Synthetic chenodeoxycholic acid derivative HS-1200-induced apoptosis of p815 mastocytoma cells is augmented by co-treatment with lactacystin

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The antitumor activity of a synthetic chenodeoxycholic acid derivative, HS-1200, on the p815 mastocytoma cell line was investigated. We present several lines of evidence indicating that HS-1200 at 35 μM induced apoptosis of p815 cells. Reduction of mitochondrial membrane potential, the release of cytochrome c to cytosol, activation of caspase-3, nuclear condensation, production of poly(ADP-ribose) polymerase cleavage, generation of DNA fragmentation and nuclear condensation were demonstrated. Importantly, HS-1200 inhibited proteasome activity. Next, the combination treatment of HS-1200 or a proteasome inhibitor lactacystin was undertaken. Although the single treatment of 20 μM HS-1200 or 1 μM lactacystin induced apoptosis slightly, the combination treatment of them augmented prominently the extent of apoptosis. The combination therapy of HS-1200 and lactacystin could be potentially a therapeutic strategy reducing the extent and severity of treatment-related toxicity. ***Anti-Cancer Drugs*** **14:219–225 © 2003 Lippincott Williams & Wilkins.**

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Introduction

Bile acids are polar derivatives of cholesterol essential for the absorption of dietary lipids and regulate the transcription of genes that control cholesterol homeostasis. Different bile acids exhibit distinct biological effects. Importantly, natural bile salts were reported to inhibit cell proliferation and induce apoptosis in various cancer cells [1,2]. We developed several ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) derivatives, and have been studying their biological activity. We have demonstrated that they had apoptosis-inducing effect in various cancer cells, such as leukemia and breast cancer cells [3–6].

Cells undergoing apoptosis usually develop characteristic changes, including nuclear condensation and degradation of DNA into oligonucleosomal fragments [7]. Apoptotic cell death is thought to result ultimately from the proteolytic actions of caspase [8] and alterations in mitochondrial function play a key part in the regulation of apoptosis [9]. Recently, the proteasome system has been shown to be implicated as a negative or positive mediator of apoptosis. Generally, in rapidly proliferating cells, the proteasome-mediated hydrolysis of certain proteins has to occur to prevent apoptotic death [10].

This study was conducted in order to investigate the *in vitro* antitumor activity of synthetic CDCA on p815 mastocytoma cells as it pertains to both its basic drug mechanism and the potential therapeutics of the pathologic conditions accompanying mast cell proliferation. For this study we employed a CDCA derivative, HS-1200, which showed the strongest activity among various synthetic bile acid derivatives on p815 mastocytoma in our preliminary study. As will be shown, HS-1200-induced apoptosis of p815 mastocytoma cells is mediated via mitochondria, caspase and proteasome. In addition, combination treatment with lactacystin and HS-1200 potentiates the induction of apoptosis.

Materials and methods

Reagents

Rabbit polyclonal anti-human caspase-3 and anti-horse cytochrome *c* antibodies were obtained from Santa Cruz

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Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-human poly(ADP-ribose) polymerase (PARP) antibody was from Oncogene (Cambridge, MA). Goat FITC–anti-rabbit IgG antibody was from Vector (Burlingame, CA). TUNEL reaction mixture was from Boehringer Mannheim (Mannheim, Germany). ECL Western blotting detection reagents were from Amersham International (Little Chalfont, UK). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR). Caspase inhibitor, z-VAD-fmk, was from Kamiya Biomedical (Seattle, WA). Suc-LLVY-AMC and lactacystin were from Calbiochem (San Diego, CA).

Preparation of HS-1200

HS-1200 was synthesized as described previously [5].

Cell culture

The p815 murine mastocytoma cell line was purchased from the ATCC (Rockville, MA). Cells were maintained at 37°C with 5% CO_2 in air atmosphere in DMEM with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

HS-1200 treatment

The original medium was removed 24 h after p815 cells were subcultured. Cells were washed with PBS and then incubated in the same fresh medium. HS-1200 from a stock solution was added to the medium to obtain various dilutions of the drug. The concentration of ethanol, 0.1–0.5% (v/v), used in this study both as a vehicle for bile acids and as a control, had no effect on p815 cell viability in our preliminary studies. Since the dose required for half-maximal inhibition of viability 7 h after treatment was about 35 μM (as will be presented in Results), this single concentration was utilized for further *in vitro* assessment of apoptosis.

Effect of caspase inhibitor

To study the effect of pan-caspase inhibitor, cells were preincubated for 1 h with 100 μM z-VAD-fmk and then HS-1200 was added while maintaining the inhibitor in the culture medium.

Trypan blue dye exclusion

Cells treated with HS-1200 over a time span of 7 h were harvested, stained with Trypan blue and then counted using a hemocytometer.

Hoechst staining

Cells were harvested and the cell suspension was centrifuged onto a clean fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/ml Hoechst 33342 at 37°C for 30 min and fixed for 10 min in 4% paraformaldehyde.

TUNEL technique

Cells were harvested and the cell suspension was centrifuged onto a clean fat-free glass slide with a cytocentrifuge. The samples were fixed for 30 min in 4% paraformaldehyde, incubated in permeabilization solution for 2 min on ice and labeled in TUNEL reaction mixture at 37°C for 60 min.

Immunofluorescent staining

The cytocentrifuged cells were fixed in 4% paraformaldehyde for 10 min, incubated with anti-cytochrome *c* antibody for 1 h and then with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS, and observed and photographed under an epifluorescence microscope.

Cell counts

Samples were observed under an epifluorescence microscope. For each time point, the number of cells which showed condensed or fragmented nuclei with Hoechst staining, demonstrated positive TUNEL reaction and had lost the punctate staining pattern for cytochrome *c* was determined by a blinded observer from a random sampling of 400 cells per each point.

Assay of mitochondrial membrane potential

Alterations in mitochondrial membrane potential were determined by staining cells with the indicator dye, JC-1. JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min.The medium was then replaced with phosphate-buffered saline, and cells were quantified for J-aggregated fluorescence intensity in a modular fluorimetric system (Spex, Edison, NJ) using excitation and emission filters of 492 and 590 nm, respectively.

Western blot analysis

Cells (2×10^6) treated with bile acids were washed twice with ice-cold PBS, resuspended in 100 μl ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris–HCl, pH 7.6, 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin) and incubated at 4°C for 30 min. The lysates were centrifuged at 14 000 r.p.m. at 4°C for 15 min, and SDS and Na-DOC (final concentration 0.2%, respectively) were added. Protein concentrations of cell lysates were determined by the method of Bradford (Bio-Rad protein assay). Equal amounts of protein were subjected to 15 or 7.5% SDS–PAGE for caspase-3 and PARP, respectively, and transferred to a nitrocellulose membrane. Western blot analyses were carried out by using appropriate antibody (as noted in the figure legends) and immunostaining with antibodies was performed using ECL Western blotting detection reagents.

Electron microscopy

Cells treated with HS-1200 were fixed in 2.5% glutaldehyde for 1.5 h. Cells were then post-fixed in 1% osmic

acid for 2 h and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and morphological changes were examined using a Hitachi H600-3 electron microscope.

Proteasome activity assay

Cells were lysed in proteasome buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol and 4 mM dithiothreitol), sonicated and then centrifuged. The supernatant (40 μg protein) were incubated with proteasome activity buffer (0.05 M Tris–HCl, pH 8.0, 0.5 mM EDTA and 50 μM Suc-LLVY-AMC) at 37°C for 1 h. The intensity of fluorescence was measured by a modular fluorimetric system (Spex) at 380 nm excitatory and 460 nm emission wavelengths. All readings were standardized using the fluorescence intensity of an equal volume of free AMC solution (50 μM).

Effect of combination treatment with lactacystin and HS-1200

A representative proteasome inhibitor lactacystin was employed. To test the effect of combination treatment, cells were incubated with 1 μM lactacystin and 20 μM HS-1200 for 24 h since lactacystin or HS-1200 at that each concentration showed only slight apoptosis-inducing activity at that time point. The effect of combination treatment was assessed by four quantification assays by Trypan blue exclusion, Hoechst staining, TUNEL assay and cytochrome *c* release, and Western blot assays for caspase-3 and PARP. To this end, we defined the kinetics of proteasome inhibition by the combination treatment. The proteasome activity was measured over a period of 7 h.

Statistical analysis

Statistical results were expressed as the mean ± SD obtained from each independent experiment. The results of the experimental and control groups were tested for statistical significance by a one-tailed Student's *t*-test or a two-tailed ANOVA.

Results

HS-1200 induced apoptosis of p815 mastocytoma cells in a mitochondria- and caspase-dependent manner

HS-1200 produced a significant dose-dependent decrease in cell viability (Fig. 1A). Treatment of p815 mastocytoma cells with 35 μM HS-1200 resulted in several alterations associated with apoptosis (Fig. 1B–J). The amount of cell death based on the alterations in membrane integrity, nuclear condensation or fragmentation and DNA fragmentation was estimated as determined by Trypan blue exclusion (Fig. 1B), by TUNEL assay (Fig. 1C) and by Hoechst staining (Fig. 1D). Nuclear condensation, which is one of the hallmarks of apoptosis, was demonstrated (Fig. 1E). The mitochondrial events were demonstrated, too. Whereas control cells had a bright punctuate cytoplasmic cytochrome *c* distribution, the cytochrome *c* staining was diffuse and uniform throughout the cytoplasm in cells treated with HS-1200, indicating that cytochrome *c* had been released from the mitochondria (Fig. 1F). Quantification assay showed that the percentage of cells showing cytochrome *c* release increased (Fig. 1G). The mitochondrial membrane potential ($\Delta\Psi_m$) decreased prominently in HS-1200-treated cells (Fig. 1H). The changes of all of the parameters quantified by the above methods were time dependent (Fig. 1B–D, G and H). As shown in Western blotting data (Fig. 1I), HS-1200 treatment induced caspase-3 and PARP degradation, and produced the processed caspase-3 p20 and PARP p85 cleaved products. The electron microscopical observation conducted to confirm the type of cellular death showed the peripheral condensation of the nucleus in HS-1200-treated cells (Fig. 1J).

HS-1200 inhibited proteasome activity independently of caspase

HS-1200 at 35 μM caused a prominent decrease in proteasome activity in a time-dependent manner over a time span of 7 h. At 7 h after treatment, the reduction of proteasome activity reached approximately 50% of the control. A pan-caspase inhibitor, z-VAD-fmk, did not abolish HS-1200-induced inhibition of proteasome activity (Fig. 2).

Combination treatment with HS-1200 and lactacystin augmented the induction of apoptosis

As determined by viability assay, Hoechst staining, TUNEL assay and cytochrome *c* release, the combination of 1 μM lactacystin and 20 μM HS-1200 induced apoptosis in approximately 40% of cells, although the each single treatment at that concentration produced apoptosis very slightly (Fig. 3A–D). A synergistic effect was also observed by Western blot analyses for caspase-3 and PARP activation, in which the co-treatment augmented the caspase-3 and PARP degradation, and the production of caspase-3 p20 and PARP p85 cleaved products (Fig. 3E).

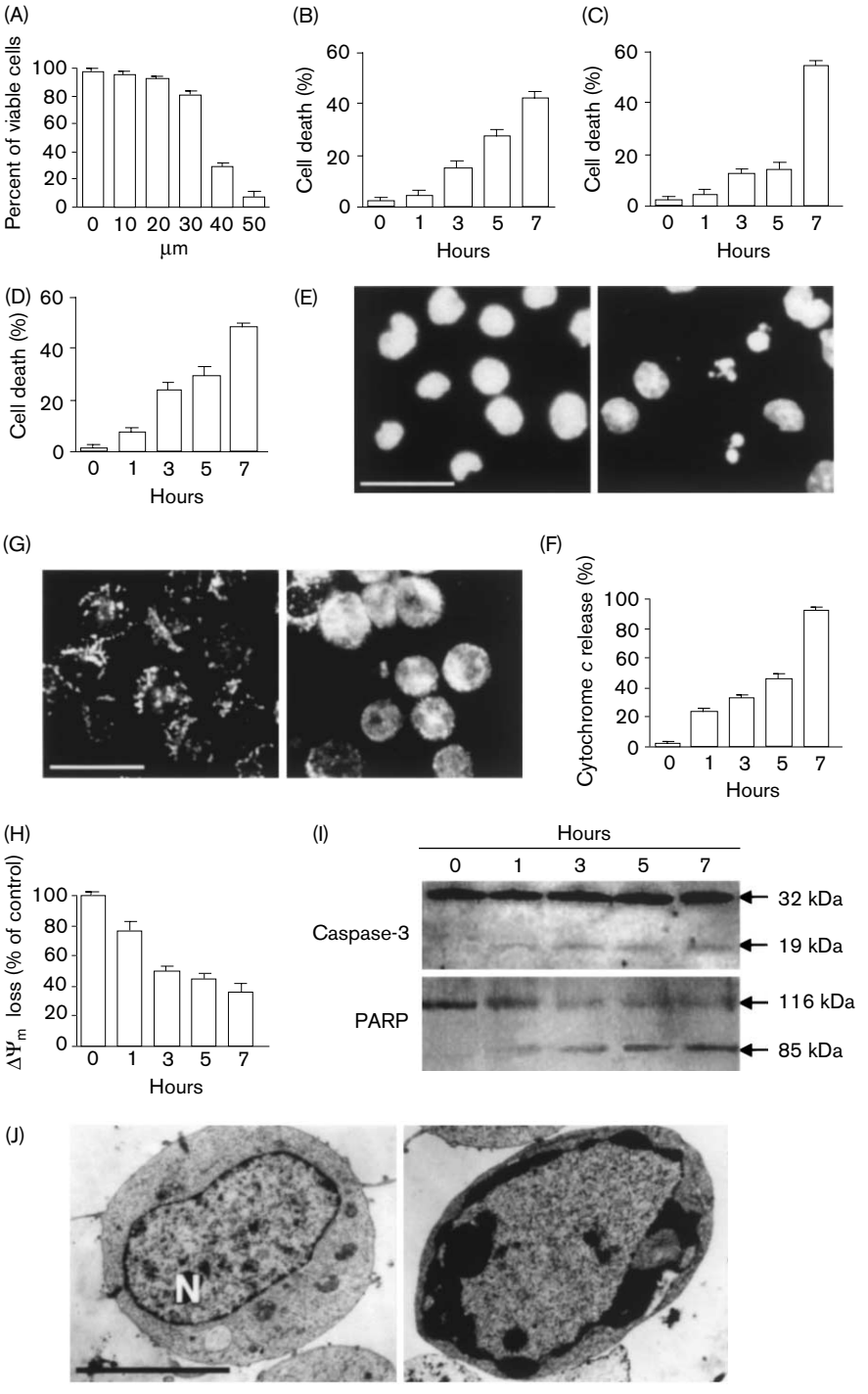
Co-treatment of lactacystin augmented the HS-1200-induced decrease of proteasome activity

The co-treatment of 1 μM lactacystin significantly potentiated the inhibition of proteasome activity by a single treatment of 20 μM HS-1200 over the time period of 7 h (Fig. 4). However the combination treatment significantly potentiated the inhibition by single treatment of 1 μM lactacystin only in the early time points (0–60 min, $p < 0.01$).

Discussion

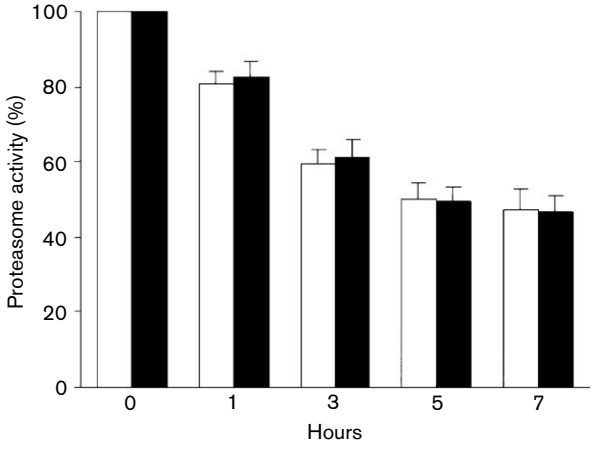
We have already reported that several synthetic bile acid derivatives induced apoptosis in various cells. A glycine methyl ester conjugate of UDCA, HS-1030, induced

Fig. 1



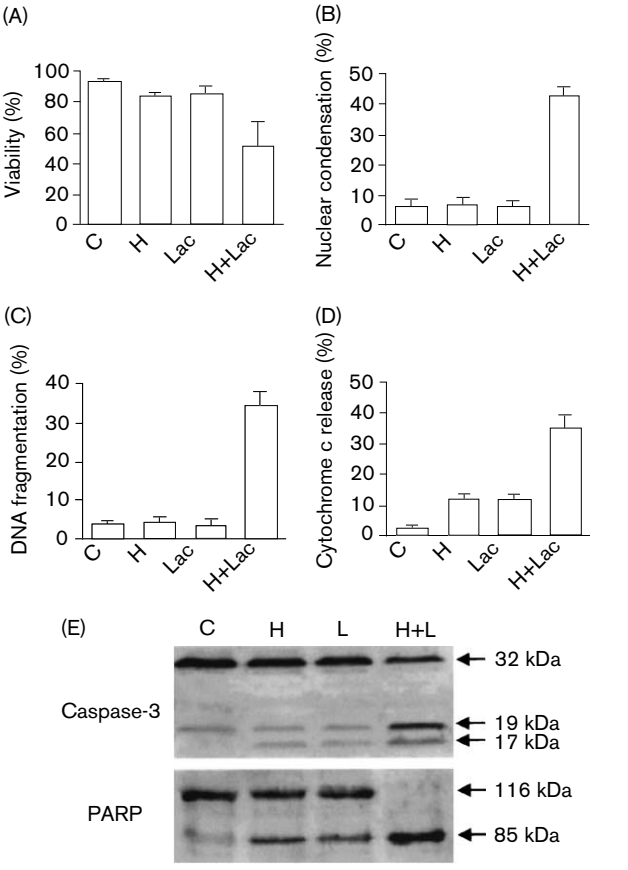
HS-1200 at 35 μM induced apoptosis of p815 cells. (A) Trypan blue exclusion. Reduction of viability in p815 mastocytoma cells treated with different HS-1200 concentrations for 7 h ($p<0.01$, 10–50 μM). (B–D) Time course of Trypan blue exclusion (B), TUNEL assay (C) and Hoechst staining (D). Three quantification assays show time-dependent increase in the amount of apoptosis ($p<0.01$, 0–7 h). (E) Hoechst staining pictures demonstrating nuclear condensation or fragmentation. Left panel, control. Right panel, 5 h after treatment. (F) Cytochrome *c* staining. Left panel, control. Right panel, 5 h after treatment of HS-1200. Scale 20 μM. (G) The time course of the percentage of cells exhibiting cytochrome *c* release. Cytochrome *c* release to cytosol increased in a time-dependent manner ($p<0.01$, 0–7 h). (H) The time course of loss of mitochondrial membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ decreased in a time-dependent fashion ($p<0.01$, 0–7 h). (I) Western blotting analyses showing caspase-3 and PARP activations. (J) Transmission electron microphotographs. Left panel, a control cell with a normal nucleus (N). Right panel, 3 h after treatment with HS-1200. An early apoptotic cell showing peripheral condensation of the nucleus. Scale 5 μM. Data of quantification assays are expressed as the percent of control. Four independent assays were performed and data shown are the mean ± SD obtained from triplicates of each experiment. Analyzed by two-tailed ANOVA test.

Fig. 2



HS-1200 reduced the proteasome activity in p815 cells. HS-1200 (35 μM; open bars) exerted a time-dependent inhibitory effect ($p < 0.01$, 0–7 h). A pan-caspase inhibitor, z-VAD-fmk (solid bars), did not abolish HS-1200-induced inhibition of proteasome activity ($p > 0.05$). Data are expressed as the percent of control. Four independent assays were performed and data shown are the mean ± SD obtained from triplicates of each experiment. Time course changes were analyzed by two-tailed ANOVA test and the effect of z-VAD-fmk by one-tailed Student's *t*-test.

Fig. 3



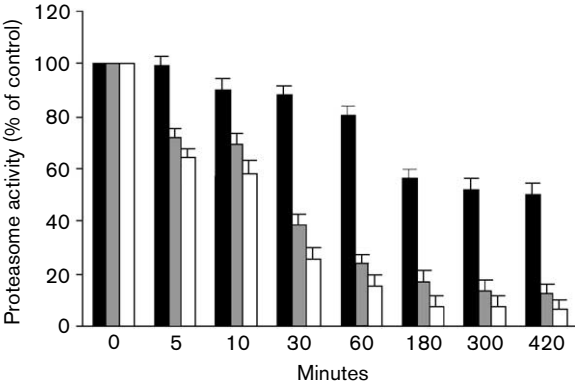
Augmentation of apoptosis by combination treatment with 20 μM HS-1200 (H) and 1 μM lactacystin (Lac) for 24 h. (A) Trypan blue exclusion. (B) Hoechst staining. (C) TUNEL assay. (D) The percentage of cells exhibiting cytochrome *c* release. Four quantification assays show that the combination treatment of HS-1200 and lactacystin augments the extent of apoptosis compared to the single treatment of HS-1200 or lactacystin ($p < 0.01$). Data are expressed as the percent of control. Four independent assays were performed and data shown are the mean ± SD obtained from triplicates of each experiment. Analyzed by one-tailed Student's *t*-test. (E) Western blotting analyses showing that the combination treatment of HS-1200 and lactacystin augments caspase-3 and PARP activation compared to the single treatment of HS-1200 or lactacystin.

apoptosis in HepG2 human hepatocellular carcinoma cells and MCF-7 human breast carcinoma [4,11,12]. L-phenyl alanine benzyl ester conjugate (HS-1199) and L-alanine benzyl ester conjugate (HS-1200) of CDCA and HS-1183 induced apoptosis in human breast carcinoma cells [5]. HS-1199 and HS-1200 also showed apoptotic activity in human leukemic T cells [6]. In this study, we demonstrated for the first time that HS-1200 induced apoptosis of p815 mastocytoma cells.

Our previous reports already showed that synthetic bile acid derivative-induced apoptotic cells showed the characteristic manifestations observed normally in a 'cellular stress' or 'mitochondrial pathway' [3–6]. Degradation of PARP and lamin B, DNA ladder formation, and morphological alterations were shown in the synthetic bile acid derivative-induced apoptosis of breast cancer cells [3,5]. Caspase-mediated signaling was also shown to contribute to the synthetic bile acid-mediated apoptosis in human leukemic T cells [6]. Moreover, the expression level of Bax was observed to increase in apoptosis of breast cancer cells [5]. Synthetic bile acid derivatives also induced loss of $\Delta\Psi_m$ in human retinal pigment epithelial cells [13]. The present study demonstrates that HS-1200 induces apoptosis of p815 mastocytoma cells in a mitochondria- and caspase-dependent manner.

The proteasome is a fundamental non-lysosomal tool that cells use to process or degrade a variety of short-lived proteins. Proteolysis mediated by the ubiquitin–proteasome system has been reported to be implicated in the regulation of apoptosis [10]. Not only did some studies demonstrate that apoptotic stimuli induced apoptosis by inhibiting the proteasome activity of the target cell [14], but others reported that the proteasome inhibitor itself induced apoptosis in a certain cells [10]. We here elucidated that the bile derivative HS-1200 prominently inhibited proteasome activity of p815 mastocytoma cells, which resulted in induction of apoptosis. The proteasome pathway is mostly known to work upstream of the mitochondrial alterations and caspase activation [15]. We also demonstrated the same results. It is supported by the fact that HS-1200 induced mitochondrial alterations

Fig. 4



The co-treatment of lactacystin augmented HS-1200-induced inhibition of proteasome activity. Co-treatment of 1 μM lactacystin (shaded bars) significantly potentiated the inhibition of proteasome activity by a single treatment of 20 μM HS-1200 (solid bars) over the time period of 7 h ($p<0.01$). However, the combination treatment (open bars) significantly potentiated the inhibition by single treatment of 1 μM lactacystin only in the early time points (0–60 min, $p<0.01$). Four independent assays were performed and data shown are the mean ± SD obtained from triplicates of each experiment. Analyzed by one-tailed Student's *t*-test.

and caspase activation, and caspase inhibitor did not modulate the inhibition of proteasome activity.

Several studies, showing that proteasome inhibitors exerted antitumor effects against various cancers [16,17], have firmly established the drugs as a new class of potential anticancer agents. The increasing interest in proteasome inhibitors stems not only from their direct apoptosis-inducing activity, but also from the possibility of sensitizing neoplastically transformed cells to the action of other antitumor agents. Several sets of *in vitro* data support that proteasome inhibitors confer upon tumor cells increased sensitivity to the apoptotic action of tumor necrosis factor (TNF) radio- or chemotherapy [18,19]. One of the underlying mechanism, by which proteasome inhibitors increase sensitivity to apoptotic stimuli, is known to be its activity modulating NF-κB activation [19]. On the other hand, proteasome inhibitors were known to restore the retinoic acid sensitivity of acute promyelocytic leukemia cells through the inhibition of PML/retinoic acid receptor α degradation [20]. An *in vivo* investigation studying the effect of combination treatment of a selective proteasome inhibitor and TNF in colon-26 adenocarcinoma in mice also showed that the two drugs were extremely potent when administered together. The antitumor activity was not accompanied by any signs of gross toxicity, such as weight loss, diarrhea or gastrointestinal bleeding [21]. Therefore, combination treatment of a proteasome inhibitor and a certain antitumor treatment could potentially result in optimal antitumor effectiveness with much lower drug doses, thus reducing the extent and severity of treatment-related toxicity.

In the present study we presented data indicating that the combination treatment of HS-1200 and lactacystin augmented the induction of apoptosis more than each single agent. Moreover, the combination treatment of HS-1200 and lactacystin potentiated the inhibition of proteasome activity. These results show that combination therapy of HS-1200 and a proteasome inhibitor lactacystin could be potentially a therapeutic strategy reducing the extent and severity of treatment-related toxicity. Further experiments are needed to demonstrate the soundness of this strategy.

Conclusions

The results presented here reveal that a synthetic CDCA derivative, HS-1200, induces apoptosis of p815 mastocytoma cells via mitochondria, caspase and proteasome, and this type of apoptosis is augmented by co-treatment with lactacystin. Combination therapy of HS-1200 and the proteasome inhibitor lactacystin could be potentially a therapeutic strategy reducing the extent and severity of treatment-related toxicity.

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