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PRELIMINARY COMMUNICATIONS

INHIBITION OF PROTEIN TURNOVER IN HUMAN LUNG CELLS BY PEPSTATIN AND TRIPEPTIDE ANALOGS OF PEPSTATIN

Rebecca A. Owens*, Wu-Schyong Liu, George I. Glover and J. Martyn Gunn*
Department of Biochemistry and Biophysics* and Department of Chemistry,
Texas A&M University, College Station, TX 77843, U.S.A.

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Although the importance of intracellular protein degradation has long been recognized, little is known about this pathway or its control and integration with other aspects of coordinated cell growth. This gap in our knowledge has important consequences for the treatment of diseases, such as muscular dystrophy, characterized by increased rates of protein degradation with net loss of tissue proteins.

Umezawa and coworkers (1,2) have isolated several low molecular weight proteins from Actinomycetes that are very effective protease inhibitors. Two of these inhibitors, leupeptin and pepstatin, delay the degeneration of muscle tissue in genetically dystrophic chickens and mice (3,4), and, in addition, inhibit rates of proteolysis in excised rat muscles (5), in isolated hepatocytes (6), and in hepatoma cells in culture (7). However, while these inhibitors are able to retard protein degradation both in vivo and in vitro, they do not, even in combination, fully inhibit the proteolytic pathway. There is, therefore, a need to develop new inhibitors for probing the pathway of intracellular protein degradation and for testing as potential therapeutic agents. Our approach is to synthesize analogs, based on the known structures of the peptide inhibitors, and to test their effectiveness as inhibitors of intracellular proteolysis in cultured mammalian cells. This communication describes the first successful application of this approach to analogs of pepstatin.

Pepstatin is an N-acyl pentapeptide containing two residues of statine (Sta), a novel 4-amino-3-hydroxy acid derived from leucine, and having the general structure Acyl-Val-Val-Sta-Ala-Sta. It is an effective inhibitor of the acid proteases pepsin, cathepsin D, and renin. Umezawa and coworkers (1,2) have prepared several derivatives and analogs of pepstatin that provide some interesting insights into the variation of biological activity with structure. The antipepsin and anticathepsin D activities of pepstatin do not vary significantly with the nature of the N-acyl group, but the antirenin activity increases about 10-fold over the series N-acetyl to N-capryl. What is more significant is that a peptide

containing two residues of statine, but lacking one of the valyl residues, retains full antipepsin activity but has no antirenin activity even at concentrations over 1000-fold higher than that required for pepsin inhibition. In addition, shorter peptides containing one or two statine residues retain significant antipepsin, but no antirenin, activity.

Thus, the effectiveness and selectivity of pepstatin can be enhanced by altering the length and composition of the molecule. In addition, because statine has two chiral centers (at the 3- and 4-positions), further enhancement of specificity might be achieved in one of the four possible stereoisomers of this unusual amino acid. Natural pepstatin contains two residues of statine and each residue has the (3S,4S) configuration. Here we report the effects of both parameters, size and stereochemistry, on the inhibitory effects of pepstatin analogs in vivo. We have tested the four stereoisomers of the tripeptide Cbz-Val-Val-Sta (8) for their effectiveness as inhibitors of intracellular proteolysis in human lung cells.

Normal human lung cells and their Simian virus 40 transformed variant, designated IMR-90 and AG-2804, respectively, were obtained from the Institute for Medical Research, Camden, NJ 08103. Rates of intracellular proteolysis were measured by a pulse-chase technique as described previously (9). Briefly, cell proteins were labelled by a 16-hr incubation with Minimal Essential Medium (MEM) containing 1 μCi/ml L-[4,5-^{3}H] leucine (specific activity 39 Ci/mmole). The monolayers were then rinsed twice with Earle's salts and MEM, containing 20 mM N-Tris [hydroxymethyl] methyl-2-aminoethane sulfonate (TES) pH 7.5 (Chase MEM), was added for a further 2 hr. This chase period effectively removes unincorporated ^{3}H-leucine and allows for the degradation of short-lived proteins (7,10). At the end of this time, the medium was changed to Chase MEM containing the inhibitors and the degradation of long-lived proteins was measured over the next 3 hr. Stock solutions of the inhibitors were prepared at 1 mg/ml in 10% (v/v) dimethylsulfoxide (DMSO). Leucine (2 mM) was added during both the chase and degradation periods to reduce the specific activity of ^{3}H-leucine released from labelled proteins and thus suppress any reincorporation of isotope which would cause an apparent reduction in proteolysis. Rates of proteolysis are first order over this time period and are calculated from the percentage of total radioactivity appearing as trichloroacetic acid soluble counts (10). The total radioactivity measured was approximately 5×10^{5} cpm.

The natural pentapeptide inhibitor, pepstatin, reduced the rate of proteolysis in IMR-90 cells (Table 1), indicating the relative importance of lysosomal cathepsin D, or some other acid protease, to intracellular proteolysis. Whether or not pepstatin is inhibiting only the lysosomal enzyme is not known. Indeed, it may not penetrate to the lysosome under these conditions so that inhibition of proteolysis may reflect the extent of autophagic vacuole formation with the inhibitor trapped inside the vacuole together with the protease(s) and substrate. However, in the perfused liver, Dean (11) facilitated penetration to the lysosome by incorporating pepstatin in lipid vesicles. This resulted in a 50 per cent inhibition of

proteolysis and provided evidence for lysosomal cathepsin D involvement in protein degradation. Pepstatin had no significant effect on proteolysis in the transformed AG-2804 cells (Table 1: $0.05 < p < 0.06$), presumably reflecting a lack of transportation into the cell (see below). Similar findings have been reported for hepatocytes and hepatoma cells (6,7).

All four stereoisomeric tripeptide analogs of pepstatin (8) inhibited intracellular proteolysis in both the normal and transformed cell lines and were more effective than natural pepstatin, even allowing for differences in molecular weight (Table 1). Whether or not this reflects enhanced penetration to the site(s) of inhibition or enhanced specificity remains to be seen. Thus, the different degrees of inhibition shown in Table 1 may reflect different specificities for the same enzyme (i.e. cathepsin D) or different specificities for different enzymes. Note also that while rates of proteolysis and percentage inhibition of proteolysis are less in the transformed cell line, there is an excellent correlation ($r = 0.94$) with inhibitor structure between the normal and transformed lines. In both cases, the relative order of effectiveness is pepstatin < (3R,4S) < (3S,4S) = (3S,4R) < (3R,4R). The cause of the overall differences between normal and transformed cells is unknown but may reflect differential transport of the inhibitors or changes in the type and/or distribution of proteases. The former possibility appears more likely because the transformed cells were uniformly less sensitive to all of the inhibitors.

Table 1. Inhibition of proteolysis by pepstatin and Cbz-Val-Val-Statine tripeptide analogs of pepstatin in IMR-90 and AG-2804 cells*

	Rates of Proteolysis (%)			
Addition	IMR-90		AG-2804	
None	5.20 ± 0.18		4.82 ± 0.14	
Pepstatin	4.38 ± 0.28	(16)	4.46 ± 0.12	(7)
(3R,4S)	3.40 ± 0.17	(35)	4.18 ± 0.19	(13)
(3S,4S)	3.12 ± 0.22	(40)	3.87 ± 0.18	(20)
(3S,4R)	3.04 ± 0.16	(42)	3.72 ± 0.28	(23)
(3R,4R)	2.44 ± 0.14	(53)	2.99 ± 0.20	(38)

*Rates of proteolysis were determined over a 3-hr period in Chase MEM (9). All inhibitors were tested at 10 μg/ml in 0.1% DMSO (final concentration). Values are the means ± S.E.M. of 24 determinations. The per cent inhibition of proteolysis, relative to controls with DMSO alone, is shown in parentheses.

In comparing pepstatin to the tripeptide analogs as inhibitors of pepsin hydrolysis of hemoglobin (8), we found none of the analogs to be as effective as pepstatin. Thus the "natural" (3S,4S) analog was 20 times less effective than pepstatin, while a change in configuration at either the 3- or 4-position produced inhibitors which were at least 100 times less effective again. Clearly this inhibition pattern is not seen when the inhibitors are tested in the cultured cell system. Indeed, a change in absolute configuration from S to R produces an equally effective inhibitor, while a change in stereochemistry at both chiral centers has the most profound effect, producing a significantly more effective inhibitor.

These data substantiate our claim that analogs of the natural peptide inhibitors, differing in both size and absolute configuration, may prove to be more effective inhibitors of intracellular proteolysis. Such analogs, once their specificity is known, will be useful tools with which to unravel the pathway of intracellular protein degradation and may prove to have therapeutic value in treating the muscular dystrophies and other diseases.

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