The antitumor drug aclacinomycin A, which inhibits the degradation of ubiquitinated proteins, shows selectivity for the chymotrypsin-like activity of the bovine pituitary 20 S proteasome

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The antitumor drug aclacinomycin A was previously shown to inhibit the degradation of ubiquitinated proteins in rabbit reticulocyte lysates with an IC50 of 52 μM (Ise, T., Naito, M., Shirai, A., Hirai, R., and Tsuruo, T. (1992) Biochim. Biophys. Acta 1117, 131–135). We report here that from all the catalytic activities of the 20 S proteasome tested, the chymotrypsin-like activity was the only one affected by the antitumor drug. An important requirement for inhibition of the chymotrypsin-like activity seemed to be the presence of hydrophobic non-polar residues in positions P1 to P3. Degradation of Z-E(OrBu)AL-pNA and Z-LLL-AMC at pH 7.5 was dramatically (87–98%) inhibited by 50 μM of the drug, while that of Z-GGL-pNA (containing uncharged polar residues in positions P2 and P3) and succinyl-LLVY-AMC (containing an uncharged polar residue in the P1 position) was inhibited only 11 and 24%, respectively. Aclacinomycin A had no effect on cathepsin B, stimulated trypsin, and inhibited chymotrypsin and, to a lesser extent, calpain. The aglycone and sugar moieties of the cytotoxic drug are essential for inhibition. The results presented here support a major role for the chymotrypsin-like activity in the degradation of ubiquitinated proteins. Aclacinomycin A is the first described non-peptidic inhibitor showing discrete selectivity for the chymotrypsin-like activity of the 20 S proteasome.

The eukaryotic 20 S proteasome or multicatalytic proteinase complex (MPC)† is a multicimeric 700-kDa enzyme with subunits of similar size but different charges, arranged in four stacked rings with heptameric symmetry (reviewed in Ref. 2). The subunits of the eukaryotic 20 S proteasome of known amino acid sequence have been divided into two superfamilies with similarities to either the α- or the β-type subunits of the archaebacterial MPC (3). Subunit arrangement may vary among different populations of the eukaryotic 20 S proteasome (3), but they are organized as symmetrical dimers with the α-type subunits forming the outer rings (4).

Recent x-ray crystallographic and site-directed mutagenesis studies showed that the active site of the archaebacterial 20 S proteasome resides in the β-type subunit and that its N-terminal threonine contributes to the nucleophilic attack in the active site (5, 6). Lactacystin, an irreversible inhibitor of the mammalian 20 S proteasome, was shown to covalently modify the N-terminal threonine of a β-type subunit, strongly suggesting that the residue may play a catalytic role in the eukaryotic molecule as well (7). Three of the β-type subunits in the eukaryotic 20 S proteasome lack the conserved N-terminal threonine residue and were proposed to be catalytically inactive (5, 6).

The eukaryotic MPC has numerous distinct catalytic centers, an advantage for an enzyme that plays a major role in the degradation of nuclear and cytosolic proteins and polypeptides. The catalytic activities include those that hydrolyze peptide bonds on the carboxyl side of basic (trypsin-like), hydrophobic (chymotrypsin-like), acidic (peptidylglutamyl peptide or PGP), branched chain, and small neutral amino acids (8–11). The initial breakdown of β-casein was shown to be accomplished by a catalytic center (casemolytic activity) different from the first three peptidase activities described above (12–15). Studies on dissociation and reassociation of the mammalian MPC indicate that structural integrity is required for expression of proteolytic and peptidase activities (16).

Attempts have been made to relate different catalytic activities with specific subunits of the eukaryotic 20 S proteasome. Of the 14 cloned yeast subunits, mutations in the pre1 and pre2 genes produced strains defective in chymotrypsin-like activity (17, 18), mutations in pre3 and pre4 led to a deficiency in PGP activity (19, 20), and PUP3 mutants were impaired in trypsin-like activity (21, 22). Subunit-binding studies with a specific inhibitor of the chymotrypsin-like activity showed preferential incorporation of disopropyl fluorophosphate into the smallest subunit of the chicken liver (23) and bovine lens MPC (24). Investigations with leupeptin, an inhibitor of the trypsin-like activity, identified only one (25) or two (26) mammalian MPC subunits that specifically bound the arginine peptidase aldehyde.

In eukaryotic cells, non-ubiquitinated and ubiquitinated protein substrates are respectively degraded by MPC or by a larger complex, the 26 S proteasome (ubiquitin/ATP-dependent protease), of which MPC is the catalytic core (reviewed in Ref. 27). The enzymatic centers involved in the initial degradation of different protein substrates have not been identified. Previous work from another laboratory showed that the degradation of ubiquitinated proteins, but not ubiquitination itself, was inhibited by the DNA-intercalative agent aclacinomycin A, also...
known as aclurubicin (1). We present evidence that the inhibitory potency of the antibiotic is most effective against the MPR activity measured with short synthetic substrates containing nonpolar hydrophobic residues in positions P₁ to P₃. Furthermore, we show that aclacinomycin A is the first non-peptidic drug with apparent selectivity for the chymotrypsin-like activity of MPR. Both aglycone and sugar moieties of the cytotoxic drug are essential for its inhibitory properties. These studies suggest that a rate-limiting step in the degradation of ubiquitinated proteins involves cleavage after a series of nonpolar hydrophobic residues.

EXPERIMENTAL PROCEDURES

Materials—The 20 S proteasome was isolated from bovine pituitaries as described (28). Frozen bovine pituitaries were from Pel-Freeze Biologicals, Inc. (Rogers, AR). Z-GGL-pNA, Z-DALR-NA, and Z-LLL-AMC were synthesized as described (8, 29, 30). The synthesis and some properties of Z-(OEtBu)-O-AL-NA, Z-EAL-pNA, and Z-E-OAL-pNA were recently described (15). Z-GPAGG-pAB and Z-GPAGL-pAB were a generous gift from Dr. C. Cardozo (Department of Pharmacology, Mount Sinai Medical School, New York, NY). Z-LLE-NA, succinyl-LLLVY-AMC, dephosphorylated β-casein, rabbit muscle calpain, bovine pancreas α-chymotrypsin and trypsin were from Sigma. Cathespin B was prepared in this laboratory by E. Wilk from rat liver as described (32, 33). Aclacinomycin A was a generous gift from the Drug Synthesis & Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute and from Mercian Corp., Fujisawa, Japan. Aclacinomycin B and all of the aclacinomycin A analogs were a generous gift from Mercian Corp. Other reagents were of highest purity available.

Purification of the Aglycone and Sugar Moieties of Aclacinomycin A—Hydrolysis of the aglycone and sugar moieties was accomplished by chromatography on a Sephadex LH-20 column (34).

Enzyme Assays—Enzyme activities with synthetic substrates were determined as described previously, at 37 °C (8, 9). Substrates were Z-GGL-pNA, Z-OAL-NA, and Z-LLE-NA for determination of the chymotrypsin-like, trypsin-like, and PGP activities of the 20 S proteasome, respectively. MPC hydrolysis of Z-GPAGG-pAB (branched chain amino acid preferring activity) and Z-GPAGL-pAB (small neutral amino acid preferring activity) were measured as described (11). Degradation of dephosphorylated β-casein by MPC (caseinolytic activity) and by calpain were determined by a gel electrophoretic method (12, 35) and quantified by image analysis (13). Treatment of MPC with 3,4 dichloroisocoumarin was as described (15). The activity of cathespin B was probed with Z-LLE-NA in a 0.2 M sodium acetate, 2 mM EDTA buffer, pH 4.8 (32, 33). The activity of chymotrypsin toward Z-GGL-pNA and Z-E(OEtBu)-O-AL-pNA was measured in 0.05 M Tris-HCl, pH 7.5, and trypsin activity toward Z-OAL-NA was analyzed in the presence of 1 mM CaCl₂, in 0.05 M Tris-HCl, pH 8.0.

Specific activities are expressed in terms of units/mg of enzyme, where 1 unit of enzymatic activity is defined as the amount of enzyme liberating 1 μmol of aromatic amine/h.

Inhibition Assays—Solutions of aclacinomycin A and its analogs were prepared either in H₂O or in dimethyl sulfoxide. The solutions were added directly to the assay mixtures containing buffer, enzymes, and their respective substrates in a total volume of 100 μl. The final dimethyl sulfoxide concentration was 1.5%. All activities were expressed relative to control (vehicle alone) conditions.

RESULTS

Chymotrypsin-like Activity of MPC Measured with Z-LLL-AMC and Z-E(OEtBu)-O-AL-pNA—In an effort to characterize the MPC activity toward Z-LLL-AMC and Z-E(OEtBu)-O-AL-pNA, the relationships between substrate concentration and rate of hydrolysis were determined (Fig. 1). Both reactions followed normal Michaelis-Menten kinetics with apparent Kₘ values of 2.7 and 2.2 mM, and Vₘₐₓ of 10 units/mg and 34 units/mg of enzyme with Z-LLL-AMC and Z-E(OEtBu)-O-AL-pNA, respectively, estimated by Lineweaver-Burk plots (Fig. 1). Substrate saturation could not be reached with either of the substrates because of their limited solubility.

Changes in the Chymotrypsin-like Activity of the 20 S Proteasome Produced by Aclacinomycin A—The effect of the cytotoxic drug on MPC hydrolysis of peptide bonds after hydrophobic amino acids was studied with six different substrates (Fig. 2 and Table I). Among the synthetic chromogenic substrates utilized to measure the chymotrypsin-like activity of MPC, Z-LLL-AMC and Z-E(OEtBu)-O-AL-pNA were the most sensitive to inhibition by aclacinomycin A, with IC₅₀ values of 16 and 18 μM, respectively (Fig. 2). Low concentrations of the cytotoxic drug (up to 10 μM) slightly stimulated hydrolysis of the L-pNA bond in Z-E(OEtBu)-O-AL-pNA (Fig. 2).

Surprisingly, hydrolysis of Z-EAL-pNA, a substrate identical to Z-E(OEtBu)-O-AL-pNA except for the blocked glutamate residue, was not affected by aclacinomycin A concentrations up to 100 μM (Table I). Insertion of an additional nonpolar hydrophobic group (Ile), as in Z-EAL-pNA, significantly increased the inhibitory effect of the drug. MPC hydrolysis of Z-EAL-pNA and Z-E(OEtBu)-O-AL-pNA followed normal Michaelis-Menten kinetics with apparent Kₘ values of 1.0 and 0.86 mM and Vₘₐₓ of 6.1 and 6.4 units/mg of enzyme, respectively, estimated by Lineweaver-Burk plots (not shown).

The potency of the aclacinomycin A inhibition was greatly reduced against those substrates with uncharged polar (Gly or Tyr) residues in positions P₁-P₃ (Table I, marked by -).

Effect of Aclacinomycin A on Other Catalytic Activities of MPC—The effect of the cytotoxic drug on other enzymatic activities of MPC was investigated (results not shown). Aclacinomycin A (100 μM) had little effect on trypsin-like (measured with Z-OAL-NA), PGP (measured with Z-LLE-NA), branched chain amino acid preferring (measured with Z-GPAGL-pAB), and small neutral amino acid preferring (measured with Z-GPAGG-pAB) activities. Degradation of dephosphorylated β-casein by 3,4 dichloroisocoumarin-treated MPC was decreased by 29% in the presence of 100 μM of aclacinomycin A.

Effect of Aclacinomycin A on Other Proteolytic Enzymes—Aclacinomycin A at concentrations of 100 μM stimulated 2.5-
Effect of aclacinomycin A on the chymotrypsin-like activity of the 20 S proteasome (2.5 μg).

Enzymatic activities were measured with synthetic substrates as described under “Experimental Procedures.” All activities were measured at pH 7.5. Activities are expressed relative to no treatment (control). The data represent means of at least three experiments for each condition. *p < 0.05, †p < 0.01, ‡p < 0.001.

| Substrate       | 50 μM  | 100 μM  |
|-----------------|--------|---------|
| Z-EAL-pNA       | 98     | 103     |
| Z-LLR-pNA       | 72     | 55      |
| Z-GGL-pNA       | 89     | 74      |
| Suc-LLV-AMC     | 76     | 64      |

Effect of aclacinomycin A (●, 10 μM; □, 100 μM) on the activities of trypsin (30 ng), cathepsin B (6 μg), and calpain (50 ng) measured in a total volume of 100 μl with Z-oALR-NA (trypsin), Z-LLR-NA (cathepsin B), and dephosphorylated β-casein (calpain) as described under “Experimental Procedures.” Values on the ordinate represent activities relative to control conditions, in the absence of inhibitor. Data represent mean and S.E. of at least three separate experiments.

Effect of Different Analogs of Aclacinomycin A on the Chymotrypsin-like Activity of MPC and the Activity of Chymotrypsin (Fig. 5). In both cases the inhibition was reversible.

Effect of Different Analogs of Aclacinomycin A on the Chymotrypsin-like Activity of MPC and the Activity of Chymotrypsin—Aclacinomycin A (Fig. 6) is an anthracyclic antibiotic consisting of an aglycone moiety (aklavivone) linked to a tri saccharide moiety with one aminosugar (l-rhodosamine) and two deoxysugars, namely 2-deoxy-L-fucose and L-cinerulose A (34, 36). To establish the structural requirements of the inhibitory reaction, different analogs of aclacinomycin A described by Oki et al. (34, 36) were tested for their ability to modify the chymotrypsin-like activity of MPC and the activity of chymotrypsin (Table II).

The effect of the aclacinomycin A-analogs on MPC activity toward Z-LLL-AMC and Z-E(OtBu)AL-pNA were similar. Aklavivone, the aglycone moiety of aclacinomycin A and B, had no inhibitory properties, and neither did tetracycline, another antibiotic with a similar aglycone structure but lacking sugars. No significant change in inhibitory potency was detected with the analogs that differed from aclacinomycin A by substitution of the aminosugar for a deoxysugar, such as in U5; by replacement of any of the deoxysugars, such as in aclacinomycin B and MA144-M1; or by deletion of one deoxysugar, such as in MA144-S1. However, inhibition by MA144-T1, a compound missing two of the deoxysugars, and by daunomycin, another anthracycline antibiotic with a different aglycone (daunomycinone) linked to only one aminosugar (daunosamine), was less...
pronounced. Intermediate inhibitory values were obtained in reactions run in the presence of the sugar moiety alone. Comparable results to those described above were obtained with the different aclacinomycin A-analogs on the activity of chymotrypsin toward Z-E(OTBu)-AL-pNA (Table II).

Of all the compounds listed in Table II, daunomycin was the most effective inhibitor of MPC hydrolysis toward succinyl-LLVY-AMC, decreasing it by 40%. The MPC activity toward Z-GGL-pNA was only modestly reduced by the aclacinomycin A-analogs.

**DISCUSSION**

Aclacinomycin A was found to inhibit the degradation of ubiquitinated proteins in reticulocyte lysates at a step following ubiquitin-protein conjugation (1). The antitumor drug did not interfere with ubiquitination and did not interact directly with ubiquitin (1). We explored the possibility that the targets for aclacinomycin A action were the catalytic centers of the 20 S proteasome.

From all the catalytic activities of MPC measured, only the chymotrypsin-like component tested with two substrates containing hydrophobic nonpolar residues in positions P₁ to P₃ (namely, Z-E(OTBu)-AL-pNA and Z-LLL-AMC) was highly sensitive to the antibiotic, with IC₅₀ values of approximately 18 μM. Hydrolysis of two other substrates, Z-GGL-pNA and succinyl-LLVY-AMC, frequently utilized to probe the chymotrypsin-like activity of MPC, were only repressed 11 and 24%, respectively, by 30 μM aclacinomycin A.

The catalytic activities of the 20 S proteasome toward other short synthetic substrates representing the trypsin-like (Z-GGL-NA), PGP (Z-LLE-NA), and chymotrypsin-like (Z-LLL-AMC) activities were measured with synthetic substrates (400 μM) as described under "Experimental Procedures." All activities are expressed relative to zero drug treatment. The data represent means of at least three experiments for each condition.

| Drug               | Sugar moiety | 20 S proteasome | Chymotrypsin |
|--------------------|--------------|-----------------|--------------|
|                    | (100 μM)     | No. of sugars   | % control    | % control |
| Aclacinomycin A    | 3            | 74              | 64           | 0         | 8          |
| Aklavinone         | 0            | 109             | 102          | 103       | 114        | 114        |
| Tetracycline       | 0            | 101             | 99           | 106       | 100        | 100        |
| US                 | 3            | 104             | 80           | 3         | 1          | 9          |
| MA 144-M1          | 3            | 93              | 83           | 4         | 27         | 9          |
| MA 144-S1          | 5            | 84              | 69           | 1         | 11         | 8          |
| MA 144-T1          | 2            | 91              | 73           | 5         | 2          | 12         |
| Daunomycin         | 1            | 100             | 70           | 18        | 43         | 43         |
| Sugar moiety       | Mix          | 104             | 86           | 59        | 79         | 77         |

**Enzymatic activities were measured with synthetic substrates (400 μM) as described under "Experimental Procedures." All activities are expressed relative to zero drug treatment. The data represent means of at least three experiments for each condition.**
The 20S proteasome may play an important role in different disease states, such as inflammation (reviewed in Ref. 42) or muscle atrophy (43). The discovery of a non-peptidic, cell-permeable inhibitor of the 20S proteasome, such as aclacinomycin A, may lead to the development of other, more potent inhibitors, which may be useful as novel therapeutic drugs.

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