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Inhibition of cysteine proteinases in lysosomes and whole cells

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Inhibitors of cysteine proteinases have been used extensively to dissect the roles of these proteinases in cells. Surprisingly though, little work has been performed to demonstrate unequivocally that the inhibitors reach and inactivate their target proteinases in cell culture or *in vivo*. In the present study, the permeability of lysosomes and whole cells has been studied. Benzyloxycarbonyl (Z)-[^{125}I]iodo-Tyr-Ala-diazomethane (CHN_2), an inhibitor of cathepsins L and B, has been shown to label active forms of these enzymes in lysosomes and whole cells. The ability of other cysteine proteinase inhibitors to block this labelling has been used to indicate the permeation of these compounds. All the inhibitors were able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in lysosomal extracts. In intact lysosomes or cells, however, only *N*-[*N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine ('E-64d') Z-Tyr-Ala-CHN_2, Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2. *N*-[*N*-(L-3-*trans*-Carboxyoxirane-2-carbonyl)-L-leucyl]amino-4-guanidinobutane (E-64) and leupeptin were unable to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in lysosomes or in cells. The ability to block labelling in lysosomes is an indication of the ability of the inhibitor to diffuse across membranes. Thus E-64 and leupeptin do not readily permeate membranes and therefore their uptake into cells probably only occurs via pinocytosis.

INTRODUCTION

The commonly used cysteine proteinase inhibitors, leupeptin, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino-4-guanidinobutane (E-64) and the diazomethanes benzyloxycarbonyl (Z)-Phe-Phe-diazomethane (CHN_2) and Z-Phe-Ala-CHN_2 have been shown to inhibit effectively the lysosomal cysteine proteinases *in vitro* (Kirschke & Shaw, 1981; Barrett *et al.*, 1982; Rich, 1986). The efficacy with which these inhibitors reach and inhibit their target enzymes *in vivo* or in cell culture, however, has not been determined. Studies using these inhibitors have looked at the effect of time on the onset of inhibition of protein degradation as an indication of the mode of entry of the inhibitors. This approach has produced conflicting results on their mechanism of uptake.

E-64 and the related epoxide inhibitors EP-475 {or E-64c; *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine} and E64d {*N*-[*N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine} are irreversible inhibitors of the lysosomal cysteine proteinases and the cytosolic cysteine proteinases, the calpains (Hanada *et al.*, 1978; Barrett *et al.*, 1982; Parkes *et al.*, 1985; Tamai *et al.*, 1986). Administration of both E-64 and EP-475 to live animals led to decreased activity of cathepsins B and H after extraction from the tissues (Noda *et al.*, 1981; Baricos *et al.*, 1988). When administered orally *in vivo*, EP-475 was shown to prolong the life of dystrophic animals in a dose-dependent manner (Tamai *et al.*, 1987). Despite these studies, how these compounds reach their target enzymes is unresolved.

Leupeptin, an inhibitor of both serine and cysteine proteinases, inhibits basal protein turnover by 30–40% (Dean, 1979). Initial experiments looking at the inhibitory characteristics of this compound seemed to indicate that it rapidly entered cells by diffusion, since its effect was observed without any discernible lag time (Simon *et al.*, 1977; Seglen *et al.*, 1979). In conflict with these results, Nonaka and colleagues (1982) administered [^{14}C]leupeptin orally to dystrophic chickens and looked at the distribution of [^{14}C]leupeptin. They found that negligible amounts of the inhibitor had entered the sarcolemma and concluded that leupeptin penetrates poorly in whole animals. The mode of entry of leupeptin is therefore controversial and requires further investigation.

Diazomethanes have been shown to inhibit protein turnover in isolated rat hepatocytes, showing maximal inhibition after 2 h (Grinde, 1983). Another study demonstrated, however, that inhibition of protein turnover in isolated mouse macrophages by Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 was observed after a delay of 2 h, and it was concluded that pinocytosis was the major mechanism of uptake for these inhibitors (Shaw & Dean, 1980).

The above techniques are indirect in that they usually measure the effect of the proteinase inhibitors on the enzymes after homogenization of the tissues. We already know that this approach results in the exposure of lysosomal proteinases to extracellular and cytoplasmic inhibitors and it could also result in added synthetic inhibitors that do not penetrate cells contacting the enzymes after homogenization. We have developed a more direct approach using the diazomethane inhibitor, Z-[^{125}I]iodo-Tyr-Ala-CHN_2, which covalently binds to the active-site cysteine of cathepsins B and L both *in vitro* and in live cells, but does not react with the inactive enzymes (Mason *et al.*, 1989*a*,*b*). The enzymes in cells were labelled within 30 min (Mason *et al.*, 1989*a*), which was considerably faster than the observed inhibition of proteolysis produced by the diazomethanes in the study by Shaw & Dean (1980). It seemed possible therefore that diffusion could account for the uptake of diazomethanes.

In this paper we address this problem by looking at the ability of this inhibitor to enter intact purified lysosomes and therefore determine its ability to diffuse through the lipid bilayer directly. In order to determine the ability of Z-[^{125}I]iodo-Tyr-Ala-CHN_2 and other cysteine proteinase inhibitors to cross a lipid bilayer, we have looked at the ability of these inhibitors to enter intact isolated lysosomes and whole cells during culture. This technique

Abbreviations used: Z, benzyloxycarbonyl; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino-4-guanidinobutane; EP-475, or E-64c, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine; E64d, *N*-[*N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine; CHN_2, diazomethane.

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has allowed the determination of the mode of entry for a number of cysteine proteinase inhibitors and has enabled the concentration at which they completely inhibit lysosomal cysteine proteinase activity to be determined.

EXPERIMENTAL

Materials

All chemicals were purchased from Fisher (ACS grade) unless otherwise noted. Electrophoresis chemicals and Bio-Rad protein assay reagent were purchased from Bio-Rad Laboratories (Rockville, NY, U.S.A.). Kodak X-Omat X-Ray film and ammediol (2-amino-2-methylpropane-1,3-diol) were purchased from Kodak (Rochester, NY, U.S.A.). Percoll, Protein A–Sepharose CL-4B and density-marker beads were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Normal rabbit serum, normal sheep serum and rabbit anti-sheep IgG were purchased from ICN Immunobiologicals (Lisle, IL, U.S.A.). α-NAD, papain and pig pancreatic elastase were purchased from Sigma (St. Louis, MO, U.S.A.). Iodogen was purchased from Pierce (Rockford, IL, U.S.A.). Z-Arg-Arg-NH-Mec, NH-Mec and Z-Phe-Arg-NH-Mec were purchased from Bachem Bioscience Inc. (PA, U.S.A.). $Na^{125}I$ was purchased from ICN. All tissue-culture reagents were purchased from Flow Laboratories (Rockville, MD, U.S.A.) or from Gibco (Grand Island, NY, U.S.A.). Kirsten-virus-transformed NIH 3T3 fibroblast cell line (KNIH 3T3) was a gift from Dr. M. M. Gottesman, NIH, Bethesda, MD, U.S.A. Human infant foreskin fibroblasts (HIFF), grown from human infant foreskin explants, were provided at an early passage (3–6) by S. Sarsfield, Strangeways Research Laboratory, Cambridge, U.K. Human fibrosarcoma epithelial cell line (HT1080) was kindly provided by Dr. J. Gavrilovic, formerly of Strangeways Research Laboratory (Rasheed *et al.*, 1974).

Affinity-purified sheep anti-(human cathepsin B) IgG was a gift from Dr. D. J. Buttle, Strangeways Research Laboratory. Rabbit anti-(human cathepsin L) serum was raised against purified human cathepsin L as described previously (Mason *et al.*, 1985; Mason, 1986). Rabbit anti-(mouse cathepsin L) serum was a gift from Dr. M. M. Gottesman.

E-64 and leupeptin were obtained from Sigma. Z-Tyr-Ala-CHN_2, Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were kindly provided by Dr. E. Shaw (Friedrich Meischer Institut, Basle, Switzerland). E-64d and EP-475 were gifts from Dr. K. Hanada (Taisho Pharmaceuticals, Omiya, Japan).

Purification of lysosomes

The purification procedure was based on that used by Yamada *et al.* (1984). All procedures were performed at 4 °C and the fractions stored on ice. Mouse livers (2 g) were homogenized in 10 vol. of 0.25 M-sucrose/20 mM-Tris, pH 7.4, a Potter-S homogenizer (ten strokes at 300 rev./min). The homogenate was then centrifuged at 1000 $g_{av.}$ for 10 min, and the resultant pellet was washed in 5 vol. of homogenization buffer and centrifuged at 1000 $g_{av.}$ for 10 min. The pellet was the crude-nuclear fraction. The supernatants from the two spins were pooled and centrifuged for 30 min at 10000 $g_{av.}$. The pellet was washed in 5 vol. of homogenization buffer and then centrifuged for a further 30 min at 10000 $g_{av.}$, resulting in the lysosomal/mitochondrial pellet. The lysosomal/mitochondrial pellet was gently resuspended in 3 ml of homogenization buffer containing 1 mM-$CaCl_2$; the suspension was then incubated at 37 °C for 5 min specifically to swell the mitochondria. The lysosomal/mitochondrial suspension was used as the partially purified lysosome preparation. The 'swelled' lysosomal suspension was layered on to a Percoll/sucrose solution (initial density 1.08 g of Percoll/l of 0.25 M-sucrose, pH 7.4; 1 ml of lysosomes/27 ml of gradient). The gradients were formed *in situ* by centrifugation at 50000 $g_{av.}$ for 1 h in a fixed-angle rotor. After the gradient had run, 2.7 ml fractions were collected from the bottom of the tube. The fractions were assayed for cathepsin B activity (lysosomal marker), malate dehydrogenase activity (mitochondrial marker) and urate oxidase activity (peroxisomal marker). Protein was determined by the method of Bradford (1976), by using the Bio-Rad microassay.

The density of the fractions was determined by running a gradient with density-marker beads. The mitochondria were generally found in the first three fractions at the top of the gradient, and the lysosomes were concentrated in the bottom three fractions. The fractions containing the peak of lysosomal marker enzyme activity and low malate dehydrogenase activity were pooled (generally fractions 8–10) and spun at 100000 $g_{av.}$ for a further hour to remove the Percoll. Under these conditions the lysosomes formed a band in the middle of the tube; this was removed and used in subsequent experiments as the purified lysosomal fraction.

Enzyme assays

Cathepsin B was assayed according to the method of Barrett & Kirschke (1981). Malate dehydrogenase was assayed essentially as described by Englard & Siegel (1969). An enzyme unit is defined as the amount of enzyme required to convert 1 μmol of NAD^+ into NADH in 1 min. The assay of urate oxidase was performed according to the method of Schneider & Hogeboom (1952).

β-Hexosaminidase activity was measured either with or without Triton X-100 as a measure of total and free activity respectively. This was performed essentially as described by Bird *et al.* (1987). The basis of this assessment is that the lysosome is impermeable to the synthetic fluorimetric substrate used to measure the enzyme activity. The total and free enzyme activity was measured after incubation of 0.2 ml of purified lysosomal suspension (200 μg of protein) in the presence of the following inhibitors: 10 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2, 10 μM-Z-Phe-Phe-CHN_2, 10 μM- and 100 μM-leupeptin and no additions. Lysosomal suspension (100 μl) was preincubated for 1 min at 25 °C with 200 μl of 0.16 M-sodium citrate buffer, pH 5.0, containing 0.25 M-sucrose with or without Triton X-100 (0.36%). The assay was started by the addition of 100 μl of 10 mM-4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside in 0.25 M-sucrose. The reaction was allowed to proceed for 1 min at 25 °C and then stopped by the addition of 2 ml of 1 M-Na_2CO_3. The amount of hydrolysis of the substrate was measured with a Perkin–Elmer fluorimeter set at excitation wavelength 354 nm and emission wavelength 444 nm. The fluorimeter was calibrated with water (blank) and 0.25 mM-methylumbelliferone (the product), such that 1000 units corresponded to hydrolysis of 10% of the substrate. All assays were performed in duplicate, including a reaction blank, both with and without Triton X-100. The assays performed in the presence of Triton X-100 were used to indicate the total activity of β-hexosaminidase. All values are expressed as the free activity as a percentage of the total (fluorescence in the absence of Triton X-100/fluorescence in the presence of Triton X-100).

Labelling of intact lysosomes

Lysosomes were prepared as described above. The lysosomes, from 2 g of starting material, were diluted in 0.25 M-sucrose, pH 7.4, giving a final volume of 4 ml. Samples (200 μl; 200 μg of protein/sample) were incubated at 37 °C with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for 10 min–3 h. After being labelled, the lysosomes were sedimented (10 min at 10000 g), the supernatant

was removed and the pellet resuspended in an equal volume of SDS/PAGE sample buffer.

Labelling of lysed lysosomes

Lysosomes were prepared from mouse liver and diluted into 0.25 M-sucrose, pH 7.4. They were then divided into 200 μl portions (200 μg of protein/portion), spun down and lysed in 200 μl of buffer containing 20 mM-sodium acetate (pH 5.5), 1% Triton X-100, either with or without 4 mM-dithiothreitol. The lysates were then incubated with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for times up to 3 h. The labelled lysates were then precipitated with trichloroacetic acid (final concentration of trichloroacetic acid 3.3%, w/v), the precipitate was washed twice in acetone and then redissolved in SDS/PAGE sample buffer. The lysosomes were then submitted to SDS/PAGE on 12.5% gels and the labelled proteins were visualized by using autoradiography.

Blocking of labelling of intact lysosomes

Lysosomes were prepared as described above, either intact or lysed, and labelling was blocked by preincubation with various inhibitors. The samples (200 μg of protein in 200 μl) were preincubated in 10 μM-blocking inhibitor for 1 h at 37 °C. After blocking, 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 was added and labelling continued for a further 1 h. After being labelled, the lysed lysosomes were precipitated as described above and the intact lysosomes were spun down and resuspended in SDS/PAGE sample buffer. The lysosomes were then submitted to SDS/PAGE on 12.5% gels and the labelled proteins were visualized by using autoradiography.

Immunoprecipitation

Cathepsin B. The cellular or lysosomal extract was prepared by solubilization in 1 ml of 10 mM-Tris/HCl/1 mM-EDTA/0.2% SDS/1% Triton X-100 (pH 7.5). The resultant extract was then boiled for 15 min, and cooled on ice. A pre-clear was performed by using 10 μl of normal sheep serum and 20 μl of rabbit anti-sheep IgG and 60 μl of 10% [w/v in phosphate-buffered saline (240 mM-NaCl/2.7 mM-KCl/1.5 mM-NaH_2PO_4/8.1 mM-Na_2HPO_4)] Protein A–Sepharose suspension. Specific immunoprecipitation was performed by using affinity-purified sheep anti-(human cathepsin B) IgG (5 μl), rabbit anti-sheep IgG (10 μl) and 10% (w/v in phosphate-buffered saline) Protein A–Sepharose (30 μl) for 2 h at 20 °C. The resulting precipitate was washed (×3) in solubilization buffer and resuspended in SDS/PAGE sample buffer.

Cathepsin L. The cellular or lysosomal extracts were prepared by solubilization in 10 mM-Tris/1 mM-EDTA buffer, pH 7.5. A pre-clear was performed by using 10 μl of normal rabbit serum and 30 μl of 10% (w/v in phosphate-buffered saline) Protein A–Sepharose. Specific immunoprecipitation was performed by using rabbit anti-(mouse cathepsin L) IgG (10 μl), and 10% (w/v in phosphate-buffered saline) Protein A–Sepharose (30 μl) for 2 h at 20 °C. The resulting pellet was washed once in the lysis buffer, followed by two washes in lysis buffer containing 0.2% SDS and 1% Triton X-100, and then resuspended in SDS/PAGE sample buffer.

Cell culture

The cell lines, HT1080, Balb/c 3T3, KNIH 3T3 and HIFF cells, were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. The cells were cultured in a humidified CO_2 incubator at 37 °C and 5% CO_2.

Labelling of cells in culture

The cells were washed in serum-free medium and then cultured for 30 min–3 h in serum-free medium containing 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2. The cell lysates were precipitated with trichloroacetic acid and the resulting precipitates were submitted to SDS/PAGE on 12.5% gels. The labelled proteins were visualized by autoradiography of the dried gel.

Blocking experiments in whole cells

The cells were washed in serum-free medium and then cultured for 1 h in serum-free medium containing the blocking inhibitors (these were generally used at 10 μM). After blocking, the medium was removed, the cells were washed in serum-free medium to remove any unbound inhibitor and the cells were then cultured for a further 1–3 h in serum-free medium containing 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2. The cell lysates were precipitated with trichloroacetic acid and the resulting precipitates were submitted to SDS/PAGE on 12.5% gels. The labelled proteins were visualized by autoradiography of the dried gel.

SDS/PAGE and autoradiography

SDS/PAGE was performed as described by Bury (1981). The M_r standards used were bovine and egg albumins (M_r 68000 and 45000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), bovine carbonic anhydrase (M_r 29000), soyabean trypsin inhibitor (M_r 21000) and bovine α-lactoglobulin (M_r 18000). Autoradiography of the dried gel was performed by using Kodak X-ray film, exposed at −70 °C using a phosphotungsten intensifying screen.

RESULTS

Labelling of lysed lysosomes with Z-[^{125}I]iodo-Tyr-Ala-CHN_2

To establish the conditions under which the probe, Z-[^{125}I]iodo-Tyr-Ala-CHN_2, would label the lysosomal cysteine proteinases, the initial experiments were performed on lysosomal lysates. Lysosomal lysates were incubated with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in the presence of 4 mM-dithiothreitol. Three proteins of M_r 24000, 33000 and 5000 were labelled after 10 min and the labelling of these proteins increased during the next 180 min of incubation in the presence of the inhibitor (results not shown). These proteins were identified, by immunoprecipitation, as cathepsin L (M_r 24000) and cathepsin B (M_r 33000 and 5000). In the absence of dithiothreitol no labelling was seen (results not shown).

To ensure that all of the inhibitors used in this study could inhibit cathepsins L and B, and thus prevent subsequent labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2, blocking experiments were performed with lysed lysosomes. The following inhibitors were used: leupeptin, E-64, EP-475, E-64d, Z-Tyr-Ala-CHN_2, Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2. Lysosomal lysates were incubated for 30 min in the presence of 10 μM-blocking inhibitor and then incubated with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for a further 30 min. All the inhibitors used completely blocked the labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2, indicating that they were all capable, in the absence of lysosomal membrane and in the presence of reducing agent, of inhibiting cathepsins L and B. Leupeptin is a reversible inhibitor of cysteine proteinases, but has a K_i of 6 nM for cathepsin B and less than 5 pM for cathepsin L, and thus will be tightly bound to both of these enzymes (Rich, 1986). One might expect to see slow labelling with Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in the presence of a reversible inhibitor, but none was seen during the 30 min labelling period in this experiment. The preincubation of lysates with no additions did not affect labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 and thus any inhibition of labelling can be attributed to the presence of the blocking inhibitor and not to inactivation of the proteinases during the preincubation.

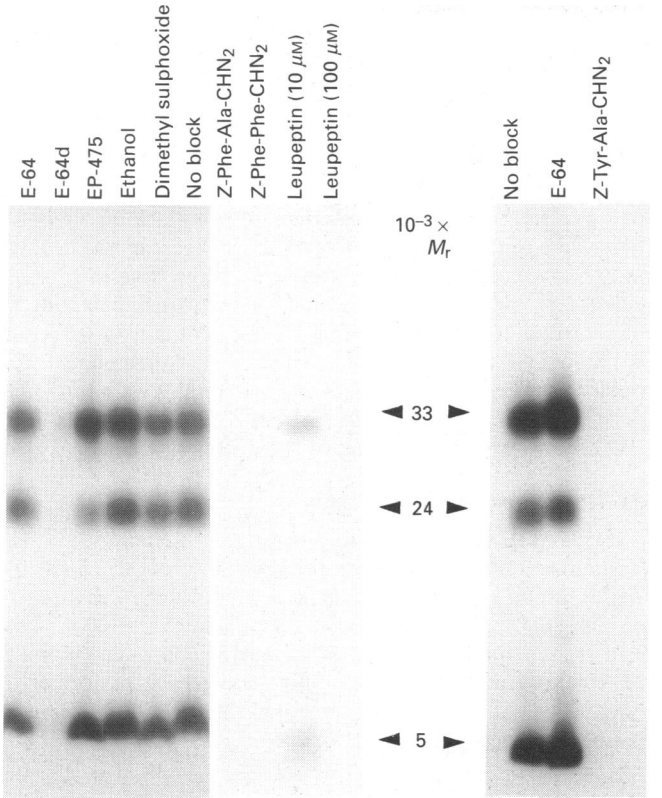

Fig. 1. Blocking of labelling of intact lysosomes by inhibitors of cysteine proteinases

Intact lysosomes were incubated at 30 °C for 1 h in the presence of the blocking inhibitor, followed by incubation with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for 1 h. The lysosomes were then sedimented by centrifugation for 10 min in a microfuge, resuspended in sample buffer, and submitted to SDS/PAGE on 12.5% gels. The inhibitors were used at 10 μM except for leupeptin which was used at both 100 μM and 10 μM. An equivalent amount of protein was loaded per lane (50 μg).

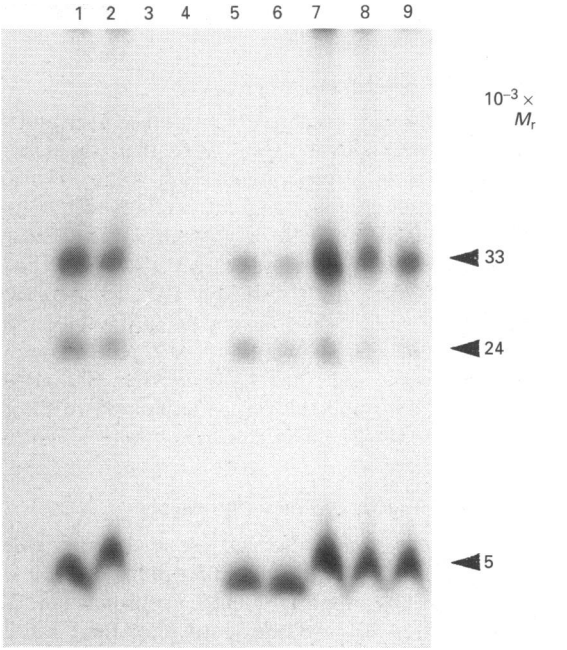

Fig. 2. Blocking of labelling in partially purified lysosomes

Partially purified intact lysosomes were incubated at 30 °C for 1 h in the presence of the blocking inhibitor, followed by incubation with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for 1 h. The lysosomes were then sedimented and submitted to SDS/PAGE on 12.5% gels. Lanes 1 and 2, no blocking inhibitors; lanes 3 and 4, 10 μM-Z-Tyr-Ala-CHN_2; lanes 5 and 6, 10 μM-E-64; lanes 7 and 8, 10 μM-leupeptin; lane 9, 100 μM-leupeptin. Lanes 1, 3, 5, 7 and 9 also contained 1 mM-cysteine. An equivalent amount of protein was loaded per lane (50 μg).

Labelling of intact lysosomes with Z-[^{125}I]iodo-Tyr-Ala-CHN_2

To assess the ability of Z-[^{125}I]iodo-Tyr-Ala-CHN_2 to enter lysosomes by diffusion, purified lysosomes were incubated for 30 min to 3 h in the presence of 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2. The labelling of lysosomal proteins was seen within 30 min of incubation with the inhibitor. Three proteins of M_r 33000, 24000 and 5000 were labelled. Each of the three proteins was labelled at a similar rate and the labelling of the three proteins increased during the time of incubation. In contrast with the labelling of lysosomal lysates, the labelling of proteins in intact lysosomes did not require the addition of the reducing agent, dithiothreitol, indicating that they were fully reduced under these conditions. To study this further, the labelling was performed in the presence of 1 mM-cysteine. Cysteine is a reducing agent which is essential for the activity of cysteine proteinases *in vitro* (Barrett & Kirschke, 1981) and it has been shown to exhibit carrier-mediated entry into the intact lysosomes (Pisoni *et al.*, 1990). The addition of cysteine did not affect labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2, thus demonstrating that the enzymes had fully reduced active sites (see Fig. 2).

To identify the labelled proteins, intact lysosomes were labelled for 1 h and the lysates subjected to immunoprecipitation using specific antisera raised against cathepsins L and B respectively. The 24000-M_r protein was specifically immunoprecipitated by the rabbit anti-(mouse cathepsin L) serum, indicating that it is cathepsin L. The proteins of M_r 33000 and 5000 were specifically immunoprecipitated by the sheep anti-(human cathepsin B) IgG, indicating that these proteins are two forms of cathepsin B.

Blocking of Z-[^{125}I]iodo-Tyr-Ala-CHN_2 labelling by cysteine proteinase inhibitors

To establish the ability of other commonly used cysteine proteinase inhibitors to enter isolated lysosomes, blocking experiments were performed. The principle of such experiments is that if the inhibitor is able to enter the lysosomes it will bind to the active sites of the cysteine proteinases and prevent the subsequent labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2. Intact purified lysosomes were incubated for 1 h in the presence of 10 μM-blocking inhibitor; the incubation was then continued for a further hour in the presence of 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2. The following controls were included and shown not to affect labelling: preincubation with 0.1% dimethyl sulphoxide, 0.5% ethanol or no additions (Fig. 1). Z-Tyr-Ala-CHN_2, Z-Phe-Phe-CHN_2, E-64d and Z-Phe-Ala-CHN_2 were able, when used at 10 μM, to prevent labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 completely, indicating that they freely enter intact lysosomes (Fig. 1). E-64 and EP-475 were unable to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 and therefore do not enter intact lysosomes under the conditions of this experiment (Fig. 1).

In this particular experiment, leupeptin blocked labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2, indicating penetration into the lysosome. This effect, unlike the effect of the other inhibitors, was not consistent with different preparations of lysosomes, suggesting that the ability of leupeptin to block inhibition may be due to reasons other than binding to the enzyme. Using the β-hexosaminidase assay, we found that, after a 2 h incubation, the amount of activity measured as 'free' increased from 40% to

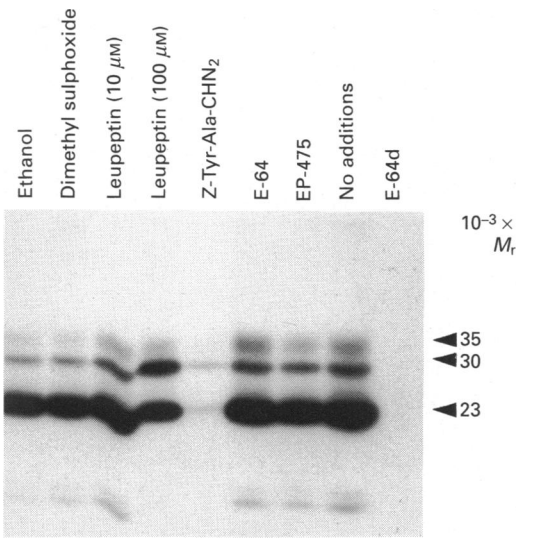

Fig. 3. Permeability of KNIH 3T3 fibroblasts to inhibitors of cysteine proteinases

KNIH 3T3 cells were grown for 2 days or until they formed a monolayer and then cultured in the absence of serum with 10 μM-blocking inhibitor for 1 h (as shown in the Figure), followed by culturing with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for 3 h. The cell lysates were precipitated with trichloroacetic acid, submitted to SDS/PAGE and the labelled proteins were visualized by autoradiography. Protein from an equivalent number of cells was loaded per lane (10^5 cells/lane).

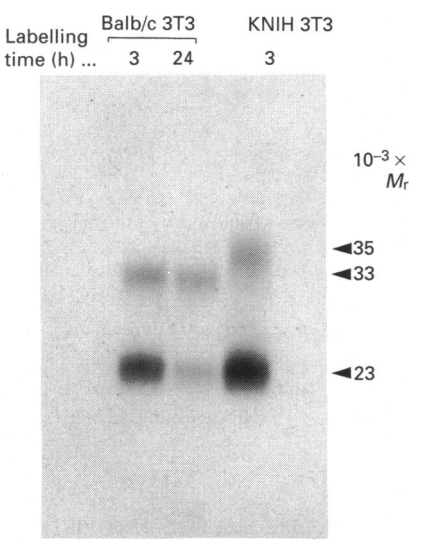

Fig. 4. Comparison of the labelling of cysteine proteinases in Balb/c 3T3 cells and KNIH 3T3 cells

Autoradiogram of Z-[^{125}I]iodo-Tyr-Ala-CHN_2-labelled proteins. Balb/c 3T3 cells were labelled at confluence with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2 for 3 h and 24 h. The cells were lysed, precipitated with trichloroacetic acid and the resulting precipitates submitted to SDS/PAGE on 12.5% gels. KNIH 3T3 cells were labelled for 3 h with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN_2. An equivalent amount of protein from 10^5 cells was loaded per lane.

70% in the absence of additions. The presence of the inhibitors, or ethanol used to solubilize the inhibitors, did not affect this. However, most of the lysosomes were still pelleted by centrifugation (determined by activity of β-hexosaminidase and cathepsin B), so it is more likely that the measurement of 'free' β-hexosaminidase activity was due to the β-hexosaminidase substrate entering lysosomes that were leaky or fragile, but not lysed. This could be attributed to the somewhat lengthy purification procedure which could lead to increased lysosomal fragility. If the purification of lysosomes leads to increased fragility of the membrane, then some of the inhibitors may be able to penetrate more easily and appear to cross the lysosomal membrane rapidly. We therefore repeated the experiments with a partially purified lysosomal preparation.

Permeability of partially purified lysosomes to cysteine proteinase inhibitors

To assess the integrity of the partially purified lysosomes, the latency of the β-hexosaminidase activity was measured after purification. Generally, these preparations were 85% intact at the start of the experiment. The preparation was incubated under the conditions shown in the legend to Fig. 2. After a 2 h incubation in the absence of inhibitors, 30–40% of the β-hexosaminidase activity was determined to be 'free'. The presence of leupeptin, Z-Tyr-Ala-CHN_2, dimethyl sulphoxide or ethanol did not significantly increase this value. This preparation was then used to investigate the permeability of lysosomes to the inhibitors.

Z-Tyr-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 (Fig. 2). E-64 and leupeptin were unable to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 (Fig. 2). This indicates that in these experiments leupeptin did not enter lysosomes, thus clarifying the earlier results with the purified lysosomes. The results for the other inhibitors studied were identical in both sets of experiments.

Permeability of cells to inhibitors of cysteine proteinases

The ability of an inhibitor to block the labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in intact lysosomes should indicate whether or not such inhibitors can freely diffuse into cells. To assess this proposal, the ability of the inhibitors to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 in cells during culture was tested. Three cell lines were chosen for this study: KNIH 3T3 (Kirsten-virus-transformed mouse fibroblasts), HT1080 (a human sarcoma cell line) and HIFF (human infant foreskin fibroblasts).

Labelling of active cysteine proteinases in KNIH 3T3 cells has been demonstrated in a previous study (Mason *et al.*, 1989*a*). The pattern of labelling was identical with that of the previous study. Three proteins were labelled, corresponding to cathepsin L (M_r 23000 and 30000) and cathepsin B (M_r 35000). The ability of the following inhibitors to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 was tested: leupeptin, Z-Tyr-Ala-CHN_2, E-64, EP-475 and E-64d. Z-Tyr-Ala-CHN_2, and E-64d completely blocked labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2, indicating their ability to penetrate cells rapidly (Fig. 3). E-64, EP-475 and leupeptin at either 10 μM or 100 μM did not block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN_2 and therefore these inhibitors do not rapidly enter this cell line.

A comparison of the labelling of the KNIH 3T3 cells with the labelling of the non-virus-transformed mouse cell line, Balb/c 3T3, shows that cathepsin B has a higher M_r in the transformed cells (Fig. 4). The cathepsin B in the non-transformed cells is therefore more similar to cathepsin B in mouse liver lysosomes, which exists as both a single-chain form of M_r 33000 and a two-chain form of 5000 plus 28000.

Labelling of active cysteine proteinases was performed in HT1080 cells and the ability of other inhibitors of cysteine proteinases to block this was assessed. Labelling of HT1080 cells with Z-[^{125}I]iodo-Tyr-Ala-CHN_2 demonstrated the presence of active cathepsin L and cathepsin B. Active cathepsin L was shown to exist in four forms, of M_r 34000, 32000, 23000 and

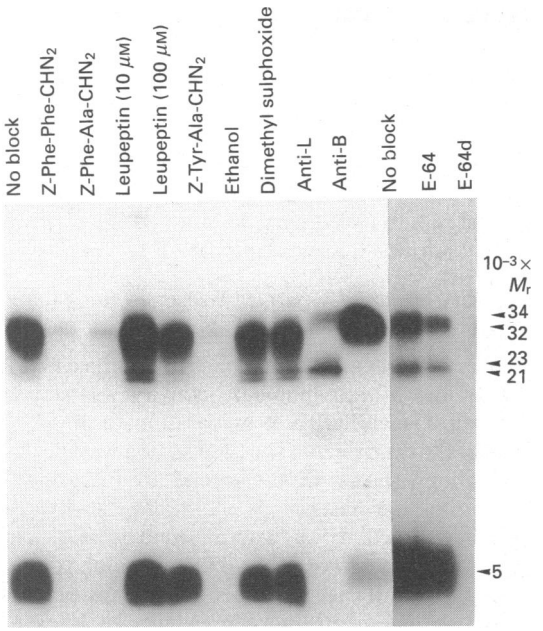

Fig. 5. Labelling of HT1080 cells with Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$

The HT1080 cells were cultured until they formed a monolayer and were labelled with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ for 3 h and then either precipitated with trichloroacetic acid or immunoprecipitated with anti-(cathepsin L) (Anti-L) and anti-(cathepsin B) (Anti-B) sera. The resultant precipitates were resuspended in SDS/PAGE sample buffer and then submitted to SDS/PAGE on 12.5% gels. Labelled proteins were visualized by autoradiography of the dried gel. The blocking inhibitors were all used at 10 μM except for leupeptin which was used at 100 μM and 10 μM. Protein from an equivalent number of cells was loaded per lane (10^5 cells/lane).

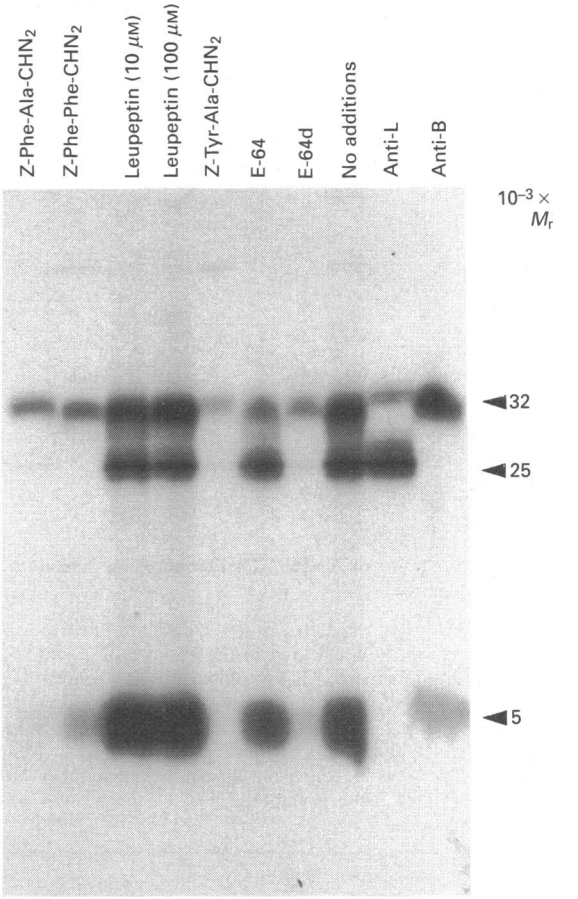

Fig. 6. Labelling of HIFF cells with Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$

Confluent monolayers of HIFF cells were cultured with 0.1 μM-Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ for 3 h. The cells were then lysed, and either precipitated with trichloroacetic acid or immunoprecipitated with either anti-(cathepsin L) (Anti-L) or anti-(cathepsin B) (Anti-B). The resultant precipitates were resuspended in SDS/PAGE sample buffer and then submitted to SDS/PAGE on 12.5% gels. Labelled proteins were visualized by autoradiography of the dried gel. All blocking inhibitors were used at 10 μM except for leupeptin which was used at 100 μM and 10 μM. Protein from an equivalent number of cells was loaded per lane (10^5 cells/lane).

21000 (Fig. 5). Cathepsin B existed in two active forms of M_r 32000 and 5000 (Fig. 5). Such multiple forms of cathepsin L have also been observed in THP-1 cells (D. Wilcox & R. W. Mason, unpublished work). The effect of the following inhibitors on labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ was investigated; leupeptin, Z-Tyr-Ala-CHN$_2$, Z-Phe-Phe-CHN$_2$, E-64, Z-Phe-Ala-CHN$_2$ and E-64d. The solvents, dimethyl sulphoxide and ethanol, which are used to prepare the stock solutions of the inhibitors, did not affect labelling when added to the cells at the same concentration that would be present in the inhibitor solutions added (Fig. 5). Z-Phe-Phe-CHN$_2$, Z-Phe-Ala-CHN$_2$ and E-64d were able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ completely (Fig. 5). E-64 and leupeptin at 10 μM were unable to block labelling (Fig. 5). Leupeptin when used at 100 μM did decrease labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$, indicating some penetration of leupeptin at higher concentrations in these cells.

HIFF cells were labelled with Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ for 3 h, with no preblocking. This demonstrated the presence of two forms of active cathepsin L of M_r 32000 and 25000 (Fig. 6) and two active forms of cathepsin B of M_r 32000 and 5000 (Fig. 6). Preincubation of cells with 10 μM-E-64 and 10 μM or 100 μM-leupeptin was unable to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$. The diazomethanes, Z-Phe-Phe-CHN$_2$, Z-Phe-Ala-CHN$_2$ and Z-Tyr-Ala-CHN$_2$, were all able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$. The putative membrane permeant analogue of EP-475, E-64d, was also able to block labelling, indicating penetration into the cell.

Thus, in the cell lines tested Z-Tyr-Ala-CHN$_2$, Z-Phe-Phe-CHN$_2$ and E-64d were able to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$. Leupeptin, EP-475 and E-64 were unable to block labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$.

DISCUSSION

It has been proposed that incubation of cells at 18 °C blocks fusion of vesicles in cells, and that incubation at 4 °C completely blocks endocytosis (Dunn *et al.*, 1980). When KNIH 3T3 cells were incubated with Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ at 18 °C and 4 °C for 3 h, labelling of cysteine proteinases was decreased by 50% and 75% respectively (results not shown). This could be due in part to a decreased rate of reaction of the enzymes at the lower temperatures and in part to decreased fluidity of the cell membranes. Such results do not rule out the possibility that entry of the inhibitor into the lysosome is by a decreased rate of endocytosis which is not completely blocked by the lower temperatures. We have therefore taken a different approach to distinguish between endocytosis and diffusion by observing the ability of Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ to cross a single lipid bilayer using purified lysosomes.

Our results have shown that reactive forms of cathepsins L and B in lysosomal lysates could be demonstrated only in the presence

of reducing agent. This is consistent with the activity of the purified enzymes [reviewed in Barrett & Kirschke (1981)]. Active cathepsins L and B in lysosomes in the absence of activating thiol reagents, however, have not previously been demonstrated. Furthermore, the addition of the reducing agent, cysteine, to the lysosomes did not significantly increase labelling of the proteinases, indicating that they were not inactivated by oxidation during purification of the lysosomes (Fig. 2).

An additional advantage of using these inhibitors with intact cells and lysosomes is that they are able to detect minor molecular forms of active proteinases, and clearly demonstrate different processing of cathepsins B and L in different cells and tissues. Thus in the KNIH 3T3 cells, cathepsin B exists as a single-chain active form of M_r 35000, whereas in mouse liver lysosomes it exists as both a single-chain active form of M_r 33000 and presumably two-chain form of 5000 plus 28000, although the 28000-M_r polypeptide does not contain the active-site cysteine and is not seen by this technique. In the non-virus-transformed mouse cell line, Balb/c 3T3, the labelled proteins were more similar to those in liver lysosomes, indicating that the processing of cathepsin B in KNIH 3T3 cells is truly different and not simply an artefact of the cell culture conditions (Fig. 4). The reason for the different M_r value of cathepsin B in the KNIH 3T3 cells is at present unknown. In the two human cell lines, processing was also different, with multiple forms seen in the HT1080 cells which are probably due to differences in glycosylation. The physiological implications of the differences in processing are not apparent at present because these different forms are all active, but these results clearly demonstrate that one cannot assume that all cells and tissues contain the same molecular forms of individual lysosomal proteinases.

We were able to discount the possibility that the labelling of cathepsins L and B by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ was caused by lysosomes being lysed and the labelling occurring outside the lysosomes. There are two reasons for discounting this: (1) all lysosomes were sedimented after labelling and (2) when lysosomes were lysed, labelling only occurred in the presence of reducing agent and therefore the observed labelling must have occurred in the intact non-oxidizing environment of the lysosome. The labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ was rapid, occurring within 30 min, and indicates that this inhibitor enters lysosomes via diffusion through the membrane.

The epoxide inhibitors E-64 and EP-475 (E-64c) were unable to prevent labelling by Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ in intact lysosomes or in cells during culture (see Figs. 1, 2, 3, 5 and 6). This clearly demonstrates that these compounds do not readily diffuse across membranes and therefore uptake of these inhibitors by cells can only occur via pinocytosis. The lack of membrane permeability demonstrated by these compounds in this study is consistent with the predictions discussed in a review by Mehdi (1991). This can be attributed to the presence of ionized side groups at neutral pH (Mehdi, 1991). E-64 contains a guanidino group which is positively charged and a negatively charged carboxy group. EP-475 (E-64c) contains only the negatively charged carboxy group which is sufficient to confer membrane impermeability. Therefore these two compounds can be considered of limited value as therapeutic agents for inhibiting cellular proteolysis, since they have poor membrane permeability and would be required at high concentration which would lead to increased non-specific reaction.

E-64d, in contrast with EP-475, is able to permeate both lysosomes and cells. This compound inhibits the lysosomal cysteine proteinases *in vitro* and is proposed to inhibit calpain, although this has not been demonstrated *in vivo* (Tamai *et al.*, 1986; McGowan *et al.*, 1989). Our study clearly demonstrates that E-64d can inhibit cathepsins B and L in intact lysosomes and in tissue culture cells. Previous studies have demonstrated that this compound is able to enter cells and it was shown to bind to at least 11 proteins, none of which were identified, and surprisingly, none seemed to correspond to the known mammalian cysteine proteinases (Shoji-Kasai *et al.*, 1988). Further clarification is required to determine all of the cellular targets of this inhibitor. The increased permeability of this compound compared with EP-475 is probably due to the esterification of the carboxy group, resulting in the loss of the negative charge at neutral pH. The broad specificity of E-64d limits the efficacy with which it can be used to study lysosomal cysteine function but it would be a good inhibitor to use when investigating both lysosomal and cytosolic protein degradation. Further development of these epoxide compounds will probably be successful, and a more specific inhibitor of cathepsin B has now been synthesized (Murata *et al.*, 1991).

Leupeptin failed to enter lysosomes and entered cells only slowly, as demonstrated by the partial inhibition of the lysosomal cysteine proteinases in cells. This is consistent with this inhibitor being unable to diffuse across membranes, probably due to the charged guanidino group present in the molecule. Any slow permeation into cells may reflect pinocytotic uptake. The lack of permeation is consistent with a previous study by Nonaka and colleagues (1982). Therefore leupeptin can be considered a poor choice of inhibitor for the investigation of lysosomal cysteine proteinase function, unless sufficient inhibitor can be used to get it into the lysosome at a sufficiently high concentration by pinocytosis.

The diazomethanes Z-Phe-Phe-CHN$_2$, Z-Phe-Ala-CHN$_2$ and Z-Tyr-Ala-CHN$_2$ have been shown to diffuse into lysosomes. These inhibitors also entered cultured cells within 1 h, and inactivated cathepsins B and L. Therefore it is proposed that these inhibitors also enter cells by diffusion. This is consistent with our earlier proposal on the mechanism of entry of Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ (Mason *et al.*, 1989*a*) and is supported by the earlier work of Grinde (1983). Although Z-Phe-Phe-CHN$_2$ and Z-Phe-Ala-CHN$_2$ clearly inhibit cathepsins B and L in intact lysosomes and in cultured cells, the possibility of additional cellular targets for these compounds cannot be eliminated at present. Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$, however, labels only the lysosomal cysteine proteinases in both intact lysosomes and cells. Z-[^{125}I]iodo-Tyr-Ala-CHN$_2$ does not react with calpain, the major cytosolic cysteine proteinase, and therefore the specificity of this inhibitor makes it the most suitable inhibitor available to study the roles of lysosomal cysteine proteinases in cells and makes it ideal for use as a model compound for therapeutic agents.

This study clearly indicates that, when choosing an inhibitor of lysosomal cysteine proteinase function, it is important to consider the ability of the inhibitor to reach its target; this study provides guidelines for the choice of such inhibitors.

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REFERENCES

Baricos, W. H., Zhou, Y., Mason, R. W. & Barrett, A. J. (1988) Biochem. J. **252**, 301–304

Barrett, A. J. & Kirschke, H. (1981) Methods Enzymol. **80**, 535–561

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, G. C., Tamai, M. & Hanada, K. (1982) Biochem. J. **201**, 189–198

Bird, S. J., Forster, S. & Lloyd, J. B. (1987) Biochem. J. **245**, 929–931

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Bury, A. F. (1981) J. Chromatogr. **213**, 491–500

Dean, R. T. (1979) Biochem. J. **180**, 339–345
Dunn, W. A., Hubbard, A. L. & Aronson, N. N. (1980) J. Biol. Chem. **255**, 5971–5978
Englard, S. & Siegel, L. (1969) Methods Enzymol. **13**, 99–106
Grinde, B. (1983) Biochim. Biophys. Acta **757**, 15–20
Hanada, K., Tamai, M., Morimoto, S., Adachi, T., Ohmura, S., Sawada, J. & Tanaka, I. (1978) Agric. Biol. Chem. **42**, 537–541
Kirschke, H. & Shaw, E. N. (1981) Biochem. Biophys. Res. Commun. **101**, 454–458
Mason, R. W. (1986) Biochem. J. **240**, 285–288
Mason, R. W., Green, G. D. J. & Barrett, A. J. (1985) Biochem. J. **226**, 233–241
Mason, R. W., Wilcox, D., Wikstrom, P. & Shaw, E. N. (1989*a*) Biochem. J. **257**, 125–129
Mason, R. W., Bartholomew, L. T. & Hardwick, B. S. (1989*b*) Biochem. J. **263**, 945–949
McGowan, E. B., Becker, E. & Detwiler, T. C. (1989) Biochem. Biophys. Res. Commun. **158**, 432–435
Mehdi, S. (1991) Trends Biochem. Sci. **16**, 150–153
Murata, M., Miyashita, S., Yokoo, C., Tamai, M., Hanada, K., Hatayama, K., Towatari, T., Nikawa, T. & Katunuma, N. (1991) FEBS Lett. **280**, 307–310
Noda, T., Isogai, K., Katunuma, N., Tarumoto, Y. & Ohzeki, M. (1981) J. Biochem. (Tokyo) **90**, 893–896
Nonaka, I., Ishiura, S., Takagi, A. & Sugita, H. (1982) Acta Neuropathol. **58**, 279–285
Parkes, C., Kembhavi, A. & Barrett, A. J. (1985) Biochem. J. **230**, 509–516
Pisoni, R. L., Acker, T. L., Lisowski, K. M., Lemons, R. M. & Thoene, J. G. (1990) J. Cell Biol. **110**, 327–335
Rasheed, S., Nelson-Rees, W. A. & Toth, E. M. (1974) Cancer **33**, 1027–1033
Rich, D. H. (1986) in Proteinase Inhibitors (Barrett, A. J. & Salvesen, G., eds.), pp. 153–178, Elsevier Science Publishers, Amsterdam
Schneider, W. C. & Hogeboom, G. H. (1952) J. Biol. Chem. **195**, 161–166
Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) Eur. J. Biochem. **95**, 215–225
Shaw, E. & Dean, R. T. (1980) Biochem. J. **186**, 385–390
Shoji-Kasai, Y., Senshu, M., Iwashita, S. & Imahori, K. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 146–150
Simon, O., Bohley, P., Munchmeyer, R., Bergner, H. & Huckel, C. (1977) Acta Biol. Med. Germ. **36**, 1923–1925
Tamai, M., Matsumoto, K., Omura, S., Koyama, I., Ozawa, Y. & Hanada, K. (1986) J. Pharamacobio-Dyn. **9**, 672–677
Tamai, M., Omura, S., Kimura, M., Hanada, K. & Sugita, H. (1987) J. Pharmacobio-Dyn. **10**, 678–681
Yamada, H., Hayashi, H. & Natori, Y. (1984) J. Biochem. (Tokyo) **95**, 1155–1160

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