Hemolytic Activity of Aminoethyl-dodecanoates

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Z. Naturforsch. **53c,** 101–106 (1998); received November 3/December 1, 1997

Lysosomotropic Substances, Aminoesters of Fatty Acids, Erythrocyte Membrane, Hemolytic Activity, Fluidity

The effect of new lysosomotropic compounds on red blood cell hemolysis and erythrocyte membrane fluidity has been investigated. In earlier studies it was shown that the compounds inhibit the growth of yeast and plasma membrane H^+-ATPase activity. The study was performed with eight aminoethyl esters of lauric acid variously substituted at nitrogen atom. Esters of dodecanoic acid were chosen for study because at that chain length dimethylaminoethyl esters showed maximum activity. The hemolytic activity of the substances studied exhibits diversified activity in their interaction with the erythrocyte membrane: they differ in hemolytic activity and affect membrane fluidity differently. Erythrocyte membrane fluidity changes under the effect of those compounds which possess highest hemolytic activity. The hemolytic activity of the aminoesters investigated was found to follow a sequence that depended on basicity (i.e. ability of the protonated form formation) of the compound and its polar head group size. The most active are the compounds that possess not more than four carbon atoms substituted at nitrogen and highest pK_a value.

Introduction

Lysosomotropic substances are weak organic bases that can pass through biological membranes and concentrate in the cell fragments of low pH (lysosomes, vacuoles); the pKa values of such lysosomotropic amines lie in the range of 5 to 9 (de Duve *et al.,* 1974). After trapping a proton from the milieu they acquire a charge on nitrogen atom. Those of the compounds that have long alkyl chain behave – after protonation – as typical cationic detergents and interact with the membrane causing a change in its structure and function and at higher concentrations even causing its destruction (Boyer *et al.,* 1993). The biological activity of lysosomotropic compounds depends on their basicity and molecular structure (Dubowchik *et al.,* 1995). Application to tumour cells control was demonstrated because of lower (of about 0.5 unit) pH in lysosomes of tumour cells than in normal tissues (Wike-Hooley *et al.,* 1984). Some lysosomotropic substances can be applied as anti-multi drug resistance (MDR) drugs (Jaffrezau and Laurent, 1993; Dubowchik *et al.,* 1994).

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During our research on new biologically active compounds we turn our attention to the group of amino-ethyl esters of fatty acids as „soft" (i.e. easly degradable in the cell to nontoxic metabolites) lysosomotropic agents. Such compounds could be useful as new fungicides and anti-MDR drugs. In earlier studies we have shown that 2-dimethylaminoethyl esters of fatty acids (DM-n) inhibit the growth of yeast *Saccharomyces cerevisiae,* minimal inhibitory concentration – (MIC) about 10 μM (Lachowicz *et al.,* 1996) and yeast-like organisms (Bień *et al.,* 1995) and that the activity depends on hydrophobic chain length with maximum at 11–13 carbon atoms, which is obviously observed for cationic detergents. The inhibition of the yeast plasma membrane H^+-ATPase by aminoethyl-alkanecarboxylates (Witek *et al.,* 1997) and their interaction with a pleiotropic drug resistance network of the yeast (Witek *et al.,* in press) was also found. Determination of the mechanism of biological action for the synthesized amioesters can be a very useful tool for desingning molecules with high biological activity.

The biological activity of lysosomotropic amines, like DM-n compounds, may depend both on their basicity and structure. The basicity and other properties of lysosomotropic aminoesters could be strongly dependent on substitution at

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nitrogen atom of the amino group. In the work presented, the effect of aminoester head-group structure on interaction with the erythrocyte membrane, which was chosen as a relatively simple and very useful model of the biological membrane, was studied. Esters of dodecanoic acid were chosen for study because at that chain length dimethylaminoethyl esters showed maximum activity. The study was performed with 8 aminoesters variously substituted at the amino group; the protonated form of the esters was used, i.e. hydrochlorides of aminoethyldodecanoates. The hemolytic activity of the compounds and the effect on erythrocyte membrane fluidity were determined.

Materials and Methods

New lysosomotropic aminoethyl dodecanoates, of structure shown in Fig. 1, were synthesized in our laboratory by acylation of proper aminoethanol with dodecanoyl chloride (Łuczynski *et al.* 1997). Hydrochlorides of aminoesters were used in the investigation, after being purified to analytical grade by crystallization (*n*-hexane/chloroform). Purity and structure of the compounds were confirmed by the ^{1}H-NMR spectra.

Hemolytic studies

The experiments were conducted on fresh, heparinized pig blood. For washing the erythrocytes and in the experiments performed an isotonic phosphate solution of pH 7.4 (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.79 mM Na_2HPO_4 $2H_2O$, 1.80 mM $Na_2H_2PO_4$ H_2O) was used. Upon removing from 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 h, each sample containing 10 ml of erythrocyte suspension of 2%, 4%, 6% and 8% 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 (Hamasaki *et al.*, 1995; Boyer *et al.*, 1993). 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.

Fluorimetric studies

Erythrocytes were prepared for modification as in the hemolytic experiments. The modification with the substances studied lasted for 0.5 h at 37 °C at 2% hematocrit. After the end of modification the modifier was removed and 5 ml samples were prepared for fluorimetric measurements, the final hematocrit being 0.05%. The modified erythrocytes in the samples were labelled with the fluorescent probe TMA-DPH at 10^{-6} M concentration. The measurements were performed with a SFM 25 spectrofluorimeter (KONTRON). On the basis of fluorescence intensity measurements the polarization coefficient was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz, 1988):

$$P = \frac{I_{II} - GI_{\perp}}{I_{II} + GI_{\perp}}$$

where I_{II}-intensity of fluorescence emitted in direction parallel to the polarization plane of the exciting light, $I_{\perp}$-intensity of fluorescence emitted in direction perpendicular to the polarization plane of the exciting light, G-diffraction constant, dependent on wavelength.

Results

The studies performed are concerned with the lysosomotropic compounds shown in Fig. 1. The effect of those substances on red blood cell hemolysis and erythrocyte membrane fluidity has been investigated. In the experiments the hemolytic activity of the compounds and also concentration ranges of their hemolytic activity for four hematocrits were determined. The hemolytic concentrations were found from the relationship between percentage of hemolyzed cells and modifier concentration for the following concentrations of erythrocytes in the solution: 2%, 4%, 6% and 8%. Such relations, in the form of sigmoidal curves, are shown in Fig. 2 for compound DM-11; the four hemolytic curves in the figure correspond to four different hematocrits. It is known (Kondo, 1976) that concentration of the compound that causes hemolysis increases linearly with the number of erythrocytes in the suspension, and thus with increased

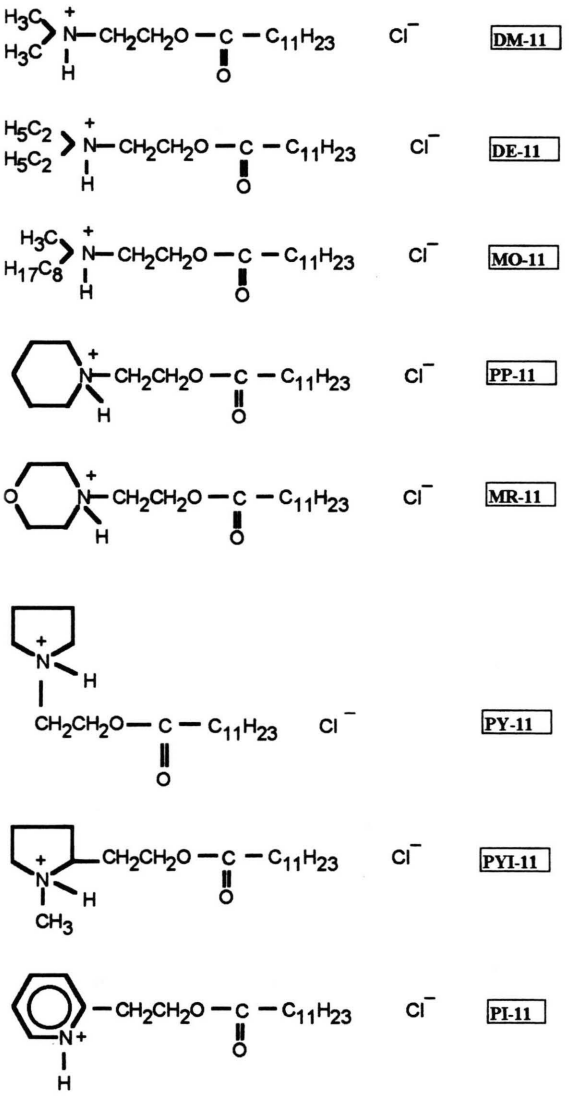

Fig. 1. The structure of lysosomotropic substances.

hematocrit the curves we get are shifted towards higher concentrations. The results of the studies can be presented as linear relations between compound and cell concentrations for 50% and 100% cell hemolysis (as shown in Fig. 2) For 50% hemolysis and all the compounds the dependence is shown in Fig. 3A, whereas for 100% hemolysis in Fig. 3B. From the plots the compound concentrations that cause 50% and 100% hemolysis for any hematocrit can be determined. The bar graphs of Fig. 4 (A, B) represent compound concentrations which cause 50% (Fig. 4A) and 100% hemolysis

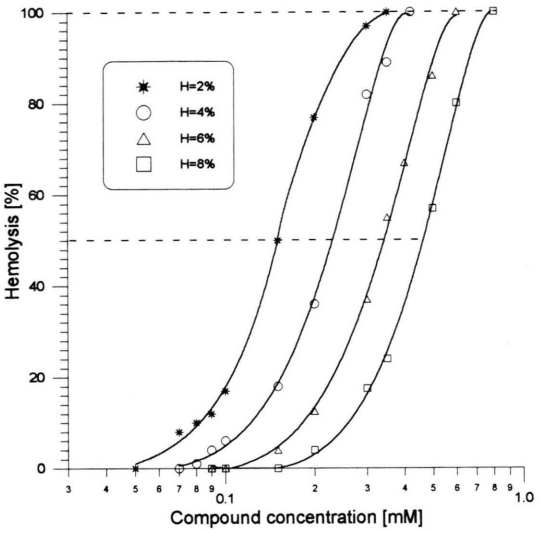

Fig. 2. Relation between percent hemolysis and concentration of compound DM-11 for four hematocrits at pH 7.4 and 37 °C.

(Fig. 4B) at 1% hematocrit. On the basis of the results shown in Figs. 4A and 4B, the sequence of hemolytic activity of the permeatoxins studied was found to be: PY > DM > DE > PP > MR > MO > PYI > PI. The compounds PY were found to be the most active, causing 50% and 100% hemolysis at lowest concentrations compared to the rest of the lysosomotropic substances.

The fluorimetric studies allowed to calculate polarization coefficient for the erythrocyte membrane modified with the compounds studied using the fluorescent probe TMA-DPH. The results represented in Table I indicate that the compounds

Table I. Polarization coefficient versus concentration of compounds studied.

Compounds studied	Polarization coefficient P Compounds concentration [μM] 1.0	2.5	5.0	7.5	10.0
PY-11	0.390	0.368	0.364	0.355	0.348
DM-11	0.392	0.388	0.386	0.383	0.368
DE-11	0.399	0.391	0.389	0.379	0.374
PP-11	0.402	0.405	0.404	0.408	0.405
MR-11	0.411	0.408	0.407	0.411	0.406
MO-11	0.409	0.414	0.410	0.413	0.411
PYI-11	0.410	0.412	0.407	0.410	0.404
PI-11	0.412	0.418	0.418	0.420	0.416
Control			0.417		

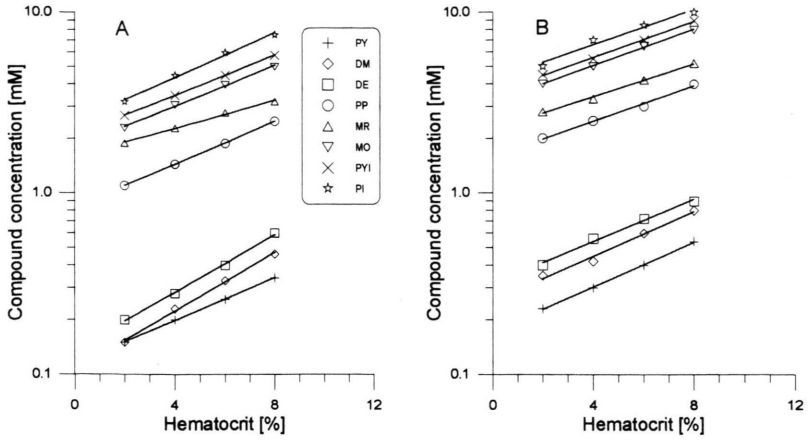

Fig. 3. Relation between hematocrit and compound concentration that causes 50% hemolysis (A) and 100% hemolysis (B), at pH 7.4 and 37 °C.

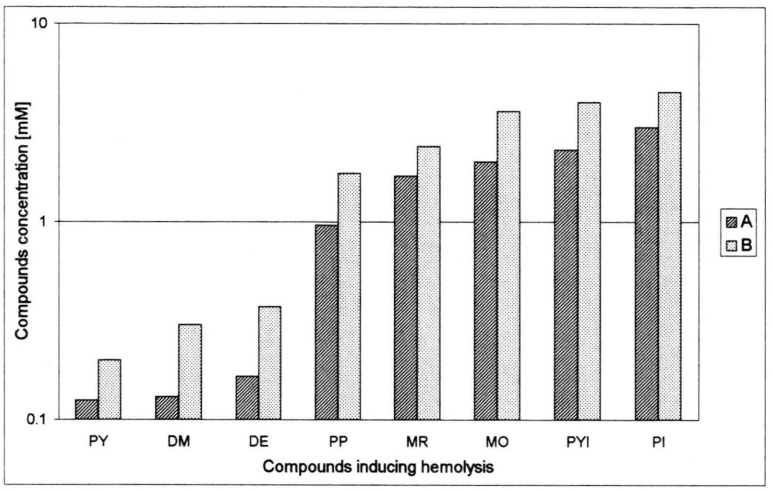

Fig. 4. Compound concentrations causing 50% hemolysis (A) and 100% hemolysis, at 1% hematocrite, pH 7.4 and 37 °C. The abbreviations refer to Fig. 1.

PY, DM and DE cause a decrease of the polarization coefficient in the concentration range 10^{-6}–10^{-5} M, with respect to unmodified cells. In addition, the polarization coefficient decreases, on the whole, with increasing compound concentration. The greatest changes are induced by PY compound. The decrease in polarization coefficient of the erythrocyte membrane induced by the compounds PY, DM and DE indicates that the red blood cell fluidity increases in the concentration range studied.

Discussion

The studies were performed with eight lysosomotropic aminoethyl dodecanoates bearing eleven – carbon alkyl chain in the ester substituent but differing in their nitrogen substitution, i.e head group structure. Dodecanoates were selected because of the highest biological activity of the aminoesters on yeast (Lachowicz *et al.*, 1996).

Our investigations allowed to determine the interaction of selected lysosomotropic compounds with the erythrocyte membrane, which was regarded as relatively simple and useful model of the biological membrane. Biological activity of the compounds studied was assessed on the basis of hemolytic activity, as it was done with other compounds (Byington *et al.*, 1974; Kondo, 1976; Kleszczyńska *et al.*, 1990; Sarapuk *et al.*, 1992; Musmeci *et al.*, 1992), and their ability to affect erythrocyte membrane fluidity. Due to the fact that all the compounds studied have alkyl chain of the same length, a change in their biological activity should

be connected with different polar head groups. These substances, as pK_a measurements showed, exhibit different acidity and therefore they can exist in solution at different pH in the form of electrically neutral molecules or in protonated form. The highest pK_a values for the compounds DE-11 and PY-11 were found to be; 8.45 and 8.41, respectively (Łuczyński *et al.,* 1997), and the basicity of the compounds studied follows the sequence: DM > PY > MO > DM > PP > PYI ≫ MR ≫ PI. The protonated form possesses detergent properties and increased biological activity. The typical for detergents course of hemolysis indicates that under the experimental conditions, i.e. at pH 7.4, the compounds occur in the protonated form mainly, with electric charge near the nitrogen atom. In this form (i.e. cationic form), as suggested for amphiphilic compounds of similar structure (Kondo, 1976; Sarapuk *et al.,* 1987; Isomaa, 1979), they incorporate into the erythrocyte membrane in such a way that the alkyl chain penetrates the hydrophobic core of the lipid bilayer whereas the polar head, having positive charge, interacts electrostatically with charged membrane elements and remains in the hydrophilic region of membrane. Both hemolytic and fluorimetric investigations confirmed the presence of such substances within membrane. However, if the compound studied occurred in the unprotonated form, they would probably permeate the red cell membrane easier, and would not concentrate in it, as was found in the case of lysosomes (Boyer *et al.,* 1993). When embedded into a membrane the lysosomotropic compounds alter its properties as a result of both electrostatic and hydrophobic interactions. Although all the compounds studied possess eleven-carbon alkyl chain, the depth of membrane penetration must depend on the size and structure of the polar head whose extent of emersion in the hydrophilic part of membrane may differ. The ratio of protonated/unprotonated form in the solution depends on pK_a value of the base and, of course, on pH of the milieu; direct dependence of hemolytic activity on basicity of the compound can be expected. The diverse hemolytic activity we observed may result from both the penetration ability of a compoud and alterations induced in the membrane hydrophilic region. The results of the investigations showed that the hemolytic activity of the compounds follows the sequence: PY > DM > DE ≫ PP ≫ MR ≫ MO ≫ PYI ≫ PI. The most active is compound PY, which causes 50% and 100% hemolysis at lowest concentrations which are 0.125 and 0.2 mM. The least active is, however, PI compound. The fluorimetric studies showed that the most hemolytically active compounds (PY, DM, DM) induce increased membrane fluidity.

As can be seen, sequencies of pK_a and those of hemolytic activity are very similar. It means that the ratio of protonated form of an aminoester is essential for its hemolytic activity. The most active were the substances with highest basicity, and the compounds of low pK_a (MR 5.89 and PI 4.92) have low activity. However, the biological activity depends also on head group size. The most active were the compounds which possess not more than four carbon atoms substituted at the nitrogen (PY, DE, DM). A piperidine derivative PP (pK_a 8.26) which has five carbon atoms and *n*-octyl MO (nine carbon atoms, pK_a 8.10) are less active than dimethyl one (pK_a 7.96). It is possible that when a head group is larger a compound cannot be incorporated into the membrane so deeply as the esters with a smaller head but the same length of the hydrophobic chain.

This work was supported by KBN grant No 7 S203 013 07

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