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THE IN VITRO EFFECTS OF THREE LYSOSOMOTROPIC DETERGENTS AGAINST THREE HUMAN TUMOR CELL LINES

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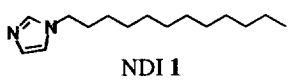
Abstract Three lysosomotropic detergents, N-dodecylimidazole (NDI) **1**, and two new analogues, serine dodecylamide (SDA) **2** and O-methyl serine dodecylamide (MSD) **3**, were tested against three human tumor cell lines in vitro: HCT116, RCA and MCF-7. All three demonstrated dose-related cytotoxicity which, except for a few cases, was also dependent on incubation time, and visually resembled cell death by apoptosis.

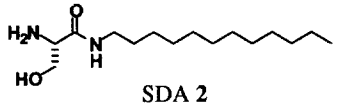
Lysosomotropic detergents,[1] such as N-dodecylimidazole (NDI) **1**, are amphiphilic agents that contain a moderately basic amino group whose pK_a is such that they remain predominantly unprotonated and inert in the cytosol at or near neutral pH, but become protonated and acquire detergent properties inside lysosomes where the pH is maintained at about 4.8. Once protonated they are much less able to cross the lipid bilayer and instead accumulate inside the lysosome until their concentration reaches a point where they are able to dissipate the lysosomal membrane. The release into the cytosol of the contents of the lysosomes, which include a variety of degradative enzymes such as proteases, esterases and endonucleases, usually results in cell death.[2]

It has been shown that certain cell lines are most sensitive to lysosomotropic detergents when they are in a rapidly growing state, and most resistant at higher cell densities.[2,3] For CHO fibroblasts, this resistance has been linked to a growth-dependent increase in P-glycoprotein levels in confluent cells.[4] Other studies have demonstrated the utility of lysosomotropic detergents as selective purging agents for leukemic bone marrow to be used for autologous transplantation based on the reduced susceptibility of cells lacking lysosomes (hematopoietic stem cells).[5] Additional selectivity may arise from an increased sensitivity of undifferentiated cell types (i.e., HL-60) to these agents.[6] Lysosomotropic detergents have also been shown to be potent antimalarials,[7] as well as broad spectrum antifungal agents.[8]

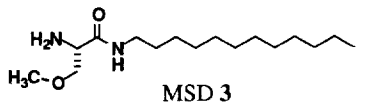
For the present study we prepared two new lysosomotropic detergents, serine dodecylamide (SDA) **2**



NDI **1**



SDA **2**



MSD **3**

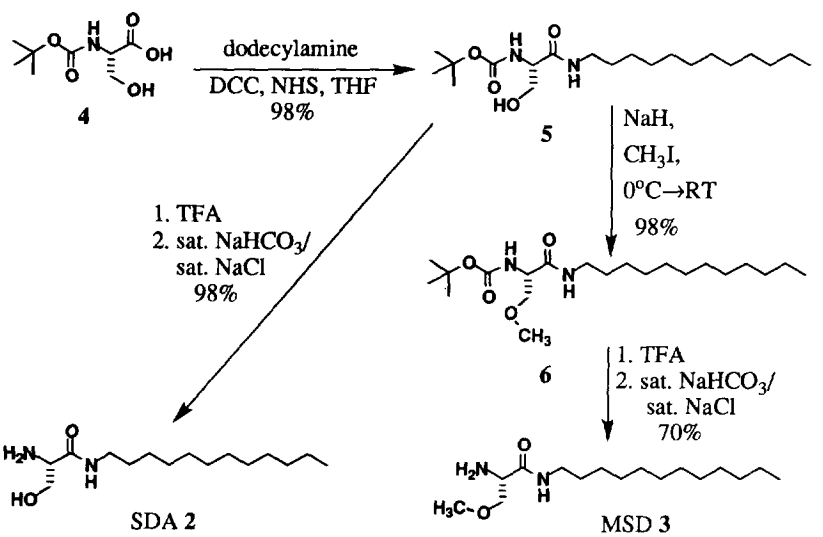
and O-methylserine dodecylamide (MSD) **3**, and examined the effect of these and NDI on three human tumor cell lines: one breast carcinoma line, MCF-7; and two colon carcinoma lines, HCT116 and RCA.

Cytotoxicity Assays The compounds were tested for their ability to inhibit DNA synthesis in three human tumor cell lines: HCT116 and RCA colon carcinomas (provided by Dr. Mike Brattain, Baylor Univ.), and MCF-7 breast carcinoma (ATCC). The compound of interest was diluted to various concentrations with RPMI medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin/streptomycin. The plated cells (10^5 cells/mL) were treated with the lysosomotropic detergent and incubated at 37°C for various times and then washed. They were then labeled with [^{3}H]-thymidine (1 μCurie/well), trypsinized, harvested and counted. One cell line (HCT116) was examined under the microscope to determine the effects of these agents visually.

Results and Discussion Although NDI, as its free base, is one of the most effective lysosomotropic detergents known, its practical use may be limited by a lack of significant aqueous solubility. Successful dissolution involves careful addition of a DMSO solution of NDI to well-stirred aqueous medium. One of our purposes in designing serine-based detergents was to improve solubility as well as to try to increase potency.

The two serine-based detergents were prepared as shown in scheme 1. N-Boc-serine was coupled with n-dodecylamine using DCC/NHS to give amide **5**. For SDA **2** the N-protecting group was removed with trifluoroacetic acid in methylene chloride. The mixture was evaporated and the free base released by treatment of the resulting oil with a 2:1 mixture of sat. sodium bicarbonate and sat. sodium chloride. The product **2** was extracted with 1:1 ether/ethyl acetate and required no further purification after evaporation of the solvents. For MSD **3**, alcohol **5** was deprotonated with sodium hydride in THF and alkylated with methyl iodide. The crude product **6**, which was pure by NMR, was deprotected as described above for **5**, giving MSD **3** after silica gel chromatography, eluting with 4% methanol/ethyl acetate.[9]

Scheme 1



We expected the pK_a of the free amino groups of SDA and MSD to be within the higher range of known lysosomotropic detergents (7-7.5), based on literature values for serine and simple dipeptides.[10] However, the pK_a of MSD, measured in water, were found to be 5.9. The values for SDA and NDI could not be determined with accuracy, in the first case because the compound began to crystallize out of solution at pH 5.3, and in the second because of oiling out at pH 5.9. From the pH of their hydrochloride salts in water the pK_as of SDA and NDI were estimated to be 5.8 and 6.3, respectively.[11] The basicities of most lysosomotropic detergents are depressed in comparison with their parent bases, and the effect is normally dependent on alkyl chain length.[1] The pK_a of N-methylimidazole (7.0), for example, is reduced to 6.6 for N-nonylimidazole,[1] and to 6.3 for NDI. The change of ca. 1.4 pK_a units from serine amide (7.3) to MSD and SDA (5.8-5.9) is twice that for the imidazoles and is unexpectedly large. To determine whether SDA and MSD have detergent properties at lysosomal, but not at neutral, pH they were dissolved in methylene chloride (10 mg/mL) and sonicated in a two-phase system with chloride-containing phosphate buffer solutions of pH 4.8 and 7.0. In both cases sonication at pH 4.8 produced thick, white suspensions that did not resolve soon afterwards, while at pH 7.0 the mixtures separated cleanly into two layers, demonstrating selective detergency at lysosomal pH. Neither compound was soluble as its free base in water but we found that the hydrochloride salts dissolved well.[12] Indeed, concentrated aqueous solutions (≥10 mg/mL) of these agents as their HCl salts provided an excellent vehicle for dispersion in culture medium without the need for DMSO. In this way, homogeneous solutions of MSD•HCl in medium could be prepared up to at least 500 μg/mL (no limit was reached). Similarly, NDI•HCl could be dissolved up to ca. 350 μg/mL before cloudiness signaled oiling out. SDA•HCl began to precipitate out of medium at >50 μg/mL. It is interesting that the most polar compound was least soluble, but this behavior is not unknown in peptide chemistry and probably results from methyl capping of a hydrogen bonding source in MSD that in SDA interacts to form a very stable solid.[13] Moreover, from the pK_a experiments it was clear that MSD was also the most soluble agent in neutral and basic solution (> 5 mg/mL up to pH 11).

The biological activities of compounds **1-3** against three tumor cell lines are shown in figures 1-3. NDI was clearly the most potent in all cases. At the 24 hr. exposure time NDI was so active that no IC_{50} was reached in this study, except against the HCT116 cells, which showed a special resistance to NDI, in comparison with the other cell lines, that SDA and MSD did not. At 30 min. exposure times IC_{50}s for NDI were typically 8-10 μM (1.9-2.4 μg/mL) with potency increasing on going to 2 hr. and then again to 24 hr. (except against HCT116 where it showed no improvement). IC_{50}s for the serine detergents were typically in the 70-100 μM (ca. 19-29 μg/mL) range at 30 min. for all cell lines. RCA cells, treated with SDA, showed a striking resistance to prolonged exposure, while the others were from five to ten-fold more susceptible to SDA and MSD at the 24 hr. incubation. Microscopic examination of HCT116 cells treated with all three detergents revealed effects similar to those reported elsewhere: a granular appearance followed by vacuolization of much of the cytosol and then blebbing of the membrane preceding cell death.[2] In addition to these visible characteristics, a study by Bradley and co-workers of the effects of NDI on BHK and CHO cells showed double strand DNA lesions and chromosomal aberrations at cytotoxic concentrations induced apparently by lysosomal enzymes diffusing to the nucleus from ruptured lysosomes.[14] Blebbing and DNA digestion are also characteristic of apoptotic cell death,[15] and, indeed, inhibition of serine proteases has been shown to prevent apoptosis.[16]

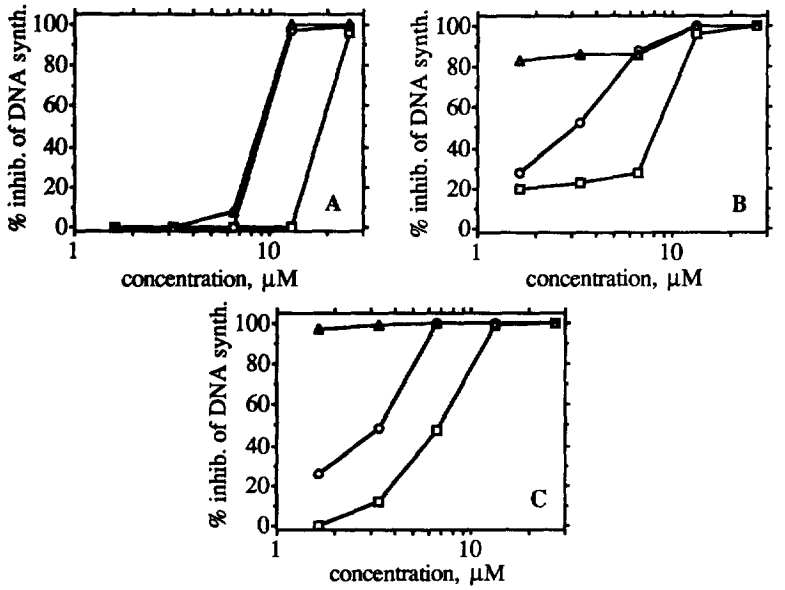


Figure 1. Inhibition of DNA synthesis of tumor cell lines: **A**, HCT116; **B**, RCA; **C**, MCF-7; exposed to different concentrations of N-dodecylimidazole (NDI) **1** for □, 30 min.; O, 2 hr.; △, 24 hr.

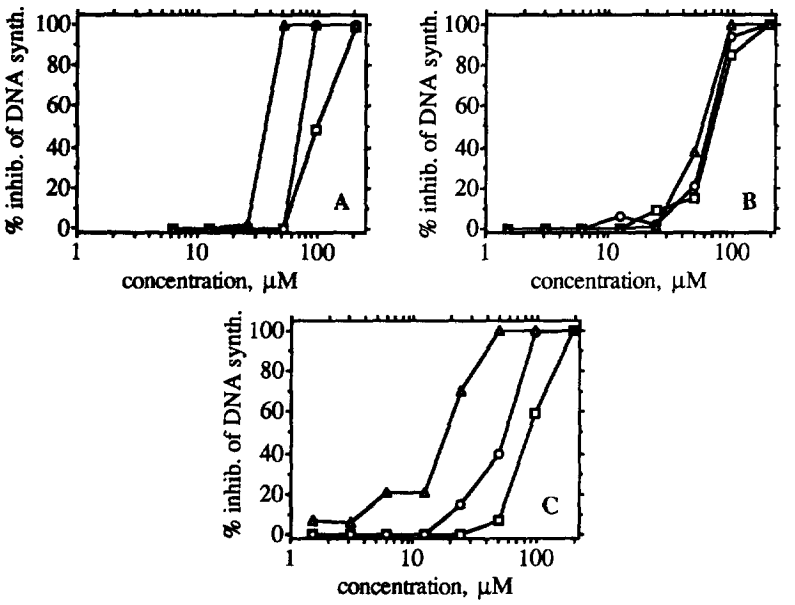


Figure 2. Inhibition of DNA synthesis of tumor cell lines: **A**, HCT116; **B**, RCA; **C**, MCF-7; exposed to different concentrations of serine dodecylamide (SDA) **2** for □, 30 min.; O, 2 hr.; △, 24 hr.

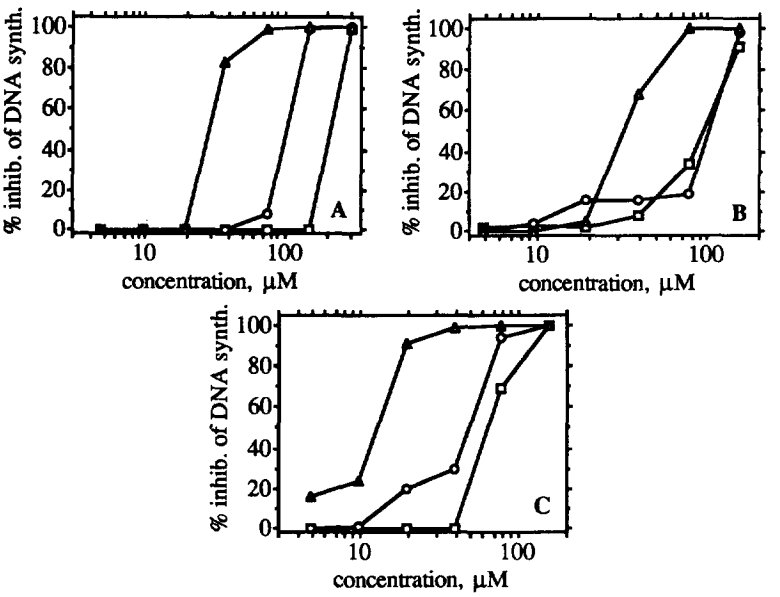


Figure 3. Inhibition of DNA synthesis of tumor cell lines: **A**, HCT116; **B**, RCA; **C**, MCF-7; exposed to different concentrations of O-methylserine dodecylamide (MSD) **3** for □, 30 min.; O, 2 hr.; △, 24 hr.

At least two factors may be involved in the greater potency of NDI over the serine detergents. First, the pK_as of MSD and SDA (5.8-5.9) are lower than those of NDI (6.3) as well as the other most potent classes of lysosomotropic detergents, morpholines and difluoroethylamines (7.4-7.5).[1] This means that more of the serine-detergents must enter the lysosome before the concentration of protonated detergent is high enough to lyse its membrane. A second factor is the ability of the detergent free base to cross two bilayer membranes, cell and lysosomal; which it must do before it can exert its effect. It has been shown that peptide drugs pass through a layer of intestinal mucosal cells with increasing ease as amide protons are systematically replaced by methyl groups.[17] The explanation, which relates to the difficulty of desolvation of NH versus NCH_3 that must occur before the compound can pass through the cell membrane, could apply here as well. NDI in its neutral form is not a hydrogen bond donor while SDA contains four donatable hydrogen bonds and MSD three. Therefore, cell and lysosomal penetration of the serine detergents may suffer because of the very features that are designed to make them more soluble and easier to deliver. In that sense, NDI may represent the ideal compromise, especially since we have also shown that it is much easier to dissolve as its hydrochloride salt.

Abbreviations used: ATCC, American Type Culture Collection; BHK, baby hamster kidney; Boc, t-butyloxycarbonyl; CHO, Chinese hamster ovary; DCC, dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; MSD, O-methyl serine dodecylamide; NDI, N-dodecylimidazole; NHS, N-hydroxysuccinimide; SDA, serine dodecylamide; THF, tetrahydrofuran.

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9. For **2**: ^{1}H-NMR ($CDCl_3$) δ 0.83 (3H, t, CH_3), 1.21 (18H, brs, CH_2), 1.47 (2H, quint., CH_2), 2.20 (3H, br, NH_2 & OH), 3.19 (2H, m, NCH_2), 3.39 (1H, m, CH), 3.66 & 3.78 (each 1H, m, OCH_2), 7.42 (1H, br, NH); ^{13}C-NMR ($CDCl_3$) δ 174.2 (CO), 66.2 (OCH_2), 55.9 (CH), 39.5 (NCH_2), 32.0, 29.7, 27.5, 22.6 & 14.2 (CH_2); Mass spec. 273 $(MH)^+$; Anal. calc. for $C_{15}H_{32}N_2O_2$: C-66.13, H-11.84 N-10.28 found: C-65.88, H-12.30, N-10.25.
For **3**: ^{1}H-NMR ($CDCl_3$) δ 0.82 (3H, t, CH_3), 1.21 (18H, brs, CH_2), 1.46 (2H, quint., CH_2), 1.62 (2H, brs, NH_2), 3.20 (2H, m, NCH_2), 3.32 (3H, s, OCH_3), 3.53 (3H, m, CH & OCH_2), 7.37 (1H, br, NH); ^{13}C-NMR ($CDCl_3$) δ 173.4 (CO), 74.9 (OCH_2), 59.3 (OCH_3), 55.1 (CH), 39.7 (NCH_2), 32.2, 29.6, 27.3, 22.8 & 14.4 (CH_2); Mass spec. 287 $(MH)^+$; Anal. calc. for $C_{16}H_{34}N_2O_2$: C-67.09, H-11.96, N-9.78; found: C-66.63, H-12.01, N-9.68.
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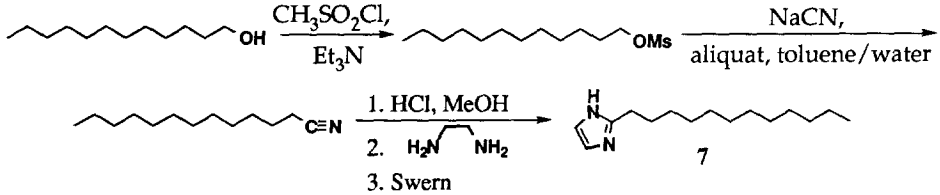
11. The pK_a values were estimated using the following equation for weak acids:

$$\frac{[H]^2}{[HA]} = K_a$$

The value for MSD obtained using it was the same as that found by titration.
12. The hydrochloride salts were prepared by treatment of the free base in chloroform with 1M HCl in ether (1.2 equiv.) and evaporation of the solvents followed by flushing with methylene chloride and drying in vacuo.
13. More recently we prepared 2-dodecylimidazole **7** as shown below. It begins to precipitate out of RPMI medium at 20 μg/mL. This can be improved to 40 μg/mL by first making a complex of **7** with hydroxypropylated cyclodextrin.



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