Effects of the proteasome inhibitor ritonavir on glioma growth \textit{in vitro} and \textit{in vivo}

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Abstract

Glioblastoma is a therapeutic challenge as a highly infiltrative, proliferative, and resistant tumor. Among novel therapeutic approaches, proteasome inhibition is very promising in controlling cell cycle and inducing apoptosis. This study investigated the effect of ritonavir, a protease inhibitor of the HIV and a proteasome modulator, on glioma cells. The hypothesis was that proteasome modulation, mainly by only inhibiting proteasome chymotrypsin-like activity, could be sufficient to control tumor progression. The experiments were done on a human glioblastoma-derived GL15 cell line and a rat nitrosourea-induced gliosarcoma 9L cell line. Culturing conditions included monolayer cultures, transplantations into brain slices, and transplantations into rat striata. The study demonstrates that ritonavir, by inhibiting the chymotrypsin-like activity of the proteasome, has cytostatic and cytotoxic effects on glioma cells, and can induce resistances \textit{in vivo}. Ritonavir was unable to control tumor growth \textit{in vivo}, likely because the therapeutic dose was not reached in the tumor \textit{in vivo}. Nevertheless, ritonavir might also be beneficial, by decreasing tumor infiltration, in the reduction of the delerious peritumor edema in glioblastoma. [Mol Cancer Ther. 2004;3(2):129–136]

Introduction

Glioblastoma is a therapeutic challenge as an infiltrative, proliferative, and highly resistant tumor. Glioblastoma is classified as the highest malignancy grade of astrocytomas by the WHO (1–4). Diagnosis is based on anatomo-pathological and imaging criteria. Classical therapy consists in a surgical resection, followed by radiotherapy and nitrosourea-based chemotherapy (5–8). Therapy, however, has a limited and transitory effect (9–13) that encourages innovating therapeutic researches.

Pharmacological approach remains one of the best because it theoretically targets all tumor cells even infiltrative cells. Common targets of the current anticancer therapies comprise the replicative DNA or mitotic machinery, but identification of other subcellular targets can help design drugs with complementary or synergistic effect. One of the novel therapeutic targets is the ubiquitin/proteasome pathway, a key element in cell cycle progression and cell survival (14–19). Protein degradation via the proteasome is often deregulated in tumors, including glioblastoma (20–22).

Many proteasome inhibitors are available in research and have demonstrated a good capacity for control tumor progression by inducing cell cycle arrest and secondary apoptosis (23–26). However, lots of them are complete inhibitors that are either toxic \textit{in vivo} or not tested in glioblastoma. Among proteasome inhibitors, ritonavir is a proteasome modulator (27, 28), already used as a HIV-protease inhibitor in the clinics (29, 30). Ritonavir inhibits the chymotrypsin-like activity of the proteasome while enhancing the trypsin-like activity. Besides, ritonavir partially inhibits the caspase-like activity of the proteasome.

GL15 cells are glioma cells, derived by Bocchini et al. (31), which provide a good model for human glioblastoma, coming as a complement to rat cells such as 9L gliosarcoma cells (32, 33). GL15 cells present characteristics of typical glioblastoma such as polyploidy with 75–90 chromosomes comprising seven to eight copies of chromosome 7 [and subsequent epidermal growth factor receptor (EGFR) amplification located on 7p12]. Glioma cells are usually cultured either on monolayer or on rat transplantation. The technique of brain slice culture is a good model at a midway between \textit{in vitro} and \textit{in vivo} techniques. It provides a three-dimensional and physiological environment, where GL15 cells are infiltrative and angiogenetic, as shown by de Bouåard et al. (34). Here, these models were adapted to test the effect of ritonavir on glioblastoma, with the hypothesis that proteasome modulation, mainly by only inhibiting proteasome chymotrypsin-like activity, could be sufficient to control tumor progression. In this way, the ability of ritonavir to inhibit the chymotrypsin-like activity of the proteasome in the GL15 cells was first verified. Then, ritonavir effects on cell cycle and cell death were investigated in the 9L and GL15 cells. Finally, it was studied whether ritonavir was able to block tumor progression in long-term slice cultures and \textit{in vivo}.

Animals, Materials, and Methods

Ritonavir

Ritonavir was kindly provided by V. Lotteau’s team (INSERM U503, France) who purified it from Norvir oral solution 80 mg/ml (Abbott Ltd., Queenborough, United
Kingdom) by diethyl ether extraction followed by liquid chromatography on silica column with 90% ethyl acetate 10% methanol as mobile phase and desiccation. Purified product was controlled by TLC showing only one spot.

For culture treatments, ritonavir was diluted in culture medium up to 100 μM final concentration (higher concentrations precipitated) after ethanol adjustment to 0.1%, from first dissolution of the purified ritonavir powder in absolute ethanol at 100 mM. Cells were thus treated every other day with medium renewal.

For animal treatments, either Norvir oral solution 80 mg/ml or the lipid solution contained in Norvir soft capsule 100 mg was used, both further diluted in sterile phosphate buffer to 10 mg/ml previous to i.p. injection.

Animal Care

Experiments on animals were done under the responsibility of M. Peschanski and J. Cadusseau (INSERM U421, France) according to French guidelines for animal good handling.

Mice and rats came from breeding of Janvier (Genest-St-Islé) or Iffa Credo (L’Arbresle) in France.

Culture Systems

Human GL15 glioma cells were kindly given by M. Tardy (INSERM U421), and grown in 45% Dulbecco’s medium (DM, Life Technologies, Inc., Gibco BRL, Paisley, Scotland), 45% MEM (Life Technologies), 7.5 mM glucose, 2 mM glutamine, 1% antibiotics (penicillin-streptomycin, Life Technologies), 10% heat-inactivated fetal bovine serum, and 0.1 mM pyruvate. Cultures were incubated at 37°C in 5% CO2 humidified atmosphere. Rat 9L glioma cells were cultivated in the same conditions.

For brain slice cultures, 6-day-old (P6) C57Bl6 mice (Janvier) were sacrificed and 400-mg brain slices were dissected on a 2-gauge needle. Culture medium was MEM with 5.5 mM d-glucose, 4 mM sodium bicarbonate, 1.5 mM bovine albumin, 1.25 mM apotransferrin, 850 nM insulin, 180 nM putrescin, 230 nM sodium selenide, 46 nM triiodothyronine, 200 nM progestosterone (Sigma, San Quentin, France), and 10% fetal bovine serum (pH 7.65). Cultures were maintained at 36°C in 6% CO2 humidified atmosphere.

Proteasome Activity

GL15 cells were plated in 35-mm-diameter Petri dishes at a density of 1 x 105 cells/dish. After 2 days, cells were treated with 0, 10, or 100 μM ritonavir for 5 h. Then cells were rinsed once with buffer I [50 mM Tris (pH 7.4), 100 mM NaCl, 5 mM MgCl2] and lysed with 100 μl of a hypotonic and cold buffer II [50 mM Tris (pH 7.4), 250 mM sucrose, 5 mM MgCl2] as adapted from previous works (36–38). Lysates were centrifuged at 10,000 × g for 20 min in cold. Protein concentrations were determined by the use of BCA kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. In a 96-well plate, 2.5 or 10 μg of protein were added to various Suc-LLVY-AMC concentrations in buffer I, final volume of 100 μl (Suc-LLVY-AMC from Sigma, specific for the chymotrypsin-like activity of the proteasome, first diluted in DMSO at a concentration of 10 mM). Released amino-methyl-coumarin (AMC) was measured at various time points between t0 and t225 min with Perkin-Elmer Luminescence Spectrometer LS50B and FLWinlab software version 4.00.02 using the well plate reader: λex = 380 nm, λem = 460 nm. AMC calibration, ranging from 0.5 to 50 μM, was used to determine the slope of the initial line [P] = f(t) (with [P]: concentration of product, i.e., concentration of AMC released, and t: time) and initial velocity of the enzymatic reaction, that is, the proteasomal activity, in micromolars per minute (or international units per liter). Pharmacological constants (catalysis: Kcat, Michaelis: Km) were evaluated using Micropharm software (INSERM, France) on a graph representing vi = f(log[S]) (with vi: initial velocity and [S]: substrate concentration). Adequation to an inhibitory model was also tested. Basal proteasome activity was investigated in untreated GL15 cells and primary culture of mouse astrocytes from C57Bl6 neonates obtained as described before.

Proteasome activity was also measured on striatum from female Wistar Han rats (Janvier) weighing 300–400 g, grafted with 150 GL15 cells, using a 5-μl Hamilton syringe 85RN 26G, and treated with 30 mg/kg ritonavir i.p. (from Norvir soft capsule) daily for 3 days. Rats were anaesthetized with 0.4 g/kg chloral hydrate and perfused with 200 ml cold buffer I,.grafted striatum were collected in HBSS and diluted 1:10 in a citrate buffer solution some, first diluted in DMSO at a concentration of 10 m M. Released amino-methyl-coumarin (AMC) was measured at various time points between t0 and t225 min with Perkin-Elmer Luminescence Spectrometer LS50B and FLWinlab software version 4.00.02 using the well plate reader: λex = 380 nm, λem = 460 nm. AMC calibration, ranging from 0.5 to 50 μM, was used to determine the slope of the initial line [P] = f(t) (with [P]: concentration of product, i.e., concentration of AMC released, and t: time) and initial velocity of the enzymatic reaction, that is, the proteasomal activity, in micromolars per minute (or international units per liter). Pharmacological constants (catalysis: Kcat, Michaelis: Km) were evaluated using Micropharm software (INSERM, France) on a graph representing vi = f(log[S]) (with vi: initial velocity and [S]: substrate concentration). Adequation to an inhibitory model was also tested. Basal proteasome activity was investigated in untreated GL15 cells and primary culture of mouse astrocytes from C57Bl6 neonates obtained as described before.

Thymidine Incorporation

GL15 cells were plated into 35-mm-diameter Petri dishes at a density of 2 x 105 cells/dish. After 3 days, cells were treated with 0, 1, 10, and 100 μM ritonavir during 24 h. Then, 1 μCi/dish 3[H]thymidine (Amersham, Orsay, France) was added to the medium and, 6 h later, DNA was extracted using a 0.1% SDS solution, precipitated by 5% cold trichloroacetic acid and filtrated. Radioactivity of the filter was measured using a beta liquid scintillation counter (Wallac 1409) and expressed in counts per minute (CPM). These experiments were also done on rat 9L glioma cells. Some dishes also received 5 μg/ml 59788 (Servier, Orléans, France), an inhibitor of multidrug resistance-related transporters.

Cell Cycle Profile

Analysis of cell cycle profiles was carried out on GL15 cells plated into 60-mm-diameter Petri dishes at a density of 3 x 105 cells/dish. The next day, cells were synchronized with low serum (0.5%) medium for 48 h. Then cells were treated with 0, 1, 10, and 100 μM ritonavir in fresh medium (10% serum, 0.1% ethanol) for 30 h. Trypsinized cells were collected in HBSS and diluted 1:10 in a citrate buffer solution (3 mM sodium citrate (pH 6), 10 mM NaCl, 0.1% NP40,
50 μg/ml RNase A, and 50 μg/ml propidium iodide]. After a 2-h incubation at 4°C, a minimum of 10^5 cells were analyzed using an Epics XL cytometer (Beckman-Coulter, Miami, FL) and the proportion of cells in each cycle phase computed by Multicycle software (Flow Phoenix System, San Diego, CA).

**MIB Labeling on Slices**

GL15 in organotypic cultures were treated with 0, 1, 10, or 100 μM ritonavir for 7 days. Tissue slices were fixed in paraformaldehyde 4% for 4 h at 4°C, rinsed thrice with PBS 0.1 M, permeabilized with PBS-0.2% Triton-5% rabbit serum for 1 h, treated in pre-boiling citrate buffer 10 mM (pH 6) for 4 min, and replaced in the previous solution for 1 h again. Then they were incubated with the monoclonal antibody raised against the human Ki-67 antigen (clone MIB-1, Dako, The Netherlands), diluted 1:400 in PBS-0.1 M, permeabilized with PBS-0.2% Triton-5% rabbit serum and incubated with FITC-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA), diluted 1:400 in PBS. They were rinsed thrice in PBS and incubated with an anti-GFP (made in rabbit, Molecular Probes, Eugene, OR and Leiden, The Netherlands), diluted 1:400 in PBS-0.2% Triton, overnight at 4°C under slight shaking. Slices were rinsed thrice in PBS and incubated with Cy3-conjugated goat anti-mouse IgG (Vector, Burlingame, CA), diluted 1:1000 in PBS. Slices in PBS thrice and incubated with an anti-GFP and Cy3-conjugated goat anti-mouse IgG (Vector, Burlingame, CA), diluted 1:1000 in PBS. They were rinsed in PBS thrice, in PB 0.1 M once and mounted. The presence of MIB-1 immunoreactive cells, human specific, co-labeled with GFP, was investigated by epifluorescence in the tumor mass (microscope Zeiss Axioplan 2 used with magnifications of ×400 and ×1000 and RS image software, RS photometrics). Cells were counted either as proliferative cells (normal mitotic nuclei) or apoptotic nuclei (nucleus condensation and fragmentation). The negative controls were first-antibody deleted or made of additional ungrafted slices. Counts were reported to high proliferation areas as measured with GFP labeling at a magnification of ×50 using KS400 software from Zeiss (Jena, Germany). The latest procedure also allowed determining the area of the tumor core.

**Lactate Dehydrogenase Release**

Evaluation of cytotoxicity kinetics was carried out using lactate dehydrogenase (LDH) kits (Boehringer Mannheim, Meylan, France). For cell cultures, GL15 cells were plated on 24-well plates at a density of 25 × 10^3 cells/well and treated with 0, 1, 10, 40, and 100 μM ritonavir for a maximum of 5 days. Levels of LDH were measured in supernatants (500 μl/well) or after lysis of surviving cells (500 μl medium-2% Triton per well) by the colorimetric method detailed in the manufacturer’s protocol using a spectrophotometer (λ = 490 nm; Dynex MRX, Dynatech Laboratories, Guyancourt, France). Results are expressed in percentage with 

\[
P = \frac{LDH_{\text{supernatant}}}{LDH_{\text{supernatant}} + LDH_{\text{cells}}} \times 100%
\]

For organotypic cultures, LDH release was only measured in the supernatant at various time points before (5 days) and after (7 days) ritonavir treatment.

**Caspase Activity**

GL15 cells were plated into 35-mm-diameter Petri dishes (10^5 cells/dish) and treated with 0 or 100 μM ritonavir. At 0 and 60 h, proteins were extracted in a cold Tris-2% Triton buffer containing 1 mM phenylmethylsulfonyl fluoride and 5 μl/ml Protease Inhibitor Cocktail (Sigma); aliquots of whole cell lysates were kept frozen. Thirty micrograms of total proteins were incubated for 2 h at 37°C with 100 nM z-DEVD-AFC (Calbiochem, La Jolla, CA), a specific fluorogenic substrate of caspase-3, using the dilution buffer specified by the manufacturer and supplemented or not with 0.2 mM caspase-3 inhibitor I. Amino-trifluoromethyl-coumarin (AFC) release was measured after a 2-h incubation, with Perkin-Elmer Luminescence Spectrometer LS50B and FLWinlab software version 4.00.02 (2001) using the well plate reader: λ_{exc} = 400 nm, λ_{em} = 505 nm. Results were expressed in FOR: “fold over a reference” with 

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F = \frac{\text{fluorescence of the well}}{\text{fluorescence of the reference well}} \times 100%
\]

Similar experiments were performed with various caspase substrates (z-YVAD-AFC, z-VEID-AFC, z-IETD-AFC, Ac-LEHD-AFC, for caspases 1, 6, 8, and 9, respectively).

**Survival Experiments**

9L tumor cells (10^5; in 1 μl RPMI) were implanted into the brain of male Fischer rats (Iffa Credo) as previously described (39). Rats were treated daily with 40 mg/kg, i.p. ritonavir from Norvir oral solution diluted in PBS and maintained on standard laboratory chow and water ad libitum until their death, to draw Kaplan-Meier survival graphs.

**Statistics**

Results are expressed as mean ± SEM. Groups were compared with Kruskal-Wallis followed by Bonferroni/Dunn with the StatView statistical package. Statistical significance was set at 5% and expressed with asterisks on illustrations (*).

**Results**

**Measurement of the Chymotrypsin-Like Activity of the Proteasome**

GL15 cells are human glioblastoma-derived cells that share main characteristics of glioblastoma such as polyploidy and supernumerary chromosome 7 (31, 40). They are heterogeneously GFAP- and vimentin-positive, and also express multidrug resistance-related transporters, like multidrug resistance-related protein (MRP) (10). To investigate whether ritonavir is able to enter tumor cells and target the proteasome in vivo, GL15 cells were treated with ritonavir at different doses for 5 h and lysates, obtained in soft conditions, were then tested with a specific fluorogenic substrate of the chymotrypsin-like activity of the proteasome. Kinetic curves were used to determine enzymatic activity and pharmacological constants. \( K_m \) values were 84 ± 28, 89 ± 41, and 113 ± 28 μM, and \( K_{cat} \) values were 4.68 ± 0.6, 3.9 ± 0.7, 2.58 ± 0.3 s^-1, for controls, 10 μM ritonavir, and 100 μM ritonavir, respectively (Fig. 1A). This corresponds to a ritonavir-induced noncompetitive inhibition of the proteasome chymotrypsin-like activity with a correlation to the theoretical noncompetitive model of 0.96. The concentration corresponding to 50%
inhibition of the activity (Cl_{50}) was at around 50 μM ritonavir, but this inhibition was not sufficient to recover basal activity of astrocytes (not shown). Ritonavir inhibition was not seen to induce polyubiquitylated protein accumulation considering that GL15 cells constitutively have lots of them (not shown).

As ritonavir is described not to cross the blood-brain barrier in vivo (41), its penetration in rat tumor-bearing brain was evaluated through the measurement of proteasome activity in GL15 cell-transplanted striata from rats treated with 30 mg/kg, i.p. ritonavir daily for 3 days. A 34% inhibition of the chymotrypsin-like activity from these pieces of dissection compared to controls suggests that ritonavir entered the tumor in vivo (Fig. 1B). These studies thus show that ritonavir is a moderated noncompetitive inhibitor of the chymotrypsin-like activity of the proteasome, in the GL15 cells, in monolayer cultures, and in vivo when transplanted into the rat striatum.

**Effects of Ritonavir on Proliferation and Cell Death**

Proteasome is required for cell cycle progression (14, 15, 19). To determine the ability of ritonavir to induce cell cycle arrest by inhibiting the chymotrypsin-like activity of the proteasome, GL15 were treated with crescent doses of ritonavir for 30 h and exposed to [3H]-thymidine during the last 6 h. [3H]-thymidine incorporation in the newly synthesized DNA decreased, as shown by decreased radioactivity, especially at 100 μM ritonavir, with values of 14,645 ± 723 and 7,710 ± 1,008 CPM for controls and 100 μM ritonavir, respectively (Fig. 2A). These results were reproduced with 9L cells (not shown). As ritonavir is a substrate of multidrug resistance-related transporters (42), the same experiment was done with a specific inhibitor S9788 (43). The effect of ritonavir on GL15 cells did not change in a significant manner when S9788 was added (not shown). To see in which phase of the cell cycle GL15 cells were blocked, cell cycle profile was drawn on synchronized cells, treated for 30 h with crescent doses of ritonavir, and labeled with propidium iodide. High doses of ritonavir enhanced the proportion of cells in the G1 phase from 59% for controls to 84% at 100 μM ritonavir (Fig. 2B).

Because cell cycle arrest often precedes apoptosis in tumor cells, GL15 cells were examined for apoptotic

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**Figure 1.** Ritonavir inhibits the chymotrypsin-like activity in vitro and in vivo. Chymotrypsin-like activity of the proteasome was measured using a specific fluorogenic substrate on lysates of GL15 cells previously treated with 0, 10, or 100 μM ritonavir for 5 h (A) or of GL15 cell-transplanted striata from rats treated daily with ritonavir 30 mg/kg, i.p. for 3 days (B). A, Log(S), that is, logarithm of substrate concentrations, in function of enzymatic activity, that is, initial velocity of the reaction (vi), expressed in international units per liter (IU/l) and leads to calculation of pharmacological constants of Michaelis (K_m) and of catalysis (K_cat): K_m = 4.68 ± 0.6 s^{-1} and K_cat = 84 ± 28 μM for controls; K_m = 3.9 ± 0.7 s^{-1} and K_cat = 89 ± 41 μM with 10 μM ritonavir; and K_m = 2.58 ± 0.3 s^{-1} and K_cat = 113 ± 28 μM with 100 μM ritonavir. Correlation with a noncompetitive inhibition model was 0.964. B, chymotrypsin-like activity of the proteasome was also found to be inhibited by 34% in vivo with a fluorogenic substrate concentration of 80 μM: 0.067 ± 0.001 IU/l for controls and 0.044 ± 0.002 IU/l for the treated group (n = 4, P < 0.001).

**Figure 2.** Ritonavir induces a cell cycle arrest of GL15 cells. GL15 cells were treated with 0, 1, 10, or 100 μM ritonavir for 30 h and were exposed in the end to [3H]-thymidine (A) or propidium iodide (B). In A, radioactivity (expressed in counts per minute (CPM)) corresponds to [3H]-thymidine incorporation in the cell DNA and decreased with crescent doses of ritonavir: 14,645 ± 723, 14,257 ± 1,105, 12,013 ± 516, and 7,710 ± 1,008 CPM at respective concentrations of 0, 1, 10, and 100 μM ritonavir (n = 6, P < 0.001). This tendency was not significantly changed with the addition of S9788, an inhibitor of the multidrug resistance-mediated transporters (P = 0.79). B, corresponding cell cycle profile, with the following percentages in G_1-S-G_2 phases: 59 – 20 – 21% for controls (14,604 events); 54 – 23 – 23% at 1 μM ritonavir (16,547 events); 53 – 22 – 25% at 10 μM ritonavir (21,338 events); and 84 – 7 – 9% at 100 μM ritonavir (12,080 events). Note that ritonavir induced a G_1-block at the 100-μM dose.
Features under ritonavir treatment. First it was investigated whether cell death occurred at all, using the measure of LDH release as a marker of cell membrane permeability and cell death. This measure of LDH release was related to LDH total amounts in cells, and expressed in percentages of cytotoxicity. One hundred micromolars ritonavir induced an increase in cytotoxicity up to 45 ± 2% and 61 ± 1% at 3 and 5 days, respectively, whereas normal values turned around 10% (Fig. 3A). To explore which mechanisms underlie this cell death induction, GL15 cells were treated with 100 μM ritonavir and caspase activity was determined using fluorogenic substrates. Caspase activities increased in general except for caspase-1 (Fig. 3C). Caspase-3 activity had the highest increase within 60 h by around 2-fold over t₀ (Fig. 3B). Therefore, these studies show that high concentrations of ritonavir induce a cell cycle arrest in the G1 phase followed by apoptosis of GL15 cells.

Analysis of Three-Dimensional Tumor Growth under Ritonavir Treatment

It is reported that cells can develop resistance over time to proteasome inhibitors (37). To investigate eventual emergence of molecular resistance to ritonavir, the model of brain slice cultures was selected. First described by Stoppini et al. (35) and further adapted to tumor culture by de Bouard et al. (34), brain slice cultures provide a unique model for culturing glioma cells in a physiological and long-term manner. To verify that brain slices could survive to high doses of ritonavir, slices were exposed to 100 μM ritonavir during 1 week. No increase of LDH release could be detected in treated slices compared to controls (not shown). Then murine slices transplanted with human GL15 cells were treated with crescent doses of ritonavir for 7 days (Fig. 4). Proliferative cells were labeled with a human specific MIB-1 antibody directed against the Ki-67 antigen. Because this antibody can also label apoptotic nuclei (44), which will have condensed and fragmented chromatin, nuclei were counted at a magnification of ×1000 in the zone of highest glioma cell proliferation. One hundred micromolars ritonavir induced a doubling of the density of proliferative GL15 cells as compared to controls. But numbers of apoptotic MIB-positive nuclei increased in parallel at this concentration, corresponding to around 40% of the proliferative cell count. Surprisingly, tumor areas were much smaller at any concentration of ritonavir tested.

Finally, to test the efficacy of ritonavir on tumor growth in vivo, the help of Fischer rats allowed the setup of the syngeneic model of 9L cells for brain transplantsations (33, 45). Compared to GL15 cells, 9L cells are not infiltrative, so ritonavir was only tested for its ability to block tumor proliferation in vivo. Rats were treated daily with 40 mg/kg, i.p. ritonavir until their death, to draw a Kaplan-Meyer survival graph. Although, cytostatic effect of ritonavir on 9L cells was similar to its effect on GL15 cells in monolayer cultures, no improvement in rat survival was observed. Thus, the cytostatic effect of ritonavir, described in vitro, led to the emergence of a highly proliferative and resistant tumor subpopulation and was unable to control tumor cell proliferation in vivo.

Discussion

The present study demonstrates that ritonavir, by inhibiting the chymotrypsin-like activity of the proteasome, has cytostatic and cytotoxic effects on glioma cells that can however induce resistances in vitro and are unable to control tumor proliferation in vivo.
Effects of Ritonavir on Glioma Growth

The idea that ubiquitin/proteasome pathway is an attractive target for novel anticancer therapies is actively explored (24, 46). Indeed tumor cells, including glioma cells, not only progress through up-regulation of oncogenes, but also through increase of proteolytic degradation of tumor suppressors and cell cycle inhibitors, which mostly requires the ubiquitin/proteasome system (20, 22, 47). The constitutively high proteasome basal activity and the presence of many ubiquitinylated proteins, as observed here in GL15 cells, suggests that protein degradation occurs through increase of both protein targeting and endopeptidase activity.

A commonly used proteasome inhibitor, lactacystin, inhibits the proteasome by binding covalently to the NH2-terminal threonine residue of any active site of the proteasome (48). Most proteasome inhibitors used in research are also full and irreversible inhibitors, lethal, and not to be used in humans: most pharmacophores with a lactone, vinylsulfone, or epoxiketone. Although aldehydes (e.g., LLnL or MG132) are competitive reversible inhibitors, they are still complete inhibitors and most of them are toxic in vivo. Few aldehydes (e.g., PSI) or boronates (e.g., PS-341) are inhibitors of the proteasome that are usable in vivo because they are reversible and more selective (24, 49). Here, ritonavir, used in clinics as a HIV-protease inhibitor, appeared to inhibit the chymotrypsin-like activity of the proteasome in a noncompetitive manner when exposed to GL15 cells, which may explain the possibility of a proteasome modulation by enhancing or inhibiting its activities. However, Schmidtke et al. (28) showed that ritonavir could have a competitive inhibition site, considering that it partially protects LMP7 and PSBM-1(X) from a covalent binding with a specific inhibitor; LMP7 and PSBM-1(X) are β-subunits of the proteasome, implicated in trypsins- and chymotrypsin-like activities (50, 51). Other HIV-protease inhibitors, like saquinavir or indinavir, share these properties of proteasome modulation and ritonavir seems to have the greatest affinity among them (52, 53), but these effects are not considered to be involved in their therapeutic action on HIV infection.

Most proteasome inhibitors have demonstrated strong cytostatic and proapoptotic activities in various cell lines (23, 47, 54–56). Few studies were done on glioma cells, but proteasome inhibitors induced apoptosis in all cases (57–59). In this study, ritonavir also lead to cell cycle arrest in the G1 phase and subsequent apoptosis. MIB-1 (anti-Ki-67 antibody) labeling of apoptotic cells suggests that these cells were proliferating when they died (44). The relationship between ritonavir-induced proteasome inhibition and cytostatic/cytotoxic effect is only suggested here, but it is supported by recent data with saquinavir inducing apoptosis in U373 glioma cells in vitro (38). However, implication of other subcellular targets is not excluded, for example, metalloproteases of the extracellular matrix (60, 61). Thus, it might be considered that proteasome inhibition is only an indirect phenomenon in cells. In this sense, it has been shown by Beyette et al. (62) that chymotrypsin-like activity can specifically be inhibited in lymphocytes during apoptosis in an endogenous way, as an apoptotic step by itself.

Glioma resistances to chemotherapy have often been reported, in particular through low folylypolyglutamyl synthetase activity and multidrug resistance-related transporters (9, 11). For instance, P-glycoprotein (P-pg) is known to exclude ritonavir out of the cytoplasm, although weakly compared to other molecules (42). However, GL15 cells are P-pg-negative (10). They do express MRPs, but MRPs are inhibited by S9788 and ritonavir itself (43, 63, 64); the combination of the two, as studied here, did not enhance ritonavir effect in the cells at short term. The study on slice cultures revealed that a resistance to ritonavir cytostatic effects eventually emerges. It can be explained by previous works of Glas et al. (37) showing the induction of other proteolytic pathways to compensate for long-lasting proteasome inhibition.

Figure 4. Ritonavir effects on GL15 cells in brain slice culture. GFP-expressing GL15 cells were cultured on brain slice and treated with 0, 1, 10, and 100 μM ritonavir for 7 days. Immunohistochemistry with MIB-1 anti-Ki-67 antibody was then used to label specifically human GL15 cells, depending on their nuclear morphology as observed at a magnification of ×1000. GFP-labeling allowed the measurement of tumor areas (A) and tumoral highly proliferative areas (mainly tumor borders) (A). The latest values were used to calculate the proliferating cell density, as reported in (A); results are 23 ± 4, 30 ± 4, 24 ± 6, and 53 ± 3 cells/mm² at 0, 1, 10, and 100 μM ritonavir, respectively (n = 3, P < 0.01). The table (B) indicates that lots of apoptotic nuclei were seen at a ritonavir concentration (Rv[μM]) of 100 μM, as compared to controls and low doses of ritonavir (apoptotic line: −, +, ++, and +++ correspond to <10%, 10–30%, and 30–60% apoptotic cells as compared to proliferative cell count, respectively). C, tumor mass area: 414,000 ± 66,000 μm² for controls; 147,000 ± 5,000 μm² at 1 μM ritonavir; 83,000 ± 17,000 μm² at 10 μM; and 185,000 ± 24,000 μm² at 100 μM (n = 3, P < 0.001 at 10 μM, and P < 0.01 at 1 and 100 μM).
Ritonavir is known not to cross the normal blood-brain barrier (41, 42), but the results presented here suggest that ritonavir could cross the blood-tumor “barrier” in vivo. However, studies with radioactive ritonavir are required to confirm this result. Other substances, which did not cross the blood-brain barrier, were successfully tried in the treatment of glioblastoma in humans (65) because the blood-tumor barrier is permeable (66–68). The main problem with ritonavir is that high concentrations are probably not reached in vivo. When used in an oral dosage of 600 mg bid (chronic use twice daily), concentration of ritonavir is below 50 ng/ml in the human cerebrospinal fluid (less than 0.1 μM), whereas it could reach up to 17 μg/ml (24 μM) in the plasma at 2 h (41, 69). The increase of vessel permeability within the tumor is probably not sufficient to locally reach high concentrations of ritonavir which can explain the inability of ritonavir to prolong survival of 9L cell-transplanted rats, as observed in this study. However, the 9L cell line is not infiltrative, and the results are not sufficient to exclude an effect of ritonavir on infiltrative tumors, which is just suggested here with the reduction of tumor mass areas of GL15 cells in brain slice cultures at any tested concentrations, including at low concentrations. In this sense, HIV-protease inhibitors are very promising in the treatment of Kaposi’s cancer by inhibiting tumor cell infiltration and angiogenesis (60, 61) although this effect is surely not related to proteasome inhibition. By immunomodulation and anti-infiltration, ritonavir might also be favorable in the reduction of the deleterious peritumor edema in glioblastoma, in association with other local cytotoxic strategies.

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References


Molecular Cancer Therapeutics

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Nathalie Laurent, Sophie de Boüard, Jean-Sébastien Guillamo, et al.


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