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Hemolysis of Erythrocytes and Erythrocyte Membrane Fluidity Changes by New Lysosomotropic Compounds

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This work contains the results of studies on the influence of newly synthesized lysosomotropic substances (lysosomotropes) on human erythrocytes. Six homologous series of the compounds differing in the alkyl chain length and counterions were studied. They were found to hemolyse erythrocytes and to change their osmotic resistance. The observed hemolytic effects were dependent both on the compound's structure (polar head dimension and alkyl chain length of compound) and its form (the kind of the counterion). In parallel, the influence of lysosomotropes on fluidity of the erythrocyte membrane was studied. Three different fluorescent probes were used; 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan). Their anisotropy (DPH and TMA-DPH) or general polarization (laurdan) values after incorporation into ghost erythrocyte membranes were measured. The results obtained show that fluidity changes accompanied the effects observed in hemolytic experiments both quantitatively and qualitatively.

KEY WORDS: Hemolytic activity; osmotic resistance; membrane fluidity.

INTRODUCTION

Lysosomes are small cell organelles filled with hydrolytic enzymes for intracellular digestion and surrounded by membranes separating them from the rest of the cell and thus protecting against the action of cell components and *vice versa*. Their interior acidity is kept at about pH 5 that ensures optimal activities of enzymes. Due to this acidity, many compounds, including drugs, may enter and accumulate in lysosomes in concentrations several hundreds higher than those in extracellular fluid [1–3]. When accumulated in lysosomes in high enough concentrations they may break the lysosomal membrane which, following uncontrollable leakage, may lead to cell death [3]. Some of them remain in lysosomes and are expelled out of cell before reaching the target and exerting their therapeutic action [4]. Such phenomenon is called multidrug resistance (MDR) and is, at least partially, the result of a protonation of acidic drugs that significantly limits their permeation through lysosomal membranes [3,4]. In some cases it is useful, especially if a compound is to act inside a lysosome. The MDR effect is just a contrast example. To act one or another way the compounds must fulfill some specific requirements described in detail by de Duve *et al.* [2].

It was shown that there are many compounds that, after accumulating in lysosomes, start behaving as detergents. They are amphiphiles and contain at least one basic nitrogen [4,5]. Some of them were checked for biological activity and found efficiently killing tumor cells and microorganisms [3,5–7] and some were applied as anti-MDR drugs [4,8].

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Recently, we have studied the activity of such detergents on the yeast *Saccharomyces cerevisiae* and yeast-like organisms and found that it was dependent on the size of the polar head group and alkyl chain length of the compounds [9–11] and that they influenced MDR transporters [12]. Additional experiments were performed in order to determine hemolytic efficiencies of the compounds and it was shown that these efficiencies followed the observed inhibitory activities against *Saccharomyces cerevisiae* [11]. They also agreed quite well with pK_a values of individual compounds [13]. These results justify the use of erythrocytes as biological models for studying the activity of various compounds including lysosomotropic ones. Also, they are commonly regarded as paradigmatic and well-established models [14].

In this work we present the results of studies on new lysosomotropic compounds. Their hemolytic efficiency was measured, the influence on osmotic resistance of erythrocytes and on fluidity of erythrocyte ghosts. The assumption was that the results obtained may prove to be good indicators of biological activity of the compounds studied. It is worth mentioning that the compounds belong to the so-called "soft" lysosomotropic compounds that easily degrade in the cell to nontoxic metabolites.

EXPERIMENTALS

New lysosomotropic substances were derived by quaternization of the appropriate tertiary amino compounds with methyl bromide, dimethyl sulphate or *n*-alkyl chloromethyl ethers. They were alkyl *N,N*-dimethylalaninates methobromides (DMALM-*n*), alkyl *N,N*-dimethylglycinates methobromides (DMGM-*n*, fatty acids *N,N*-dimethylaminoethylesters methobromides (DMM-*n*), fatty acids *N*-methyl-*N*-alkoxymethylaminoethylesters metochlorides (DME$_x$-11), fatty acids *N,N*-dimethylaminoethylesters methylsulphates (DMMS-*n*) and alkyl *N*-methyl-*N*-alkoxymethylglycinate methochloride (DMGE$_{10}$-10). Their structures are presented in Fig. 1. Purity of the compounds was checked by ^{1}H-NMR (Brucker instrument 300 MHz, $CDCl_3$, TMS as internal standard). The compounds synthesis is described elsewhere [9,15,16].

The experiments were conducted on fresh, heparinized pig blood. Erythrocytes were washed in the experiments performed by an isotonic phosphate solution of pH 7.4 (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.80 mM $Na_2HPO_4{\cdot}2H_2O$, 1.80 mM $Na_2H_2PO_4{\cdot}H_2O$). On removal from the plasma, the erythrocytes were washed four times in phosphate buffer and then incubated in the same solution but containing proper amounts of the compounds studied. The modification was conducted at 37°C for 0.5 hr, each sample containing 10 mL of erythrocyte suspension of 2% hematocrit, stirred continuously. After modification 1 mL samples were taken, centrifuged and the supernatant assayed for hemoglobin content using a spectrophotometer (Spekol 11, Carl Zeiss, Jena) at 540 nm wavelength. Hemoglobin concentration in the supernatant, expressed as percentage of hemoglobin concentration in the supernatant of totally hemolyzed cells, was assumed as the measure of the extent of hemolysis.

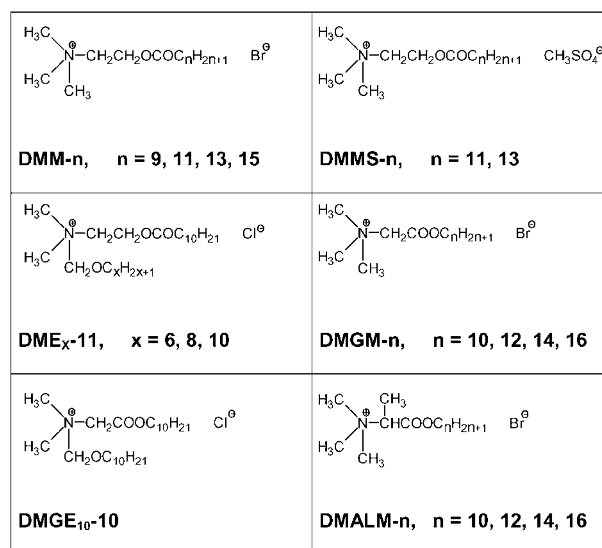

Fig. 1. Chemical structure of studied compounds.

Osmotic resistance of erythrocytes modified for 0.5 hr in solutions containing 10 μM lysosomotropic substances were measured. The hematocrit was 2%. The modified erythrocytes were then suspended in hypotonic solutions of NaCl at 0.4 to 0.9% and hemolysis was measured as described.

Fluidity experiments were done on erythrocyte ghosts, which were subjected to the action of the compounds studied. Three fluorescent probes were used; 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexa-triene, p-toluenesulfonate (TMA-DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan), all at 10 μM concentration. Erythrocytes were incubated with the probes for 24 hr at 23°C. The concentration of compounds in samples was 25 μM. Protein concentration in the samples was ca. 100 μg/mL. The measurements were performed with an SFM 25 spectrofluorometer (KONTRON, Zurich, Switzerland) at 20°C. In the case of DPH and TMA-DPH the excitation and emission wavelengths were 354 and 429 nm, respectively; excitation wave-length for laurdan was 360 nm and emission

wave-lengths were 440 (in the gel phase) and 490 nm (in the liquid phase).

Fluorescence anisotropy A was calculated according to the formula [17]:

$$A = \frac{I_{\mathrm{II}} - \mathrm{GI}_{\perp}}{I_{\mathrm{II}} + 2\mathrm{GI}_{\perp}} \quad (1)$$

where I_{II} and $I_{\perp}$ are vertical and horizontal polarized fluorescence emission intensities, respectively, and G is the grating correction factor.

The GP (general polarization) values were calculated according to the formula [14]:

$$\mathrm{GP} = \frac{I_{\mathrm{b}} - I_{\mathrm{r}}}{I_{\mathrm{b}} + I_{\mathrm{r}}} \quad (2)$$

where I_b and I_r are the corresponding emission intensities at 440 and 490 nm (the blue and red edges of the spectrum) and excitation wave-length was 360 nm.

All reagents used were of analytical grade. The fluorescent probes were purchased from Molecular Probes Inc. (Eugene, USA).

RESULTS AND DISCUSSION

The results of the studies conducted are presented in Table I. It contains the concentrations of the lysosomotropic compounds studied that induce 50 and 100% hemolysis (C_{50} and C_{100}), percent concentration of hypotonic sodium chloride solutions (C_{NaCl}) inducing 50% hemolysis of erythrocytes, coefficients of anisotropy (A) of fluorescent probes DPH and TMA-DPH and values of general polarization (GP) of the fluorescent probe laurdan, all incorporated in erythrocyte ghosts membranes at 10 μM concentrations.

As expected, hemolytic efficiencies of the compounds of particular series depended on the number of lipophilic groups a compound had. The dependencies were not linear and tended to reach a maximum. This was especially evident in the case of DMM-n compounds. However, the comparison of the efficiency of DMM-11 with efficiencies of DME_x-11, compounds with additional side alkyl chains of different lengths ($x = 6$, 8, and 10; see Fig. 1), shows that differences in the lipophilicity between a particular series of compounds is not the only factor deciding the hemolytic efficiencies of the compounds studied. There are two factors that make the compounds DME_x-11 worse hemolysing agents: bigger dimensions making it more difficult to incorporate into the erythrocyte membrane and the kind of their counterion. While DMM-n compounds were used as bromides, DME_s-11 were chlorides. It is known that compounds with different counterions interact with biological and model membranes with different efficiency. The bromide and chloride ions belong to so-called chaotropic ions affecting (increasing)

Table I. Concentrations of the Compounds Studied Inducing 50% (C_{50}) and 100% Hemolysis (C_{100}) of Erythrocytes, NaCl Concentrations Inducing 50% Hemolysis (C_{NaCl}) and Values of the Anisotropy Coefficients Measured for Probes 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-Toluenesulfonate ($A_{TMA-DPH}$) and 1,6-Diphenyl-1,3,5-hexatriene (A_{DPH}) and Values of Great Polarization measured for Probe Laurdan (GP)

Compound	C_{50} (mM)	C_{100} (mM)	C_{NaCl} (%)	$A_{TMA-DPH}$ 10 μM	A_{DPH} 10 μM	GP 10 μM
DMALM–10	1.0	4.00	0.70	0.267	0.284	0.497
DMALM–12	0.23	0.40	0.71	0.246	0.268	0.432
DMALM–14	0.054	0.10	0.74	0.231	0.242	0.425
DMALM–16	0.055	0.09	0.76	0.219	0.232	0.412
DMGM–10	0.57	1.00	0.69	0.256	0.271	0.484
DMGM - 12	0.27	0.60	0.70	0.239	0.248	0.463
DMGM–14	0.15	0.45	0.74	0.211	0.226	0.408
DMGM–16	0.18	0.35	0.75	0.192	0.218	0.399
DMM–9	2.70	4.00	0.68	0.267	0.281	0.488
DMM–11	0.24	0.45	0.72	0.252	0.272	0.487
DMM–13	0.08	0.20	0.74	0.241	0.267	0.467
DMM–15	0.06	0.20	0.74	0.233	0.245	0.422
DME_6–11	>5.00	>5.00	0.68	0.269	0.263	0.491
DME_8–11	>2.50	>2.50	0.68	0.174	0.208	0.499
DME_{10}–11	0.35	2.00	0.72	0.155	0.165	0.453
DMMS–11	0.24	0.50	0.69	0.258	0.277	0.495
DMMS–13	0.09	0.20	0.70	0.233	0.249	0.498
DMGE_{10}–10	>5.0	>5.00	0.66	0.202	0.228	0.488
Control			0.68	0.269	0.286	0.494

Note. Standard deviation for C_{50}, C_{100} and C_{NaCl} determination did not exceed 5%, and for A and GP did not exceed 4%.

solute solubility. The effect brought by bromides is significantly stronger than that observed in the presence of chlorides [18–20] and is seen as a very intensive interaction of bromide compounds with membranes. Even the high lipophilicity of DME_x-11 compounds, roughly twice that of corresponding DMM-*n* compounds if one apply simple criterion of counting the lipophilic groups, do not compensate the counterion effect which is additionally enhanced by smaller dimensions of DMM-*n* compounds. The same approach can be applied to $DMGE_{10}$-10 compound that was used as chloride, and its hemolytic potency was found to be weak. The DMMS-*n* compounds activities are very similar to those found for DMM-*n* compounds. Because these compounds differ only in counterions, the conclusion must be that bromide and methyl sulphate counterions are facilitating the interaction of these series in the same degree.

Hemolytic activity of compounds is accompanied by changes in osmotic resistance of erythrocytes. The more hemolytically active compounds, the greater the changes they induce in osmotic resistance, the measure of which being sodium chloride concentration inducing 50% hemolysis. However, these changes were relatively small and no differences between the values of C_{NaCl} for corresponding compounds of a particular series were found.

Thus, the hemolytic experiments have shown that the efficiency of the interaction of lysosomotropic compounds with the erythrocyte membrane depended on their structural features and the kind of their counterion, while the measurements of osmotic resistance seem to indicate that it depends only on lipophilicity of the compound. To elucidate a possible mechanism of the interaction, fluorescence measurements were performed. Three different fluorescent probes were used to detect changes in erythrocyte ghosts membrane fluidity. They were loosely constrained in the lipid bilayer: DPH, anchored to the bilayer surface, TMA-DPH and water-sensitive laurdan. Since each of them preferentially localized in membrane they were expected to monitor fluidity changes to a large extent.

The results obtained show that there are no differences in the values of anisotropy coefficients (A) determined with the use of probes DPH and TMA-DPH for the same compounds. Under the experimental conditions both the probes exhibited the same sensitivity. It must be emphasized that the greatest changes in A were found for the compounds with substituted side alkyl chains (DMM_x-11 and $DMGE_{10}$-10). These results, when confronted with obtained in hemolytic experiments, suggest that such big molecules strongly disturb membrane structure, although their hemolytic efficiency is not very big. It seems that possible structure disturbance is limited to the neighborhood of membrane surface and compensate the fact that their counterions (chloride) do not facilitate incorporation into membranes. This is especially well seen when one compares DMALM-10 and DMGM-10 with $DMGE_{10}$-10, i.e., compounds with same alkyl chain. Fluorescence anisotropies found for these compounds are 0.267, 0.256, and 0.202 (TMA-DPH probe), respectively.

The situation is not so complex in the case of single chain compounds. The changes in fluidity are similar in the case of corresponding compounds and this is true for both bromides and methylene sulfates. Some differences appear for compounds of longer alkyl chains ($n = 14$ and 16) and are probably the result of substitution of an additional methylene group at the beginning of the alkyl chain of DMALM-*n* compounds (see Fig. 1), which makes them more hemolytic than DMGM-*n*. These latter, not possessing that group, probably incorporated deeper into the membrane bilayer as evidenced by the fluorescence parameters measured.

The lipophilic probe laurdan was used to determine the general polarization. The results obtained show that the GP parameter changed similar to A in the case of incorporated single-alkyl-chain bromide compounds. It was not the case when chlorides (DME_x-11 and $DMGE_{10}$-10) and methylene sulfates (DMMS-*n*) were studied. The observed changes in the great polarization were significantly lower for these compounds, which suggests smaller sensitivity of laurdan to changes induced at the membrane surface.

All fluidity experiments were performed at concentrations of the lysosomotropic compounds far below their concentrations causing 50% hemolysis of erythrocytes (25 μM). To determine how the fluorescence parameters vary with increasing concentrations of the compounds, some additional experiments were done with chosen most active lysosomotropes (DME_{10}-11, DMGM-16, and DMM-15). In each experiment, different probe was used and concentrations of the compounds were 30 μM, 40 μM, and 50 μM, respectively. It was found that fluorescence parameters changed about 10% in the case of highest concentration for TMA-DPH and DPH probes and about 5% for laurdan. The results obtained after additional 24 h incubation were not different. For instance, fluorescence anisotropies (TMA-DPH probe) found for specified concentrations of DME_{10}-11 were 0.160, 0.149, and 0.138 (24 hr incubation) and 0.155, 0.139, and 0.140 (48 hr incubation); control was 0.269. The corresponding changes of GP parameter found for DMM-15 (laurdan) were 0.424, 0.410 and 0.402 (24 h) and 0.418, 0.405 and 0.407 (48 hr). Similar qualitative results were obtained in the case of DPH probe used in experiments with DMGM-16. Such results prove validity of the results obtained.

The fluorimetric experiments clearly showed that the perturbation caused in membranes by the lysosomotropic compounds incorporated into them was smallest in the polar region of membranes and increased with depth of incorporation.

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