Proteasome Inhibitors Abolish Cell Death Downstream of Caspase Activation During Anti-microtubule Drug-induced Apoptosis in Leukemia Cells

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Abstract. Purpose: Anti-microtubule drugs and proteasome inhibitors are currently among the most intensively studied anti-tumor agents, however little is known about their pharmacological interactions at the cellular level. Materials and Methods: The human promyelocytic leukemia cell line, HL-60, was exposed to nocodazole or etoposide in combination with proteasome or caspase inhibitors. Apoptotic cell death was detected by flow cytometry as sub-G1 population. Caspase and proteasome activities were monitored by the fluorogenic substrates Ac-DEVD-AMC and Suc-LLVY-AMC, respectively, in cell lysate. Heat shock protein 70 (HSP70) expression was determined by Western blotting. Results: Nocodazole, a microtubule inhibitor, induced caspase-dependent apoptosis in the HL-60 cell line. At sub-cytotoxic concentrations, proteasome inhibitors, including MG-132 or clasto-‘-lactone, decreased nocodazole-induced apoptotic DNA fragmentation without affecting the induction of caspase-3 activity. In contrast, MG-132 decreased both DNA fragmentation and caspase activation induced by etoposide, a topoisomerase-II inhibitor. HSP70 had previously been found to inhibit apoptosis independently from caspase activation. In this study, MG-132 up-regulated HSP70 protein expression, both in the presence or absence of nocodazole. Conclusion: Proteasome inhibitors decreased anti-microtubule agent-induced apoptotic DNA fragmentation downstream of caspase-3 activation, possibly due to increased HSP70 expression. The results indicate that combination treatment with these novel anti-tumor agents in leukemia requires careful evaluation of their molecular interaction at the level of apoptosis induction.

Drugs which interfere with microtubules, such as taxol, have been among the most successful chemotherapeutic agents of recent years (1), and have been used in the treatment of many solid tumors including breast and ovarian cancers (2, 3). However, considerably less is known about their therapeutic value in leukemias (4, 5). Interestingly, anti-tumor cytotoxicity induced by arsenic, a novel and effective anti-leukemia agent, is mediated via the inhibition of microtubule function (6).

Proteasome inhibitors can affect apoptotic signaling in several ways (7). Inhibition of the proteasomal degradation of p53 can enhance apoptosis induced by chemotherapeutic drugs (8). Activation of Nuclear Factor-Î·B (NF-Î·B) requires proteolytic degradation of Inhibitor-Î·B (I-Î·B), therefore proteasome inhibitors can abolish the NF-Î·B-induced up-regulation of several anti-apoptotic proteins such as Inhibitor of Apoptosis Protein (IAP-1/2) and X chromosome linked IAP (XIAP) by preventing degradation of I-Î·B (9). This could also be the molecular rationale for the anti-tumor activity of proteasome inhibitors in tumors with high constitutive NF-Î·B activity. Further, this is the molecular basis of the synergistic interaction between proteasome inhibitors and death ligands, which induce NF-Î·B activation in parallel to the activation of the caspase

Abbreviations: Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin; CBL, Galsto-‘-lactone; DMSO, dimethylsulfoxide; Eto, etoposide; HSP70, heat shock protein 70 kD; MG-132, benzoyloxycarbonyl-Leu-Leu-Leu-aldehyde; Noc, nocodazole; Z-VAD-FMK, benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluorome-thylketone.

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cascade (10). However, in certain cell types, NF-ÎB can be pro-apoptotic by up-regulating death ligands themselves in response to chemotherapeutic drugs (11). It has also been reported that proteasome inhibitors can up-regulate the expression of HSP70, (12, 13) which, in turn, can inhibit cell death by interacting with various components of the death pathways (14, 15).

Both microtubule and proteasome inhibitors are valuable targets for the development of novel anti-leukemic drugs. In the current experiments, HL-60 leukemia cells were treated with the microtubule inhibitor nocodazole, either alone or in combination with the proteasome inhibitors MG-132 or clasto-Îβ-lactone. Combinations of cytotoxic agents may be synergistic or antagonistic at the level of apoptosis induction (16). In this study, nocodazole was found to induce apoptosis in leukemia cells, but low concentrations of proteasome inhibitors interfered with this microtubule inhibition-induced apoptosis, downstream of effector caspase activation but upstream of apoptotic DNA fragmentation. These findings indicate that certain combinations of microtubule and proteasome-inhibitors in the treatment of leukemia can be antagonistic.

Materials and Methods

Materials. The materials were purchased from the following vendors: Z-VAD-FMK (Z-Val-Ala-Asp(OMe)-FMK) from Enzyme System Products (Livermore, CA, USA); nocodazole (Noc), etoposide (Eto), MG-132 (MG), clasto-Îβ-lactone (CBL), Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarine), Suc-LVLY-AMC (succinyl-Lys-Lys-Val-Tyr-AMC) from Sigma (St. Louis, MO, USA). Stock solutions were made from all these compounds by DMSO (dimethylsulfoxide, from Sigma) at the concentration that the final dilutions contained less than 0.1% DMSO. Ethidium bromide from Calbiochem (San Diego, CA, USA); Triton X-100 from Serva (Heidelberg, Germany); D(+)-glucose from Reanal (Budapest, Hungary); FCS (fetal calf serum) from Gibco (Invitrogen, Carlsbad, CA, USA); plastic materials from Sarstedt (Nümbrecht, Germany); mouse monoclonal antibody to human HSP70 from Transduction Laboratories (Becton Dickinson, San Jose, CA, USA); HRPO-conjugated goat anti-mouse IgG1 from Southern Biotechnology (Birmingham, AL, USA); Vectastain ABC Kit from Vector Laboratories (Burlingame, CA, USA); DAB (3,3'-diaminobenzidine) from DAKO (Carpinteria, CA, USA); all other compounds, salts or solutions were purchased from Sigma.

Cell culture. The HL-60 promyelocytic leukemia cell line was obtained from Dr. Balazs Sarkadi (National Medical Center, Budapest, Hungary) and was maintained in RPMI-1640 medium supplemented with 10% FCS, L-glutamine and penicillin-streptomycin. The cell line was cultured in a humidified atmosphere of 95% air and 5% CO2. For all experiments, cells with 10% FCS, L-glutamine and penicillin-streptomycin, at 37ÆC, in an atmosphere of 95% air and 5% CO₂. For all experiments, cells growing in the exponential phase were used.

Apoptosis assay. HL-60 cells were plated at a density of 5x10⁵ cells/ml in 24-well plates. The cells were treated overnight (usually 12 h) with nocodazole (200 nM) and were subsequently further incubated for 8 h in the presence or absence of either of the protease inhibitors: MG-132 (3 µM); clasto-Îβ-lactone (10 µM) or the caspase inhibitor: Z-VAD-FMK (100 µM). In separate experiments, cells were treated with etoposide (10 µM) for 6 h, with or without 30 min pretreatment with the same protease inhibitors. Control samples were exposed to either DMSO (as vehicle) or protease inhibitors only. Finally, the cells were harvested and fixed in 70% ethanol (~–20°C) for 30 min at room temperature, and stored at ~–20°C until analysis. Fragmented DNA was extracted with alkaline buffer (200 mM di-sodiumphosphate, pH 7.8, adjusted with 200 mM citric acid) supplemented with 100 µg/ml RNase A. Samples were left at room temperature for 30 min followed by the addition of 5 µl ethidium bromide (final concentration 10 µg/ml). After a further 15 min, samples were measured with a flow cytometer (FACScan, Becton-Dickinson) and the cell cycle and proportion of sub-G1 (apoptotic) cells were calculated (17). Gating was evaluated as described previously (18). In the case of nocodazole, the presented percentage of sub-G1 cells represents the difference between the results obtained after 20-h and 12-h treatment.

Caspase and proteasome activity assays. Assays were performed as described earlier (19), with slight modifications. The cells were treated as described above, and subsequently washed twice in PBS containing 5 mM glucose (PBS+G), then resuspended in 100 µl of protease activity buffer (PAP) composed of PBS+G and 10 mM dithiothreitol (DTT). Caspase activity was detected with Ac-DEVD-AMC (50 µM final concentration) added to the cell suspension in addition to 100 µl PAP containing 1% Triton X-100. Proteasome activity was detected by Suc-LVLY-AMC (50 µM final concentration) added to the cell suspension in 100 µl PAP containing 1% Triton X-100 and 5 mM EGTA. Lysates were incubated for 3 min at room temperature, and fluorescence was detected by a fluorescence plate reader (Fluoroskan Ascent, Thermo Electron Corp., www.thermo.com) at 380 nm excitation and 460 nm absorption for 20 min. All activity was linear in this time range. Activity was calculated as released AMC/min/10⁶ cells, calibrated by free AMC dilutions.

Western blot analysis. 1 x 10⁶ HL-60 cells were treated with drugs, as described above. At the end of the incubation, the cells were washed twice in PBS and pelleted (300 g, 3 min). The pellet was resuspended in 40 µl ice-cold water then 10 µl 6x lysis buffer (300 mM Tris-HCl pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromphenol blue and 60% glycerol) was added. Each sample was boiled for 3 min, and the protein content was determined by the Bradford method. Electrophoresis of total protein (20 µg) was performed in a 12.5% polyacrylamide gel. Mouse monoclonal antibody was used to detect both inducible and constitutive HSP70, (12, 13) which, in turn, can inhibit cell death by interacting with various components of the death pathways (14, 15).

Statistical analysis. All experiments were repeated at least three times and presented as mean±SD. Statistical analysis was performed by unpaired, two-tailed Student’s t-test, and p values <0.05 were considered significant. The inhibition percentage of caspase activity by protease inhibitors in anti-tumor drug-induced samples was calculated by the following formula: (value of caspase activity in the drug-treated sample – value of caspase activity in the drug+protease inhibitor treated samples)/value of caspase activity in the drug-treated sample x 100.
Results

Cell cycle arrest and subsequent apoptosis induced by nocodazole. HL-60 cells were treated with nocodazole (200 nM) for incubation times from 0-20 h (Figure 1B). Cells with apoptotic DNA fragmentation were detected as a sub-G1 population by FACS analysis. In time-course experiments, the percentage of apoptotic cells increased sharply from 14% at 12 h to 38% at 20 h. Cell cycle analysis of nocodazole-treated cells demonstrated that most cells were in the G2/M-phase of the cell cycle at 12 h (Figure 1A). These results indicated that nocodazole-induced events can be divided into two phases, namely arrest of cells in G2/M by 12 h, with subsequent induction of apoptosis.

Both proteasome and caspase inhibitors reduced nocodazole-induced apoptotic DNA-fragmentation. Cells were incubated in the absence or presence of nocodazole for 12 h, followed by incubation in the presence of Z-VAD-FMK (100 ÌM) or proteasome inhibitors (MG-132; 3 mM, or clasto-β-lactone; 10 ÌM) for an additional 8 h. The sub-G1 fraction of cells with apoptotic DNA fragmentation, determined in comparison to values measured at 12 h, revealed that nocodazole induced apoptosis (24%) during 8 h of incubation. Apoptosis was completely inhibited by Z-VAD-FMK (1%, p=0.007), significantly reduced by MG-132 (7.75%, p=0.033), and slightly reduced, but not significantly, by clasto-β-lactone (16%, p=0.106; Figure 2A). The protease inhibitors were non-cytotoxic at the concentrations and incubation time employed.

Etoposide induces double-strand breaks by inhibiting topoisomerase II during unwinding of the DNA (20). However, proteasomal degradation of the enzyme-DNA complex is required to expose DNA damage and to subsequently activate the apoptotic signaling pathway.
Inhibition of proteasomal activity blocks the initiation of etoposide-induced apoptosis (21). In accordance with these results, etoposide (10 μM) induced rapid apoptosis in HL-60 cells (57% at 6 h; Figure 2B), while apoptosis was completely blocked by Z-VAD-FMK (2%, p=0.002) and significantly reduced by MG-132 (16%, p=0.004), as well as by clasto-β-lactone (19%, p=0.004; Figure 2B).

Caspase activity was not influenced by proteasome inhibitors in nocodazole-treated cells. In the following experiments, it was determined whether inhibition of DNA fragmentation occurred upstream or downstream of caspase activation. First, protease inhibitors were examined in ex vivo activity assays. Z-VAD-FMK specifically inhibited caspase activity, while the proteasome inhibitors MG-132 and clasto-β-lactone inhibited proteasome activity, without affecting caspase activity (data not shown). Subsequently, caspase-3 activity was determined in HL-60 cells treated with nocodazole in the presence or absence of Z-VAD-FMK or of the proteasome inhibitor MG-132. Nocodazole-induced caspase-3-like (DEVD-ase) activity was completely inhibited by Z-VAD-FMK (inhibition=97%, p=0.009). However, neither MG-132 (p=0.642) nor clasto-β-lactone (p=0.603) reduced nocodazole-induced caspase-3 activity (Figure 3A). In contrast, etoposide-induced caspase activity was significantly reduced by both MG-132 (inhibition=53%, p=0.008) and clasto-β-lactone (inhibition=52%, p=0.004). Z-VAD-FMK also completely inhibited (by 97%, p=0.001) etoposide-induced caspase-3 activity (Figure 3B).

MG-132 up-regulated the expression of the inducible HSP70 protein in HL-60 cells. Inhibition of caspase-3 directed DNA-fragmentation by HSP70 (14) and induction of HSP70 by proteasome inhibition have been previously described (12, 13). The monoclonal antibody used in this experiment detects both the inducible HSP72 and constitutive HSP73 forms of HSP70. Lanes: Cont: control (DMSO); Noc: nocodazole (20 h); MG-132 (8 h); Noc+MG: nocodazole (20 h) + MG-132 (8 h).

Discussion

In this study, it was demonstrated that the proteasome inhibitors MG-132 and clasto-β-lactone decreased apoptosis previously induced by the microtubule polymerization inhibitor nocodazole in the HL-60 human myeloid leukemia cell line.

Nocodazole-treated HL-60 cells accumulated in the G2/M-phase of the cell cycle prior to the induction of the characteristic apoptotic DNA fragmentation, in a fashion similar to cells treated with taxol (22). Proteasome inhibitors can induce cell cycle block in G1 via stabilization of the cyclin-
important anti-apoptotic molecules such as IAP-1/2, XIAP, The NF-κB transcription factor can increase the expression of pro-apoptotic molecules such as FasL (11) and TRAIL (30). Therefore, inhibition of NF-κB signaling can interfere with a death receptor-mediated autocrine suicide mechanism (11). All of these anti-apoptotic mechanisms can inhibit apoptosis upstream of the main effector caspases including caspase-3.

In conclusion, these findings indicate that further in vitro and in vivo investigations are warranted before combination treatments of leukemia with microtubule polymerization inhibitors and proteasome inhibitors are applied.

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