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(1) Abbreviated designation of amino acid derivatives and peptides is according to the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (IUPAC Information Bulletin No. 26). In addition, the following abbreviations were used: Prd, pyridine; βHTyr, β-homotyrosine; βHIle, β-homoisoleucine.
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Lysosomotropic Agents. 1. Synthesis and Cytotoxic Action of Lysosomotropic Detergents

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Amines whose pK values lie between about 5 and 9 are lysosomotropic because lysosomes are acidic intracellular compartments. If such amines bear long hydrophobic chains, they become detergents upon protonation inside the lysosomes, rupturing the lysosomal membrane and killing the cell. Six types of lysosomotropic amines have been prepared that all behave in the expected manner. They are cytotoxic to all lysosome-bearing cells but not red blood cells, which lack lysosomes. Their mode of action, the effect of alkyl chain length on activity, and the fact that their cytotoxic action appears only above a threshhold intracellular concentration support the belief that they behave as lysosomotropic detergents. Among the potential applications is cancer chemotherapy.

Lysosomotropic drugs are a promising medical development. To date, the most prominent application of this concept, which was originated by De Duve and his coworkers,[1] has been the treatment of leukemia with DNA complexes of adriamycin and daunorubucin.[2] The toxicity of the complexes is much lower than that of the free drugs, which are released within the target cells only after uptake of the complexes by "piggyback endocytosis", followed by intralysosomal digestion of the DNA. Selectivity greater than that of the free drug is achieved because malignant cells are often more pinocytic than normal ones.[2,3]

This is an important discovery. Nevertheless, it seemed desirable to us to avoid altogether the use of exogenous toxic agents, even as a complex, because decomplexation might not always be limited to the intralysosomal milieu. Therefore, the use of the lysosomal enzymes themselves to kill the target cells was investigated.

Lysosomes are membrane-bound organelles containing a variety of hydrolytic enzymes that would be lethal to the host cell if released within it.[1] The goal, then, was to selectively rupture the lysosomal membrane, leaving the cell membrane intact. A good way to break up phospholipid bilayers is with detergents, but ordinary detergents attack the cell membrane first and, therefore, are indiscriminate as to membrane type. Consequently, it was necessary to develop detergents that acted only on lysosomal membranes and none other.

We took advantage of the fact that intralysosomal pH is usually between 3 and 5, i.e., 2–4 pH units lower than that of the crytosol.[1,4] It has long been recognized that an amine whose basicity is such that it is substantially protonated inside but not outside lysosomes, i.e., whose pK is between about 5 and 9, will be lysosomotropic.[1] If in addition it bears a long hydrophobic chain, it will be a lysosomotropic detergent, accumulating inside lysosomes not only because it is thermodynamically more stable there, but also because ionization inhibits its passing out through the lysosomal membrane. At neutral pH in the cytosol or intercellular fluid the largely un-ionized amine will be simply an oily substance without surface-active properties, but upon ionization within the lysosome it will become a detergent, accumulating with (presumably) its hydrophobic tail buried in the hydrocarbon zone of the bilayer and its hydrophilic protonated amine head group

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Table I. Chain Length vs. Activity[a] of I[b] with MPM[c]

expt no.	control[d]	n = 3	5	8	10	12	16	18
1	5			23	36	52		
2	9	10				92	63	20

[a] Percent LDH release = ±15%, 24 h. LDH (lactate dehydrogenase) is used as a cytoplasmic marker enzyme. [b] Compounds added as 10% Me_2SO solutions, 1 μL/mL, final concentration 100 μg/mL. [c] Thioglycolate stimulated MPM, 2-4 × 10^6 cells/dish, in duplicate. The experimental procedure has already been described.[12] [d] Control = Me_2SO only, 1 μL/mL.

Table II. Comparison of NAG with LDH Release[a] with I[b] and MPM[c]

readout	control[d]	n = 5	8	10	12	14	18
LDH	4	4	7	12	10	5	5
NAG	10	14	19	32	27	25	

[a] Units released into medium, ±15%, 24 h. NAG (*N*-acetyl-β-D-glucosaminidase) is used as a lysosomal marker enzyme. [b] Compounds added as Me_2SO solution, 1 μL/mL. Final concentrations all 0.750 mM (equivalent to 200 μg/mL for n = 12). [c,d] See corresponding footnotes in Table I.

facing the aqueous interior of the lysosome. It would be expected that the membrane will gradually become more fragile, breaking up when some point related to the critical micelle concentration (CMC) of the protonated amine is reached. Release of the lysosomal enzymes into the cytosol and death of the cell would then rapidly ensue.

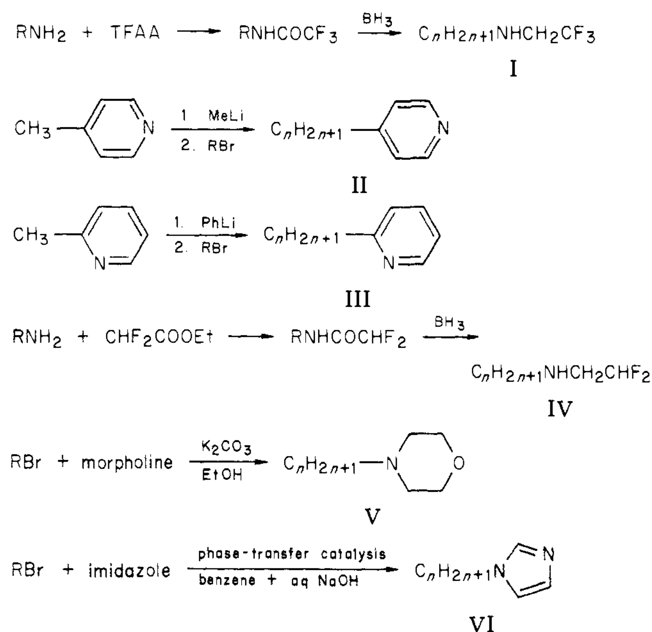
A number of long-chain lysosomotropic amines were prepared in order to test these predictions.

Chemistry. After perusal of the literature, amines of types I–VI (Scheme I) were selected for study because their reported pK values were all in the lysosomotropic range. In some cases, it was found that putting long alkyl chains onto them lowered their pK values appreciably, a phenomenon we cannot explain. However, for an intralysosomal pH of 4.75,[4] even I (n = 12) is fairly lysosomotropic, although lysosomotropism is gradually lost in ths series as n grows larger.

For series I, pK values are for n = 1, 6.0;[5] n = 8, 5.9;[6] n = 12, 5.5; n = 16, 5.2; n = 18, 4.7. For series II, pK values are for n = 1, 6.0;[7] n = 13, 5.9. For series III, pK values are for n = 1, 6.0;[7] n = 13, 5.6. For series IV, pK values are for n = 12, 7.5. For series V, pK values are for n = 1, 7.4;[7] n = 12, 7.6. For series VI, pK values are for n = 1, 7.0;[7] n = 9, 6.6.

Both di- and trifluoroethylamines are most conveniently made by hydride reduction of the corresponding amides. Use of LAH[5] failed in our hands with a trifluoroacetamide (n = 12) but borane[8] worked every time, although extended reaction times were required for the long-chain compounds. For the metalation of picolines, phenyllithium is favored for the 2 isomer,[9] but methyllithium must be used for the 4 isomer[9] because phenyllithium gives some 2-phenylation. Dodecylmorpholine was made by a published procedure.[10] Alkylation of imidazole was most conveniently done in a two-phase system with phase-transfer catalysis.

Scheme I



Results and Discussion

Amine types I–VI, n = 1–18, have been tested against a variety of lysosome-bearing normal, transformed, and malignant cells, and cytotoxicity has been observed in every compound with sufficient chain length for detergency, i.e., $n \geq 8$. Of great importance is the fact that *all six amine classes were nontoxic to all cell types at equivalent or greater concentration when* $n \leq 5$. This shows that the toxicity has a physical rather than chemical origin and is strong evidence for detergency as the mode of action. Specific data supporting lysosomotropic detergency are presented below.

Mouse Peritoneal Macrophages (MPM).[11] This cell type was chosen for initial studies because MPM are pinocytic and rich in lysosomes. The series I was prepared with a larger variety of chain lengths than any other, and the effect of chain length on cytotoxicity is shown in Tables I and II. The compounds are inert with n = 3 or 5. Activity sets in at n = 8, rises at n = 10, and is maximal at n = 10–12. This is in excellent accord with the relationship between chain length and detergency for common detergents (Table III). The activity of I then falls steadily in the series n = 12, 16, 18. This comes about because their pK values, and therefore lysosomotropism, fall (vide infra). The variability in maximum readout from one experiment to another arises because of unavoidable variations in cell density; the sensitivity to lysosomotropic detergents, both in speed of action and degree of cell kill, is inversely related to cell density, even far below cell contact. However, relative values within an experiment are significant.

Types I, II, and III produce the same effect on MPM (25–100 μg/mL): over a course of several hours, depending on concentration and cell density, there is for a time no visible change, then large vacuoles appear within the still

Table III. Chain Length vs. Activity of Common Detergents

type	n = 5	6	8	10	12	13	14	16
$C_nH_{2n+1}COOK$[a]	1.68	0.75	0.20	0.050	0.012	0.0058		
$C_nH_{2n+1}OSO_3Na$[b]				2	11		12	10

[a] Critical micelle concentration (CMC).[13] [b] Units of soil removed from cotton cloth, 30 min, 43 °C, 0.018 m.[14]

intact cells, and soon afterward only cell ghosts remain. Vacuolation, but not cell death, occurs at 5 μg/mL with I (n = 12) and at 10–25 μg/mL with II (n = 13), with cell death occurring at 25 and 50 μg/mL, respectively. In contrast, n-dodecylamine (pK = 10.6),[7] a detergent even at pH 7, kills MPM at 100 μg/mL *without* inducing vacuolation.

This observation suggests that the lysosomotropic compounds act primarily *within* the cell, in contrast with the ordinary detergent $C_{12}H_{25}NH_3^+$, which hits the *outer* membrane first. That the site of intracellular action is indeed the lysosome is indicated by the data in Table II. The release of NAG, a lysosomal enzyme, parallels that of LDH, a cytoplasmic enzyme, even with the weaker detergents in the series. If these were killing the cells elsewhere than through the lysosomes, then LDH, but not NAG, release would be observed.

In other similar experiments with MPM, cytotoxicity was also demonstrated for V (n = 12). The order of activity is V (n = 12) > I (n = 12) > II (n = 13) > III (n = 13).

P-815 Mastocytoma.[15] Cell death in this system is measured as percent ^{51}Cr release from labeled cells.[16] Experiments must be terminated at 5 h, since after that spontaneous release rises too high; thus, the less potent compounds appear to be inactive at this short time. In a typical experiment with all test compounds at 100 μg/mL, percent ^{51}Cr release data were: control (no compound added) 10; V (n = 12) 85; IV (n = 12) 45; I (n = 12) 21; II (n = 13) 10. In other experiments, VI (n = 12) was very active (93%) even at 27 μg/mL. Thus, the activity series toward P-815 is VI > V > IV > I > II (n = 12 or 13), the same as with MPM as far as the data allow. To the extent that comparisons have been made, the order is also the same with all other cell types (vide infra).

Another important observation with P-815 was that the dose–response with lysosomotropic detergents was not smooth, but rather a step function in this limited time assay. Thus, V (n = 12) produced the following percent ^{51}Cr release at 25, 50, 75, and 100 μg/mL, respectively: 13, 14, 15, 85; control 14. Similarly, VI (n = 12) at 1, 3, 9, 27, and 50 μg/mL produced 10, 11, 11, 93, and 94% release; control 11. These data show that cytotoxicity is not proportional to the amount ingested but rather that it has a sudden onset above some critical intracellular concentration. This is just the way detergents behave, in that their action also sets in abruptly only above the CMC.

Erythrocytes (RBC).[17] An important corollary of the theory is that lysosomotropic detergents should not significantly attack cells lacking lysosomes, since they require an acidic compartment to be transformed into detergents. Red blood cells (RBC) are a good test system because they have no lysosomes, and their lysis is easily measured by determining the absorbance of liberated hemoglobin.

In accord with the prediction, ox RBC are not lysed by I, n = 12, at times and concentration sufficient to kill MPM (which succumb within 1 h at low cell density); see Table IV. In contrast, RBC are rapidly lysed by the nonlysosomotropic detergent n-dodecylamine, which requires no acidic compartment for activation. The difference in activity is even greater than it appears from Table IV because the first reading was not taken until 1 h, but it was apparent to the eye that the action of dodecylamine took only a few minutes to be completed. Similar data (not reported) were obtained with rabbit RBC.

Other Cell Types. Both I (n = 12) and II (n = 13) are toxic to Raji cells down to 40 μg/mL in 3 days and also to WI38, MRC5, and RK13 cells.[18] Compound V (n = 12) is potent down to 10 μg/mL against Raji and MRC5, with the order of activity once again V > I > II. Compound I (n = 12) kills HeLa cells at 100 μg/mL in 24 h. Research on other cell systems is continuing in these and other laboratories, to extend the phenomenon and to search for selectivity between normal and malignant cells. That such selectivity is not impossible is suggested by preliminary toxicity data in whole animals.[19] Compounds I (n = 12) and II (n = 13) are nontoxic and V (n = 12) only slightly so in mice at 500 mg/kg (ip, 10-day observation), while IV (n = 12) is toxic at this level but not at 250 mg/kg.

Table IV. Activity of I (n = 12) vs. n-Dodecylamine with RBC

compd	concn, mM	% lysis[a] 1 h	4 h
I (n = 12)	0.375	5.2	6.5
	0.188	4.2	7.3
n-dodecylamine	0.375	100	100
	0.188	100	100
control[b]		1.7	1.8

[a] Hemoglobin released was measured at 541 nm.[20] Aliquots were lysed with Triton X-100 for 100% points. AD values ca. 1% of full scale. See Experimental Section for details. [b] Me_2SO only, 1 μL/mL.

Summary. The evidence taken together supports the belief that our compounds are a new type of cytotoxic agent. That their toxicity arises from physical rather than chemical action is shown by the inertness in all cell systems of I–VI with short alkyl chains. The similarity to the chain length–activity profiles of common detergents and the apparent existence of intracellular threshholds of activity both attest to their action as detergents. Finally, the evidence that they act on lysosomes is that (1) their pK values render them lysosomotropic, (2) they could not act as detergents anywhere but in lysosomes, (3) the pattern of enzyme release during cell killing shows that lysosomes are a primary and not a secondary target, and (4) RBC, which do not have lysosomes, are not lysed.

Experimental Section

NMR spectra were run in $CDCl_3$ on a Varian T-60. Chemical shifts are reported in parts per million from Me_4Si. All compounds gave satisfactory analyses for C, H, N, and F (where applicable). All compounds gave satisfactory mass spectra.

***N*-Dodecyltrifluoroacetamide.** Trifluoroacetic anhydride (21 mL, 0.148 mol) was added over 30 min to a stirred solution of 17.9 g of dodecylamine (0.097 mol) and 13.5 mL of triethylamine (0.097 mol) in CH_2Cl_2 at 0 °C, and the mixture was stirred for another 30 min. It was washed successively with water, 1 M H_3PO_4, and brine; dried over $MgSO_4$; filtered; and evaporated to yield 26.01 g (96%) of product: TLC, single spot on silica gel (10:1 cyclohexane–EtOAc), R_f 0.5; NMR 6.19 (br s, 1 H), 3.30 (q, J = 4 Hz, 2 H), 1.20 (s, 20 H), 0.92 ppm (m, 3 H). The following analogues were prepared similarly: N-propyl, pentyl, octyl, decyl, tetradecyl, hexadecyl, and octadecyl.

***N*-Dodecyl-2,2,2-trifluoroethylamine (I, *n* = 12).** To a solution of 1.12 g of N-dodecyltrifluoroacetamide (4 mM) in 6 mL of THF at 0 °C under N_2 was added 8 mL of BH_3 in THF (8 mM) dropwise. The mixture was refluxed for 16 h, cooled in ice, and carefully treated with 6 mL of concentrated HCl. The THF was distilled off, and solid NaOH was added to the residue at 0 °C until alkaline. The reaction mixture was extracted three times with hexane, and the combined extracts were washed with water, dried over K_2CO_3, filtered, and evaporated to give 2.0 g of crude product. It was chromatographed on silica gel with 10:1 cyclohexane–EtOAc (R_f 0.4), affording 870 mg of oil which was distilled at 135 °C (0.3 Torr) to give 405 mg (38%) of pure I: NMR 3.22 (q, J = 10 Hz, 2 H), 2.79 (m, 2 H), 1.40 (s, 20 H), 0.93 ppm (m, 3 H). Similarly prepared was I, n = 3, 5, 8, 10, 14, 16, and 18, with the exception that the propyl analogue, because of its

volatility, was isolated by extracting with ether after the NaOH addition and precipitated with HCl gas.

4-Tridecylpyridine (II, *n* = 13). The procedure of Osuch and Levine[9] was used: NMR 8.5 (m, 2 H), 7.1 (m, 2 H), 2.6 (t, $J = 6$ Hz, 2 H), 1.25 (s, 22 H), 0.85 ppm (m, 3 H).

2-Tridecylpyridine (III, *n* = 13). A procedure from the same paper[9] was used: NMR 8.55 (m, 1 H), 7.0–7.8 (m, 3 H), 2.8 (t, $J = 6$ Hz, 2 H), 1.25 (s, 22 H), 0.85 ppm (m, 3 H).

***N*-Dodecyldifluoroacetamide.** Ethyl difluoroacetate (24.8 g, 0.2 mol) and dodecylamine (37.0 g, 0.2 mol) were allowed to react for 16 h in 200 mL of MeCN at 25 °C. Evaporation of the solvent left a white wax which was chromatographed on silica gel with 15:1 $CHCl_3$–EtOAc (R_f 0.7), affording 50.2 g (95%) of pure product: NMR 7.2 (br s, 1 H), 5.9 (t, $J = 26$ Hz, 1 H), 3.35 (m, 2 H), 1.3 (s, 20 H), 0.9 ppm (m, 3 H).

***N*-Dodecyl-2,2-difluoroethylamine (IV, *n* = 12).** The above amide (50 g, 0.19 mol) was reduced with borane by the same method as in the preparation of I. The hexane extracts were distilled at 114–152 °C (0.3 Torr), but the product was contaminated with THF decompositon products (NMR). Therefore, the hydrochloride was precipitated with HCl gas in ether, 28.58 g. From this the pure free base was obtained by treatment with aqueous NaOH, extraction with hexane, and distillation: bp 154 °C (0.3 Torr); yield 15.62 g (31%): NMR 5.9 (t of t, $J = 4$ and 56 Hz, 1 H), 3.05 (d of t, $J = 4$ and 16 Hz, 2 H), 2.7 (t, $J = 6$ Hz, 2 H), 1.3 (s, 20 H), 0.9 ppm (m, 3 H). The *N*-propyl analogue was prepared similarly.

***N*-Dodecylmorpholine (V, *n* = 12):**[10] NMR 3.85 (m, 4 H), 2.45 (m, 4 H), 2.4 (m, 2 H), 1.35 (s, 20 H), 0.95 ppm (m, 3 H).

***N*-Dodecylimidazole (VI, *n* = 12).** A stirred mixture of 9.96 g of dodecyl bromide (40 mM), 5.44 g of imidazole (80 mM), 100 mL of 0.97 N NaOH (97 mM), 100 mL of PhH, and 336 mg of Aliquat 336 (1 mM; methyltricaprylylammonium chloride) was refluxed for 23 h. The benzene layer was separated, washed with brine containing a little NaOH, and evaporated. The residue was purified of a little front-running and some origin impurities by quick chromatography on 150 g of silica gel with 1:1 CH_2Cl_2–EtOAc (R_f 0.35), affording 7.48 g of single-spot material which was distilled at ca. 0.5 Torr (bp 144 °C) to give 6.97 g of pure product (74%): NMR 7.5 (s, 1 H), 7.1 (br s, 1 H), 6.95 (br s, 1 H), 3.95 (t, $J = 7$ Hz, 2 H), 1.85 (m, 2 H), 1.3 (s, 18 H), 1.0 ppm (m, 3 H).

Lysis of Erythrocytes (RBC).[17] For each experimental point, 1.75×10^8 ox RBC were suspended in 2.5 mL of medium 199 (Gibco no. 320-1157) and added to a 60-mm Petri dish. Separately, the same medium, 2.5 mL, containing the test compound at twice the final concentration in 5 μL of Me_2SO, was vortexed for 1 min and sonicated for 10 min and then added to the RBC. The cells were incubated at 37 °C. At the prescribed times, two 2-mL aliquots were removed. One aliquot was treated with 0.1% Triton X-100 to obtain 100% lysis, and both aliquots were centrifuged at 2000 rpm in a Beckman Model TJ centrifuge. The optical density of the supernatant fluid was read at 541 nm.[20] All points are the average of three determinations.

The data are the average ± SD. The optical densities obtained were (100% points in parentheses) at 1 h, control (Me_2SO only), 0.012 ± 0 (0.718 ± 0.005); I (*n* = 12), 0.375 mM, 0.037 ± 0.002 (0.708 ± 0.004); I, 0.188 mM, 0.030 ± 0.001 (0.709 ± 0.009); *n*-dodecylamine, 0.375 mM, 0.779 ± 0.001 (0.684 ± 0.001); dodecylamine, 0.188 mM, 0.744 ± 0.004 (0.693 ± 0.001); at 4 h, control, 0.013 ± 0.0003 (0.712 ± 0.012); I, 0.375 mM, 0.045 ± 0.001 (0.688 ± 0.0003); I, 0.188 mM, 0.050 ± 0.001 (0.681 ± 0.002); dodecylamine, 0.375 mM, 0.723 ± 0.007 (0.634 ± 0.005); dodecylamine, 0.188 mM, 0.722 ± 0.003 (0.636 ± 0.002).

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