, caused significant growth inhibition (76–79% after 7 days of treatment) of established EL4-T cell thymomas growing s.c. in syngeneic C57BL/6 mice. Unexpectedly, we found that ritonavir activates the chymotrypsin-like activity of isolated 20S proteasomes, in strong contrast to its effect on isolated 20S proteasomes. The net effect of low micromolar concentrations of ritonavir on the chymotrypsin-like activity in cells and cell lysates was a weak inhibition, consistent with marginal alterations of polyubiquitinated proteins, marginal alterations in acid-soluble proteolytic peptide levels, and a small accumulation of the tumor suppressor protein p53, in cells treated with ritonavir. In contrast, we found a relatively strong accumulation of the cyclin-dependent kinase inhibitor p21\(^{\text{WAF1}}\), a sign of deregulation of cell-cycle progression typical for apoptosis induction in transformed cells by classical proteasome inhibitors. We demonstrate that p21 accumulation in the presence of ritonavir is attributable to the inhibition of proteolytic degradation. Accumulation of p21 most likely reflects a selective inhibition of proteasomes, in line with the atypical degradation of p21, which does not require ubiquitination. These findings suggest that selective perturbation of proteasomal protein degradation may play a role in the antitumoral activities of ritonavir.

INTRODUCTION

Ritonavir is an inhibitor of the HIV protease and is used widely in the therapy of HIV infection (1–3). The compound was developed as a peptidomimetic of proline-containing peptide bonds of the HIV gag-pol polyprotein precursor and blocks the maturation of infectious virus particles (4). More recently, ritonavir has also been shown to modulate the peptidase activities of the 20S proteasome. Whereas the chymotrypsin-like activity of the 20S proteasome is inhibited by ritonavir, the trypsin-like activity is enhanced. In accordance with the role of proteasomes in antigen processing, inhibition of the presentation of some CTL epitopes in vitro and of lymphocytic choriomeningitis virus-specific CTL responses in ritonavir-treated mice has been observed (5).

Proteasomes appear to degrade most extralysosomal proteins and, together with the ubiquitin system, they determine the lifetime of many short-lived, regulatory proteins (6). Among these are proteins involved in the interface between cell cycle and apoptosis, such as the cdk\(^{\text{p}}\) inhibitors p21\(^{\text{WAF1}}\) and p27\(^{\text{KIP1}}\), as well as those involved in the regulation of cell survival and apoptosis, such as the tumor suppressor p53 and NF-\(\kappa\)B, the latter of which is activated by proteasomal proteolysis (7, 8).

Classical proteasome inhibitors are antiproliferative and induce apoptosis of tumor cells (9–11). Nontransformed cells are usually less sensitive to apoptosis induction by proteasome inhibitors. Noncycling, primary cells such as postmitotic neurons even differentiate on proteasome inhibitor treatment (12), and, like thymocytes, they can be rescued by proteasome inhibitors from apoptosis triggered by a variety of stimuli (13, 14). Thus, the apoptosis-inducing effect is largely tumor cell-specific, and significant antitumor effects of proteasome inhibitors have been observed in mice (15–17). Although proteasome inhibitors theoretically appear to be well suited as antineoplastic drugs, none has yet been licensed for use in humans for this or other purposes.

Ritonavir is a reversible modulator of proteasomal peptidase activities and is licensed for use in humans for the treatment of HIV infection. Although it was not clear to what extent ritonavir affects cellular protein degradation, we deemed it important to ask whether it has antitumoral effects similar to classical proteasome inhibitors.

MATERIALS AND METHODS

Reagents. RPMI 1640, FCS, penicillin/streptomycin, and t-glutamine were purchased from Life Technologies, Inc. Lactacystin was obtained from Dr. E. J. Corey, Harvard University, Cambridge, MA, USA. Ritonavir was used either in the form of the Norvir solution or as the purified substance (Abbott Laboratories). Ritonavir and lactacystin were dissolved in DMSO. The fluorogenic proteasome substrates Suc-LYY-AMC and Z-LYL-AMC and the proteasome inhibitors N-acetyl-leucyl-leucyl-norleucinal (LLnL) and MG132 were from Calbiochem.

Cell Lines. A Meth A-induced fibrosarcoma was originally obtained from Dr. L. Old (Memorial Sloan-Kettering Cancer Center, New York, NY). Murine T-lymphoid EL4, human T-lymphoid Jurkat, human T/B-lymphoblastoid T1, human promyelocytic HL-60, mouse P815 mastocytoma, NIH 3T3 mouse fibroblasts, mouse P815 mastocytoma cells, and the Ramos cell line (an EBV-negative immortal transformed line originally derived from a Burkitt’s lymphoma tumor specimen) were all purchased from the American Type Culture Collection (Manassas, VA). A2780 human ovarian cancer cells were from Prof. I. Runnebaum (University of Freiburg, Freiburg, Germany). Mouse bone-marrow-derived macrophages obtained from precursor cells during 10 days of culture, were prepared as described previously (18). EL4, Jurkat, T1, P815, and Ramos cells were grown in RPMI 1640 containing 10% (v/v) FCS, 20 mm HEPES, sodium penicillin G at 100 units/ml, and streptomycin sulfate at 100 \(\mu\)g/ml (Life Technologies, Inc.). HL-60, NIH 3T3, and A2780 cells were grown in DMEM supplemented with 10% FCS. For inhibitor-related assays, suspension cells were seeded into 50-ml Greiner tissue-culture flasks at a density of 5–8 \(\times\) 10\(^5\) cells/ml. 3T3 fibroblasts, A2780 cells, and primary macrophages were seeded onto Costar 6-well tissue-culture plates at a density of 5–8 \(\times\) 10\(^5\) cells/ml. 3T3 fibroblasts, A2780 cells, and primary macrophages were seeded onto Costar 6-well tissue-culture plates at a density of

Received 8/31/01; accepted 10/4/02.

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of $1 \times 10^6$ cells/well. The cells were fed with fresh medium and allowed to recover for 4–6 h.

**Proliferation Assay.** Cells were treated with the indicated concentrations of ritonavir for 8 days. The ritonavir-containing medium was exchanged daily and proliferation was assessed daily by counting the number of viable cells after trypan blue exclusion staining.

**Assays of Apoptosis.** Cells were treated with different concentrations of ritonavir or lactacystin for different periods of time as indicated. To assay apoptosis, genomic DNA was extracted and electrophoresed on 1% agarose gels. After staining with ethidium bromide, the gels were analyzed under UV light for DNA fragmentation. For flow cytometry, cells were subjected to annexin V-FITC labeling and counterstained with PI.

**In Vivo Tumor Experiments.** For tumor implantations, female C57BL/6 mice (6–8 weeks old) were inoculated s.c. with EL4 cells ($4 \times 10^5$ cells/50 µl PBS) in the midline ventral position. Ritonavir treatment commenced on day 6 or 7 after implantation, by which time the tumors had reached a volume of 50–100 mm$^3$. The mice were treated daily (for about 1 week) by oral application of ritonavir (Norvir) through an intragastric sonde. To avoid intoxication of the animals, alcohol was expelled from the original Norvir-solution at 37°C under a stream of nitrogen, whereby a viscous but still clear solution was obtained. Tumor growth was scored daily by two cross-sectional measurements and tumor size was calculated using the formula described by Kyriazis et al. (19), as follows: tumor volume = width$^2 \times$ length $\times 0.4$. The experiments were terminated when the tumors reached 1300–1500 mm$^3$, and the mice were killed under anesthesia.

**Western Blot Analysis.** After incubation with ritonavir, lactacystin, or $N$-acetyl-leucyl-leucyl-norleucinal for the indicated times (see Fig. 5), cell lysates were prepared and analyzed by Western blotting. Antibodies used in Western blot analysis were from Santa Cruz Biotechnology (p21), Oncogene Research Products (p53), and Affiniti Research Products (anti-poly-ubiquitin). The blots were developed with the enhanced chemiluminescence reagent (Amersham) according to the manufacturer’s instructions. Luminescence was recorded on Biomax MR film (Eastman Kodak). Quantitative luminescence analyses were performed on a Lumi-Imager (Boehringer, Mannheim, Mannheim, Germany).

**Proteasome Assays.** Purification of 20S and 26S proteasomes and determination of their chymotrypsin-like activity with the fluorogenic substrates Suc-LLVY-AMC (100 µM) and Z-LLL-AMC (25 µM) were performed essentially as described previously (20). For proteasome inhibition assays, purified proteasomes, cells, or cell lysates were pretreated with the protease inhibitors for 30 min.

**Assay of Intracellular Protein Degradation.** EL4 cells were incubated with $[^{14}C]$leucine in leucine-free medium for 16 h at 37°C in medium containing dialyzed FCS. Protease inhibitors (7.5–75 µM ritonavir, 20 µM lactacystin, or 10 µM MG132) were added for the last 20 min of this pulse period. The cells were then chased at 37°C with the medium containing unlabeled leucine (50 µg/ml) in the presence or absence of the inhibitors. After 2 h, trichloroacetic acid (10%) was added, and the amount of trichloroacetic acid-soluble radioactivity in clarified supernatants (15,000 × g) was determined in a liquid scintillation counter.

**Cell Cycle Analysis.** EL4 cells cultured for 0, 6, 7, 8, 10, 12, and 15 h in ritonavir (20 µM)-containing or control (DMSO-containing) medium were harvested, washed with PBS, fixed with 70% ethanol, and treated with 100 µg/ml RNase (Sigma). Cells were then stained with PI (50 µg/ml), and a cell cycle profile was determined using the software Cell Quest on a Calibur (Becton Dickinson) flow cytometer.

Fig. 1. Ritonavir inhibits the proliferation of murine and human tumor cells at low micromolar concentrations. The cells were grown under conventional cell-culture conditions in medium containing 10% FCS and various concentrations of ritonavir. The culture medium was changed daily and cell proliferation was assessed by counting the number of viable cells after trypan blue exclusion staining. **A**, proliferation of tumor cell lines; **B**, left panel, proliferation of non-transformed, immortalized 3T3 fibroblasts; right panel, percentage of dead 3T3 cells after treatment with ritonavir or lactacystin, based on viability counts using trypan blue.
RESULTS

We first examined the effect of ritonavir on the proliferation of a number of murine and human tumor cell lines in vitro. The cells were exposed to concentrations of ritonavir from 0 to 50 μM. (The blood-plasma ritonavir concentrations obtained in the therapy of HIV-infection are ~5–15 μM, but much higher maximal concentrations of up to 46 μM have been determined in individual patients; see “Discussion”). The cells were exposed for a week, with daily exchange of the ritonavir-containing medium. As shown in Fig. 1A, ritonavir had a particularly strong effect on the proliferation of lymphoblastoid cell lines.
lines such as the murine T-lymphoma EL4 and the human T/B-lymphoblastoid hybrid T1 lines, inhibiting their proliferation completely at concentrations as low as 15 μM. The human T-lymphoma Jurkat, a murine Meth A-induced fibrosarcoma line and murine P815 mastocytoma cells were all less sensitive; 15 μM or 20 μM ritonavir caused only a partial inhibition of proliferation, whereas cessation of proliferation was observed at 20 μM and 30 μM, respectively, or higher concentrations, in all three of the cases. The proliferation of nontransformed but immortalized 3T3-fibroblasts was also reduced in a dose-dependent manner, but proliferation ceased only at 40-μM or higher concentrations of ritonavir (Fig. 1B, left panel). However, ritonavir was less cytotoxic for 3T3 cells than was the strong, irreversible proteasome inhibitor lactacystin (Fig. 1B, right panel). Primary, embryonic fibroblasts, which proliferate very slowly (doubling time, >6 days), also showed a dose-dependent reduction of proliferation at concentrations ≥20 μM ritonavir (data not shown).

Next, we examined whether apoptosis contributed to the inhibition of tumor-cell proliferation observed under ritonavir treatment. To this end, the integrity of genomic DNA was investigated at 8, 16, and 24 h after a single application of ritonavir. In the case of cell lines that were not sensitive or that exhibited only low sensitivity, Ritonavir was added a second time 24 h later, and DNA was again sampled 24 h after the second addition. As shown in Fig. 2, tumor cells of lymphoblastoid origin were particularly sensitive to the apoptotic effect of ritonavir. In the case of the T-lymphoma EL4 and the Burkitt’s lymphoma line Ramos, DNA fragmentation was observed as early as 8 h after a single addition of the drug at concentrations of 20–30 μM or higher. After longer exposure to the drug (24 h), DNA fragmentation was observed in these cell lines at concentrations as low as 7.5 μM. Meth A fibrosarcoma cells and HL-60 human myeloid leukemia cells were not as sensitive. However, 24 h after the second ritonavir addition, DNA fragmentation was also observed in these cell lines at relatively low concentrations (7.5 μM). In contrast, the nontransformed, immortalized 3T3 fibroblast cell line 3T3 and primary, terminally differentiated bone-marrow macrophages were resistant to the apoptosis-inducing effect of ritonavir. Only high concentrations of the strong proteasome inhibitor lactacystin (10 μM), almost completely blocking the chymotrypsin-like activity of proteasomes, gave a weak DNA ladder in the case of the nontransformed fibroblast cell line. Bone-marrow macrophages were completely resistant even to a repeated application of lactacystin. The HIV protease inhibitor indinavir, which does not affect the chymotryptic activity of 20S proteasomes (5), did not induce apoptosis in tumor cell lines such as EL4 cells at concentrations between 0 and 50 μM (data not shown).

In view of the striking difference in sensitivity to ritonavir observed above between nontransformed cell lines or differentiated primary cells and transformed tumor cell lines, we investigated whether ritonavir has an antitumoral activity in vivo. For this, we chose the EL4-thymoma tumor model in syngeneic C57BL/6 mice. EL4 cells implanted into untreated syngeneic C57BL/6 mice grew very aggressively (Fig. 3). After 13–14 days postimplantation, the tumor size exceeded protocol limits (>1300–1500 mm3); therefore, at this stage, the mice were killed. In parallel, tumor-bearing mice were treated with ritonavir. Treatment commenced on day 6 or 7 when the tumors had a volume of ~50–100 mm3. A dose of 8.8 mg alcohol-free ritonavir (Norvir) was administered daily by an intragastric sonde as described in “Materials and Methods.” This dose was tolerated by the mice, except for the first application, after which the mice showed some signs of immobility. For this reason, in subsequent experiments, the dose of the first application was reduced to 6 mg. Other adverse symptoms were not observed, except an initial weight loss (1–3.5 g) during the first 3 days of treatment. Generally, no further loss in weight occurred during the remaining period of treatment. The retardation of tumor growth observed in the ritonavir-treated mice was strong (76–79% growth inhibition after 7 days of treatment; Fig. 3). A comparable retardation of tumor growth was also observed when a similar dose of ritonavir was administered i.p. in an insoluble form as suspension in PBS (data not shown).

Experiments were performed to address the potential mechanism(s) involved in the antitumoral activities of ritonavir. Because ritonavir has been shown to modulate the 20S proteasomal peptidase activities, we investigated its effect on isolated 20S and 26S proteasomes. Surprisingly, we found that ritonavir activates the chymotrypsin-like activity of the isolated 26S proteasome as measured with Suc-LLVY-AMC (Fig. 4). This activation was found over the wide range of 0.1–100 μM ritonavir with a maximum between 10 μM and 30/40 μM. In contrast, ritonavir has an inhibitory effect on the Suc-LLVY-AMC-hydrolyzing activity of the 20S proteasome, as reported previously (5). To assess the possible role of proteasome inhibition or modulation in the antiproliferative and tumor cell-apoptotic activity of ritonavir, we measured the influence of the compound on the chymotrypsin-like activity in tumor cell lysates with Suc-LLVY-AMC (not membrane-permeable) and in tumor cells with Z-LLL-AMC (membrane-permeable; Ref. 22). Weak inhibition of substrate hydrolysis was observed from 10 μM ritonavir upwards, reaching moderate levels at high ritonavir concentrations (Fig. 4). Similar results were obtained with 3T3 cells and cell lysates (data not shown). Consistent with the opposite effects of ritonavir on 20S and 26S proteasomes, it can be assumed that the relatively weak inhibition of the chymotryptic activities of cells and cell lysates reflects net proteasomal activities. To our knowledge, ritonavir is the only substance known that has differential effects on the peptidase activities of 20S versus 26S proteasomes.

The hydrolysis of small reporter substrates does not necessarily reflect cytoplasmic proteasomal proteolyis. To assess the effect of ritonavir on global proteosomal proteolysis, we determined the levels of poly-ubiquitinated proteins, which are classical substrates of 26S proteasomes. In contrast to classical proteasome inhibitors, ritonavir caused no significant accumulation of bulk poly-ubiquitinated proteins. Rather, a small decrease was usually found at ritonavir concentrations ≥30 μM (Fig. 5A). We also assessed bulk cellular proteolysis by following the degradation of biosynthetically radiolabeled proteins on addition of ritonavir during a 2-h chase period. In contrast to the strong, classical proteasome inhibitors lactacystin and MG132 (23), Ritonavir caused only minor alterations in the production of radioactive, acid-soluble proteolytic peptides (data not shown). These data suggest that global proteasomal proteolysis is not drastically altered at
concentrations of ritonavir that cause apoptosis in tumor cells. Nevertheless, it was possible that the intracellular concentrations of certain individual pro- or antiapoptotic proteins might be more significantly altered by ritonavir, possibly because of a selective influence on proteolysis. p53 is a classical 26S proteasomal substrate, and accumulated wild-type p53 can inhibit the cell cycle (by transcriptional induction of the cdk inhibitor p21WAF1) or induce apoptosis (24, 25). Because the transformed cell lines used in the above mentioned experiments expressed either mutant p53 (e.g., Meth A, Jurkat, P815; Refs. 26, 27) or no p53 at all (e.g., HL-60, Ramos; Refs. 28, 29), we concluded that the induction of apoptosis in these cells by ritonavir is probably not dependent on p53. We determined the effect of ritonavir on intracellular p53 levels in the p53 wild-type ovarian cancer cell line A2780. As shown in Fig. 5A, low micromolar concentrations of ritonavir induced only a marginal accumulation of p53 (maximum, 2-fold) compared with a 27-fold induction by 20 μM lactacystin. This finding is consistent with the observed weak effects of low micromolar concentrations of ritonavir on global proteasomal proteolysis.

Because ritonavir has been shown to arrest lymphoblastoid T2 cells at G1-S phase (30), we examined whether it influences the cellular levels of the antiproliferative G1 cdk inhibitor p21WAF1. Under certain conditions, p21 can also be proapoptotic (31). Consistent with these observations and with our findings described above, we found that ritonavir causes significant accumulation of p21. Accumulation was found at ritonavir concentrations of 15 μM and higher, as shown by Western blotting of Meth A fibrosarcoma cells (Fig. 5A). Low micromolar concentrations of ritonavir also induced significant accumulation of p21 in 3T3 fibroblasts (data not shown). Maximal accumulation of p21 was usually seen between 20 μM and 30/40 μM of ritonavir. The concentrations of ritonavir that induce p21 are similar to those that are antiproliferative and those that induce apoptosis in tumor cells (see also Figs. 1 and 2). It is, therefore, likely that the accumulation of p21 is related to the antiproliferative, and perhaps also to the proapoptotic, effect of ritonavir. The accumulation of p21 in the presence of ritonavir may be caused by inhibited proteolytic degradation, or by increased biosynthesis, or by regulation at the posttranscriptional level. We, therefore, performed experiments monitoring p21 levels in cells, in which protein synthesis had been inhibited by cycloheximide. These experiments were performed with 3T3 cells, which express relatively high levels of p21. Fig. 5B shows p21 levels in 3T3 cells treated with cycloheximide and for various periods of time thereafter with 20 μM ritonavir, 20 μM lactacystin, or DMSO. As shown in Fig. 5B, p21 levels are stabilized significantly on treatment with ritonavir or with lactacystin. These results suggest that the accumulation of p21 in the presence of ritonavir is caused by the
inhibition of proteolytic degradation and not by increased protein synthesis. The inhibition of p21 degradation in ritonavir-treated cells appears to reflect a selective inhibition of proteasomal proteolysis and is in line with the unusual cellular degradation of this protein that does not require ubiquitination (see “Discussion”). To find out whether the apoptosis of ritonavir-treated tumor cells is preceded by growth arrest, we performed kinetic experiments on cell cycle distribution and early signs of apoptosis after the addition of ritonavir (20 μM) by flow cytometry (PI and FITC-annexin V-staining, respectively). We observed a significant enrichment of EL4 V-positive, PI-negative) and late apoptotic or necrotic (annexin V and PI-positive) cells were calculated using Cellquest software. Significant increases in the percentages of early apoptotic (annexin V-positive, PI-negative) and late apoptotic or necrotic (annexin V and PI-positive) cells were seen only several hours later (Fig. 6B), suggesting that at low micromolar concentrations of ritonavir tumor cell apoptosis is preceded by growth arrest in the G1 phase of the cell cycle.

**DISCUSSION**

We have shown here that the HIV protease inhibitor ritonavir inhibits proliferation and induces apoptosis of tumor cells in vitro at concentrations that do not seem to affect the viability of nontransformed cells. Moreover, we have demonstrated the antitumoral activity of ritonavir in a syngeneic EL4-T-lymphoma tumor model in mice. Our data further suggest that a perturbation of proteasomal proteolysis plays a role for the antitumoral effects of ritonavir. The HIV protease inhibitor indinavir, which does not inhibit the chymotryptic activity of 20S proteasomes, is not tumor cell apoptotic. Conversely, we observed a pronounced increase in the cellular concentrations of the antiproliferative and potentially proapoptotic cdk inhibitor p21, a proteasome substrate, and demonstrated that this was attributable to inhibited proteolytic degradation.

The influence of ritonavir on the proteasomal peptidase activities is very complex. For the different peptidases of the isolated 20S proteasome, the effects range from inhibition to activation, depending on the ritonavir concentrations and the reporter substrate used (32). Proteasomes are threonine proteases unlike the HIV protease, which is an aspartyl protease. Nevertheless, ritonavir has been shown to bind reversibly to the active site of the subunit responsible for the chymotrypsin-like activity in isolated 20S proteasomes (30). The differential effects on the peptidases of the isolated 20S proteasome could be explained by postulating the binding of ritonavir to an additional, thus-far-undetected site of the 20S proteasome, the so-called “modifier site” (32). The modulation by ritonavir appears even more complex, because we have shown here that it activates the chymotrypsin-like activity of 26S proteasomes. The chymotrypsin-like activity of proteasomes is thought to be rate limiting for proteasomal protein degradation. However, given the complex and differential modulation of the peptidases of the free 20S proteasome and of the 20S core proteasome within the 26S particle, additional experiments will be necessary to elucidate the effects of ritonavir on protein degradation by isolated proteasomes.

Little information is available on how ritonavir affects the cellular concentration and stability of individual protein substrates and influences the global cellular proteolysis. For two typical model substrates of the ubiquitin-26S proteasome system (NH2-end rule substrates), no intracellular accumulation or change in degradation was observed at concentrations of up to 50 μM ritonavir (30, 33). For p53 (this study) and apolipoprotein B (34) only a marginal (up to 2-fold) accumulation has been observed at the same range of ritonavir concentrations. Only for the cytomegalovirus pp89 (30) and the cdk inhibitor p21 (this study) has significant inhibition of degradation been reported. As shown here, the overall rates of degradation of biosynthetically labeled cellular proteins and the cellular concentrations of bulk poly-ubiquitinated proteins are only marginally affected at low micromolar concentrations of ritonavir.

It is generally assumed that most cellular proteins are degraded by the ubiquitin-26S proteasome system, and, although 20S proteasomes make up a large proportion of cellular proteasomes (35), it is not clear whether they contribute significantly to cellular proteolysis. However, 26S proteasomes and 20S proteasomes can degrade non-ubiquitinated proteins, which are unfolded in vitro and perhaps also in vivo. The more or less significant accumulation of individual proteins under ritonavir treatment may be attributable to the net effects of 20S and 26S proteasomal proteolysis, or caused by the inhibition of 20S or the apparently paradoxical inhibition of 26S proteasomal proteolysis. The latter possibility is in line with our observation that the most pronounced accumulation of p21 was at concentrations of between 20 μM and 30 μM ritonavir, the range in which the chymotryptic 26S proteasomal activity is maximally activated (see Figs. 4 and 5). Our observation that the inhibition of p21 degradation by ritonavir is relatively pronounced is perhaps not surprising. p21 has recently been shown to be degraded by intracellular proteasomes without the need for ubiquitination (36). In addition, it was shown that p21 interacts directly with a subunit of the 20S proteasome and that this interaction is important for its intracellular degradation (37). It is not known whether this interaction takes place with the free 20S proteasome or is restricted to the 20S core particle within the 26S proteasome. Similar interactions also play a role in the processing of NF-κB p105 and in the proteolytic destruction of the NF-κB-inhibitor, 1κBα, (in addition to their ubiquitination; Refs. 38, 39), and in this respect, it is noteworthy that the expression of NF-κB-inducible proteins has very recently been shown to be suppressed significantly at low micromolar concentrations of ritonavir (40).

**Fig. 6.** Kinetic analyses of cell cycle distribution and early signs of apoptosis after addition of ritonavir to EL4 tumor cells. Cell cycle phase distribution and apoptotic cells were determined by flow cytometry as described in “Materials and Methods.” A, typical flow cytometric cell cycle analysis of EL4 cells after 15 h of incubation with ritonavir (20 μM) or DMSO. The G1, S, and G2/M-phase and cells in sub-G1 (apoptotic cells with subdiploid DNA content) are indicated. B, percentages of cells in G1, S, and G2/M phase as well as of early apoptotic (annexin V-positive, PI-negative) and late apoptotic or necrotic (annexin V and PI-positive) cells were calculated using Cellquest software.

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(Blockage of NF-κB-activation may also contribute to apoptosis induction.) The 20S proteasome-interaction sites in proteins have been called class I-degrons (41). Thus, it is conceivable that the “modifier site” and the “class I-degron”-binding site are identical and that competition between ritonavir and protein substrates for this site may contribute to the suppression of substrates the degradation of which depends on a class I-degron.

The precise mechanism(s) of classical proteasome-inhibitor-induced and of ritonavir-induced apoptosis are not known. The pronounced sensitivity of transformed cells may be explained by the observation that deregulated expression of oncoproteins (e.g., c-myc) in transformed cells can directly induce apoptosis or sensitize cells to proapoptotic stimuli, and by the hypothesis that conflicting signals for cell growth and cell cycle arrest may lead to apoptosis (42, 43). However, it should be taken into account that subpopulations of tumor cells may adapt to chronic proteasome-inhibitor treatment, as has been observed for the EL4 tumor-cell line treated with vinylsulfone inhibitors or lactacystin (20, 44). Conclusively, the mechanism(s) that allow survival of cells with partially compromised proteasome function should be studied in detail.

It is not yet clear whether the antiproliferative and the tumor-cell apoptotic effects contribute to the in vivo antitumoral activity of ritonavir, whether the concentrations required are reached in the therapy of HIV-infection, and/or whether certain schemes of application would lead to or enhance this activity in vivo. In the therapy of HIV infection, the ritonavir blood plasma concentrations are normally between 5 μM and 15 μM (45). In one study, patients with side effects had maximum plasma levels ranging from as high as 31 to 46 μM (46). Because of the high blood plasma-protein binding of ritonavir, the pharmacologically active free drug levels are not known (47). However, we have observed a similar sensitivity of tumor cell lines to the apoptotic activity of ritonavir in cell culture experiments with 10% FCS and 100% FCS, so that it is conceivable that antiproliferative and tumor-cell apoptotic drug levels might be reached in vivo.

While this article was in revision, two studies have appeared showing antitumoral activities of HIV protease inhibitors (saquinavir, indinavir, and ritonavir) in KS-xenotransplantation models and in another angiogenic tumor model. In one study, the effect of saquinavir and indinavir was ascribed to an (indirect) blockage of the activation of metalloproteinase 2, which is important for extracellular matrix remodeling. European-American Collaborative Ritonavir Study. N. Engl. J. Med., 333: 1528–1533, 1995.

ACKNOWLEDGMENTS

We thank Nadja Goos and Helga Kochanowski for excellent technical assistance and Prof. Klaus Eichmann and Dr. Randy Cassidy for critical reading of the manuscript. We also thank Prof. Andreas Meyerhans (University of the Saarland, Homburg, Germany) for providing inhibitors.

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