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Cytotoxicity and effect of glycyl-D-phenylalanine-2-naphthylamide on lysosomes

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Glycyl-D-phenylalanine-2-naphthylamide (Gly-D-Phe-2-NNap) is a cytotoxic agent as exemplified by its effect on Vero cells in culture. This effect is inhibited to some extent by nigericin. On the other hand, Gly-D-Phe-2-NNap induces an increase of free activity of *N*-acetylglucosaminidase when incubated with a mitochondrial fraction of rat liver at pH 7.5. The phenomenom is inhibited by chloroquine, NH_4Cl and nigericin, substances that are known to increase the intralysosomal pH. The latency of enzymes located in other subcellular structures – mitochondria, peroxisomes and endoplasmic reticulum – is not affected by Gly-D-Phe-2-NNap. Moreover, that compound does not cause a release of FITC-Dextran present in endosomes. Apparently Gly-D-Phe-2-NNap is a specific lytic agent for lysosomes. It is proposed that the molecule behaves like a lysosomotropic substance that is able to attack the lysosomal membrane from the interior of the organelle. Its cytotoxic properties could be explained by its effect on lysosomes.

Introduction

We have shown previously that glycyl-L-phenylalanine-2-naphthylamide (Gly-L-Phe-2-NNap) was able to specifically disrupt lysosomes by a mechanism involving the penetration of the compound into the organelles and its intralysosomal hydrolysis by cathepsin C [1]. The phenomenon can be profitably used in investigations on subcellular localization, particularly in studies on endocytosis [2–4] and lysosome biogenesis [5]. The process requires a pH of at least 6 to be well apparent and is enhanced as the pH increases. Recently, we found that Gly-L-Phe-2-NNap was cytotoxic. However, its relatively quick hydrolysis when added to cell culture, probably by some cathepsin C present in the culture medium made difficult to assess the relationship between its injurious effects on cells and on lysosomes.

In the work reported here, we show that the D isomer Gly-D-Phe-2-NNap, that cannot be split by cathepsin C or an other lysosomal peptidase, can also specifically disrupt the lysosomal membrane but at higher pH than that needed by the L derivative. The mechanism of the phenomenon is probably different from that described for Gly-L-Phe-2-NNap although the penetration of the compound into lysosomes is also required. On the other hand, we found that Gly-D-Phe-2-NNap was a cytotoxic agent. A relationship between the cytotoxicity of the compound and its effect on lysosomes is proposed.

Material and Methods

Experiments were performed on male Wistar rats. Liver total mitochondrial fraction (ML fraction), light mitochondrial fraction (L fraction) and microsomal fraction (P) were prepared as described by De Duve et al. [6]. To measure the effect of Gly-D-Phe-2-NNap, granule preparations were incubated at 37°C in a medium containing 0.25 M sucrose, 5 mM acetate buffer (pH 6) or Tris-HCl (pH 7.5) and the naphthylamide at the indicated concentration. Dimethylsulfoxide used as solvent for the naphthylamide did never exeed 1% in the medium. Nigericin was added to the incubation medium from a 8 mM stock solution in ethanol. The stock solution of chloroquine was prepared in 10 mM Tris-HCl and brought to pH 7.5. The controls always contain the solvent at the same concentration. Gly-D-Phe-2-NNap and Gly-L-Phe-2-NNap were obtained from Bachem (Bubendorf, Switzerland). Enzymes were assayed according to the following references: *N*-acetylglucosaminidase [7], sulfite–cytochrome-*c* reductase [8],

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malate dehydrogenase [9], catalase [10], mannose-6-phosphatase [11]. The measurements of total activities were achieved by adding 0.1% Triton X-100 to the incubation medium [12].

For assays on intact cells, we made use of Vero cells grown in MEM medium supplemented with 10% foetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 25 mM Hepes, and seeded in 96 wells plates (Falcon) 15 h before the experiment. The viability of the cells was assayed according to a slight modification of the method described by Mosman [13]. Briefly, we replaced the culture medium of the cells to be tested by a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (1 mg/ml in HBS; 100 µl/well) and led them stand for 120 min at 37°C. We then discarded the MTT and solubilized the blue formazan cristals produced by living cells and sticking to the bottom of each well with DMSO. Afterwards, the plate was read at 490 nm with a microplate reader (MR 600 Dynatech). HBS is composed of 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$ and adjusted to pH 7.4 with NaOH.

Results

Resistance of Gly-D-Phe-2-NNap to hydrolysis

To check if Gly-D-Phe-2-NNap is resistant to cathepsin C, it was incubated with Vero Cells, a mitochondrial fraction or purified lysosomes from rat liver. As shown by Fig. 1, no hydrolysis was detectable while in the same conditions, Gly-L-Phe-2-NNap is extensively split.

Cytotoxicity of Gly-D-Phe-2-NNap. Effect of nigericin

Fig. 2 illustrates the cytotoxicity of Gly-D-Phe-2-NNap. Vero cells were incubated in the presence of

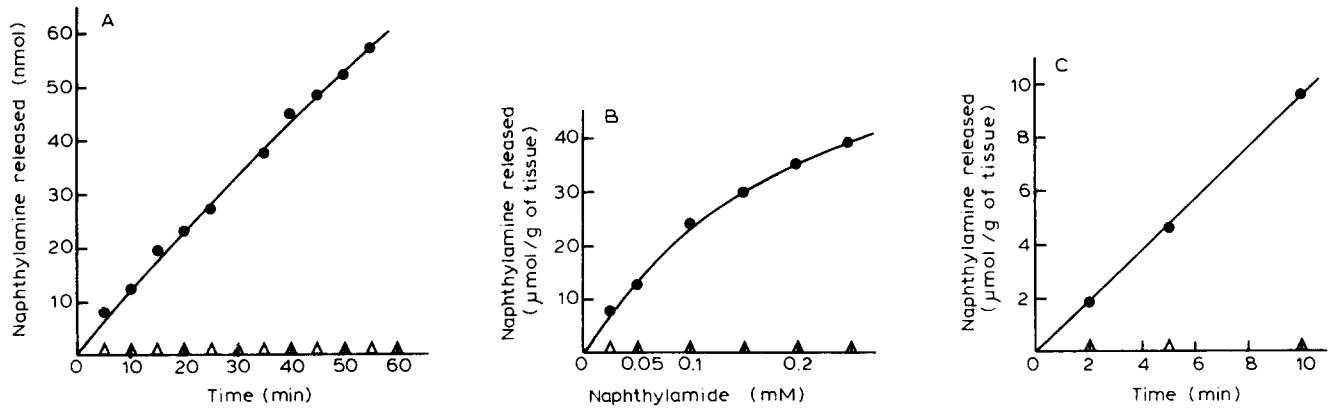

Fig. 1. Resistance of Gly-D-Phe-2-NNap to hydrolysis. Intact Vero cells (1 cm^2) grown to confluence (A), a total mitochondrial fraction from rat liver (B) and rat liver lysosomes purified on a Metrizamide gradient (C) were incubated at 37°C and for increasing times in presence of 0.25 mM dipeptidylnaphthylamide (A, C) or for 10 min in presence of increasing concentrations of dipeptidylnaphthylamide (B). Released naphthylamine was measured. ●, Gly-L-Phe-2-NNap; △, Gly-D-Phe-2-NNap.

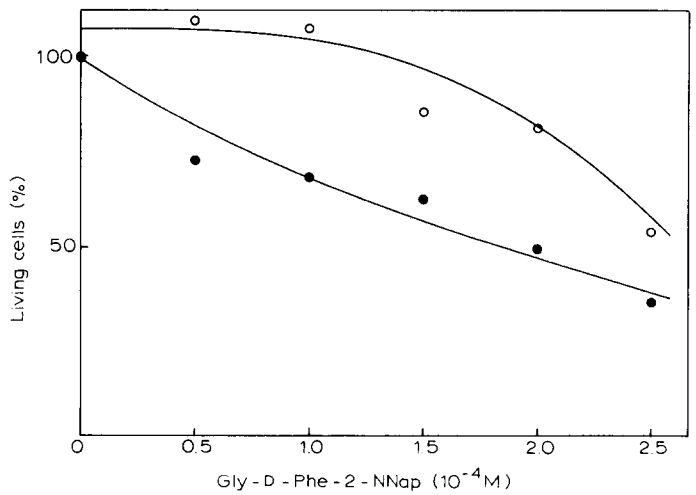

Fig. 2. Cytotoxic effect of Gly-D-Phe-2-NNap. Effect of nigericin. Vero cells grown to confluence in 96 wells plates were preincubated for 30 min with (○) or without (●) 10^{-5} M nigericin. The medium was then removed and replaced by fresh medium containing various concentrations of Gly-D-Phe-2-NNap, with (○) or without (●) 10^{-5} M nigericin. After a 4 h incubation, the cell viability was assessed as described in Materials and Methods.

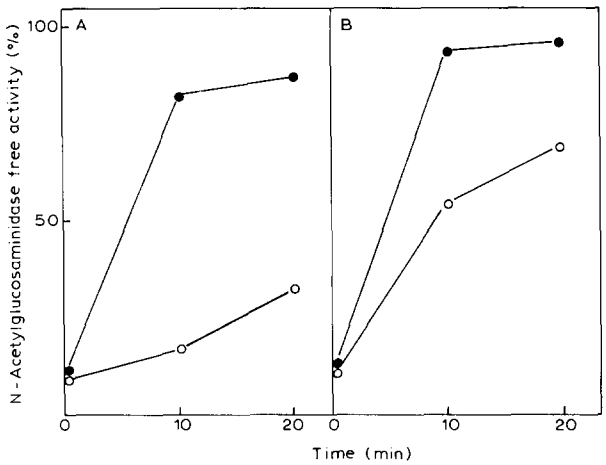

Fig. 3. Latency loss of *N*-acetylglucosaminidase caused by Gly-L-Phe-2-NNap and Gly-D-Phe-2-NNap. A mitochondrial fraction (ML) was incubated at 37 °C for the times indicated in abscissa, in presence of 0.25 mM Gly-L-Phe-2-NNap (●) or Gly-D-Phe-2-NNap (○), 0.25 M sucrose, 5 mM acetate buffer pH 6 (A) or 5 mM Tris-HCl pH 7.5 (B). After that, samples were withdrawn for assays of free and total activity of *N*-acetylglucosaminidase. The samples were incubated at 37 °C in a total volume of 1 ml containing 4 mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, 0.1 M citrate buffer (pH 5), and 0.25 M sucrose in the absence (free activity) or in the presence (total activity) of 0.1% Triton X-100. The assay was terminated after 10 min by adding 1.5 ml of 2.75% (w/v) trichloracetic acid. Free activity is given as percentage of total activity.

various concentrations of Gly-D-Phe-2-NNap, and their viability assessed by the tetrazolium test. The percentage of living cells decreases as a function of the naphthylamide concentration. The same experiment was performed in presence of 10^{-5} M nigericin. It is obvious that the protonophore significantly protects the cells against the toxic effect of Gly-D-Phe-2-NNap.

Effect of Gly-D-Phe-2-NNap on lysosomes

Fig. 3 reports the free activity of *N*-acetylglucosaminidase when an ML fraction of rat liver was incubated in presence of 0.25 mM Gly-L-Phe-2-NNap or 0.25 mM Gly-D-Phe-2-NNap at pH 6 or at pH 7.5. In agreement with previous results [1] the latency of the lysosomal hydrolase is considerably decreased by Gly-L-Phe-2-NNap at pH 6 and at pH 7.5. The effect of the D derivative is markedly less pronounced at pH 6 but is well apparent at pH 7.5. The free activity of cathepsin C increases with the incubation time of the ML fraction in presence of Gly-D-Phe-2-NNap and with the concentration of that compound (Fig. 4). The hydrolase free activity increase results from a true release of the enzyme in the medium (results not shown).

Gly-D-Phe-2-NNap may be considered as a relatively weak base and accordingly could penetrate and accumulate in lysosomes as a result of the pH difference existing between the lysosomal matrix and the medium [14]. To see if Gly-D-Phe-2-NNap had to enter lysosomes to disrupt their membrane, incubation with the naphthylamide was achieved in the presence of molecules known to increase the intralysosomal pH. Chloroquine and NH_4Cl are weak bases accumulating in lysosomes thereby decreasing the proton gradient. Nigericin causes lysosomal alkalinisation by inducing an exchange between the intralysosomal H^+ and the extralysosomal K^+. As shown by Fig. 5, NH_4Cl and chloroquine as well as nigericin are able, although to a somewhat different extent, to protect the lysosomes against the loss of latency of *N*-acetylglucosaminidase induced by Gly-D-Phe-2-NNap. It is to be noted that only chloroquine is able to totally abolish the naphthylamide effect. That observation could be explained by a dual effect of the molecule: its ability to increase the lysosomal pH

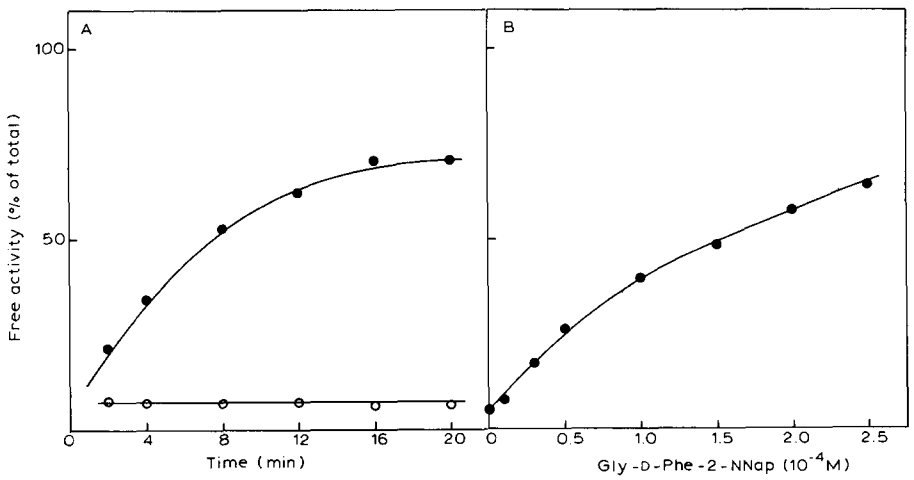

Fig. 4. Latency loss of *N*-acetylglucosaminidase caused by Gly-D-Phe-2-NNap. Effect of incubation time and of Gly-D-Phe-2-NNap concentration. (A) A mitochondrial fraction (ML) was incubated for increasing times at 37 °C in presence of 5 mM Tris-HCl (pH 7.5), 0.25 M sucrose without (○) or with (●) 0.25 mM Gly-D-Phe-2-NNap. After incubation, free and total *N*-acetylglucosaminidase activities were measured. (B) A mitochondrial fraction (ML) was incubated for 20 min at 37 °C in presence of various concentration of Gly-D-Phe-2-NNap, 5mM Tris-HCl (pH 7.5), 0.25 M sucrose. After that, free and total *N*-acetylglucosaminidase activities were measured. Free activity is given as percentage of total activity.

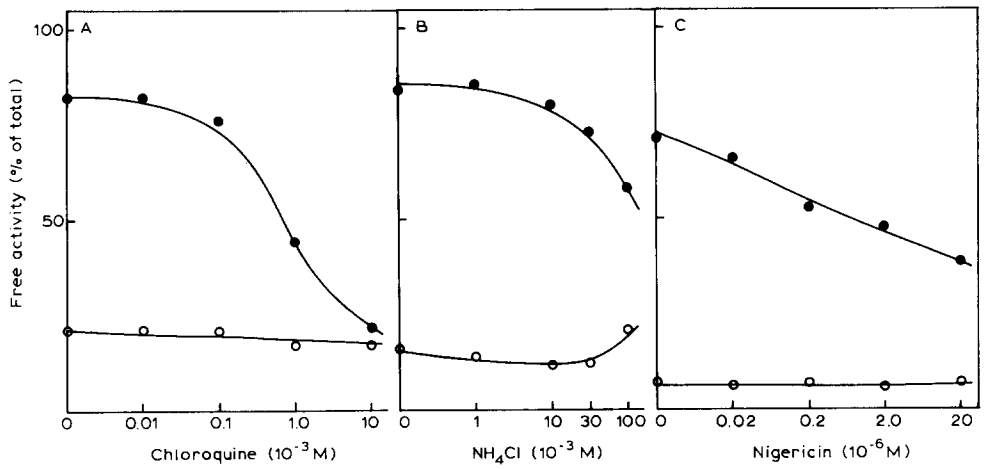

Fig. 5. Latency loss of *N*-acetylglucosaminidase caused by Gly-D-Phe-2-NNap. Effect of chloroquine. NH_4Cl and nigericin. For testing the effect of chloroquine (A) or NH_4Cl (B), an L fraction was incubated for 10 min at 37 °C in presence of these compounds at the concentration indicated in the abscissa, 0.25 M sucrose, 5 mM Tris-HCl (pH 7.5). After that, the mixture was diluted 10-times with the same medium but devoid of chloroquine or NH_4Cl, in the presence (●) or absence (○) of 0.25 mM Gly-D-Phe-2-NNap. After 20 min incubation at 37 °C, free and total activities of *N*-acetylglucosaminidase were determined. To assess the nigericin effect (C), the L fraction was kept for 20 min at 37 °C in presence of that substance at the concentration indicated in the abscissa, 0.25 M sucrose, 5 mM Tris-HCl (pH 7.5), 25 mM KCl with (●) or without (○) 0.25 mM Gly-D-Phe-2-NNap. After that free and total activities of *N*-acetylglucosaminidase were measured. Free activity is given as percentage of total activity.

and its known effect as stabilizer of biological membranes [15,16].

Effect of Gly-D-Phe-2-NNap on non lysosomal subcellular structures

The effect of Gly-D-Phe-2-NNap on the latency of enzymes located in other subcellular structures than lysosomes was tested. Sulfite–cytochrome-*c* reductase (intermembrane space) and malate dehydrogenase (matrix space) were selected for mitochondria [8,9], catalase for peroxisomes [10] and mannose-6-phosphatase for endoplasmic reticulum [11]. As illustrated by Fig. 6, Gly-D-Phe-2-NNap does not cause an increase of the free activity of these enzymes. A similar experiment was performed to see if endosomes were not susceptible to Gly-D-Phe-2-NNap. To this aim, the rat was injected with FITC-Dextran (20 mg/100 g of body weight) and killed 10 min later. A microsomal fraction that contains most of the endosomes [17] was prepared and the release of FITC-Dextran measured after incubating the microsomes with Gly-D-Phe-2-NNap. Results show that no release of FITC-Dextran occurs under these conditions. In addition, we have found that Gly-D-Phe-2-NNap is not haemolytic at concentrations that induce a lysis of lysosomes (results not shown).

Discussion

Gly-D-Phe-2-NNap may be considered as a weak base relatively hydrophobic able to cross the lysosomal membrane. Our results strongly suggest that the penetration of the molecule in the lysosomes is required for its lytic effect on these organelles. Indeed, a rise of the pH that increases the proportion of unprotonated diffusible form of Gly-D-Phe-2-NNap favors the effect of the compound on lysosomes. On the other hand, chloroquine, NH_4Cl and nigericin that, by lowering the pH gradient inhibit the transfer of the molecule into lysosomes, prevents lysis of these organelles.

How the presence of Gly-D-Phe-2-NNap in the lysosomes does induce a disruption of the particles? Two mechanisms have to be considered. First, accumulation of the molecule in the lysosomal matrix could cause an osmotic unbalance, a swelling and a rupture of the granules [14]. Such a mechanism is improbable since the amount of Gly-D-Phe-2-NNap that would have to be located inside the lysosomes to bring about a significant osmotic unbalance would largely exceed the solubility of the molecule in aqueous media. To this respect it is also worthwhile to mention that chloroquine, a lysosomotropic weak base, does not cause lysosome lysis at concentrations similar to that of Gly-D-Phe-2-NNap (results not shown). The second possibility is a direct attack of the lysosomal membrane by that compound. The processus would be promoted when Gly-D-Phe-2-NNap is inside the lysosomes thanks to acid intralysosomal pH. At such pH, not only the total concentration of the substance will be higher inside than outside the particle, but the proportion of the protonated (amphiphilic) form, more potent to disturb the lipid bilayer will be increased.

Other subcellular structures are resistant to the lytic effect of Gly-D-Phe-2-NNap. In the case of peroxisomes, endoplasmic reticulum vesicles and mitochondria, that probably results from the fact that the pH inside the structure is similar to the external pH,

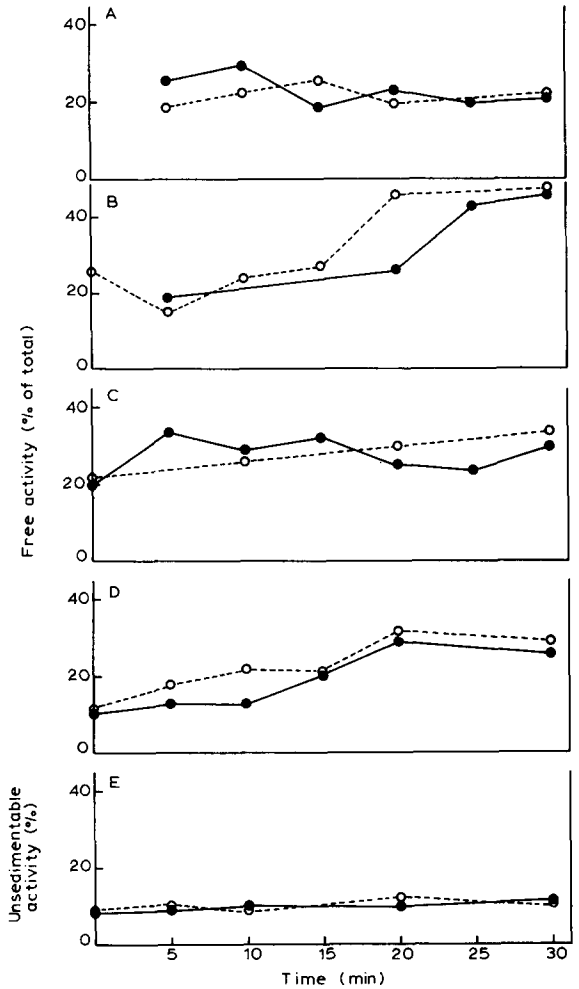

Fig. 6. Effect of Gly-D-Phe-2-NNap on different subcellular membranes. A mitochondrial fraction (ML) was incubated for increasing times at 37 °C in 5 mM Tris-HCl (pH 7.5), 0.25 M sucrose with (●) or without (○) 0.25 mM Gly-D-Phe-2-NNap. After that free and total activities of sulfite–cytochrome-*c* and reductase (A), malate dehydrogenase (B), catalase (C) and mannose-6-phosphatase (D) were determined. Results presented in (E) were obtained after incubating in the same conditions a microsomal fraction P isolated from the liver of a rat injected with FITC-Dextran (20 mg/100 g of body weight) 10 min before killing. After incubation, the preparation was centrifuged for 20 min at 25000 rpm in the Spinco rotor 40.3 and the FITC-Dextran released in the supernatant was assessed by a fluorescence assay. The total amount of FITC-Dextran associated with the granules was determined by measuring the fluorescence released after treating the preparation with 0.1% Triton X-100.

what precludes an accumulation of Gly-D-Phe-2-NNap in the interior of the particle. With respect to endosomes that are also acidic [17], resistance to Gly-D-Phe-2-NNap could be caused by the absence of osmotic behaviour of these organelles in sucrose solutions (Ref. 18, and personnal results). It is possible that to exert its action, Gly-D-Phe-2-NNap has first to increase the permeability of the lysosome membrane so that external solutes like sucrose, could enter the particles and cause an osmotic unbalance. Such an osmotic unbalance could not take place in the case of endosomes owing to their permeability to sucrose. In any way, the lytic properties of Gly-D-Phe-2-NNap is apparently very specific for lysosomes and could be profitably used for investigations on these organelles.

Our results strongly suggest that the cytotoxicity of Gly-D-Phe-2-NNap originates from its lytic effect on lysosomes. First, both phenomena occur in the same concentration range; second, amongst the subcellular structures investigated, only lysosomes are disrupted by that compound; third, nigericin opposes both lysosome lysis and cell killing by the naphthylamide. Gly-D-Phe-2-NNap could be a convenient substance to investigate the connection between lysosome activation and cell injury.

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References

1 Jadot, M., Colmant, C., Wattiaux-De Coninck, S. and Wattiaux, R. (1984) Biochem. J. 219, 965–970.
2 Jadot, M. and Wattiaux, R. (1985) Biochem. J. 225, 645–648.
3 Oude Elferinck, R.P.J., Van Doorn-Van Wakeren, J., Hendriks, T., Strijland, A. and Tager, J. (1986) Eur. J. Biochem. 158, 339–344.
4 Misquith, S., Wattiaux-De Coninck, S. and Wattiaux, R. (1988) Eur. J. Biochem. 174, 691–697.
5 Oude Elferinck, R.P.J., Van Doorn-Van Wakeren, J. (1985) Eur. J. Biochem. 153, 55–63.
6 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617.
7 Jadot, M., Wattiaux-De Coninck, S. and Wattiaux, R. (1989) Biochem. J. 262, 981–984.
8 Wattiaux-De Coninck, S. and Wattiaux, R. (1971) Eur. J. Biochem. 19, 552–556.
9 Wattiaux-De Coninck, S., Dubois, F. and Wattiaux, R. (1977) Biochim. Biophys. Acta, 471, 421–435.
10 Baudhuin, P., Beaufay, H., Rahman-Li, O., Sellinger, Z., Wattiaux, R., Jacques, P. and De Duve, C. (1964) Biochem. J. 92, 179–184.
11 Godelaine, D., Beaufay, H., Wibo, M. and Ravoet, A.M. (1983) J. Cell Biol. 97, 340–350.
12 Wattiaux, R. and De Duve, C. (1956) Biochem. J. 63, 606–608.
13 Mosman, T. (1983) J. Immunol. Methods 65, 55–63.
14 De Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1 974) Biochem. Pharm. 23, 2495–2531.
15 Ignarro, L.J. (1971) Biochem. Pharm. 20, 2847–2860.
16 Smith, R.J., Crawford, S., Gilchrest, H. and Williams, S. (1976) Biochem. Pharm. 25, 2171–2177.
17 Fuchs, R., Male, P. and Mellman, I. (1989) J. Biol. Chem. 264, 2212–2220.
18 Park, R.D. and Storrie, B. (1989) 5th FAOB Congress, Abstracts of papers, p. 227.