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A New Fluorescent Probe, ABM: Properties and Application in Clinical Diagnostics

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Properties of benzanthrone aminoderivative ABM (conditional name) as a potential fluorescent probe were investigated. Spectral characteristics of the compound in different solvents as well as their binding to model lipid membranes (liposomes) and human peripheral blood mononuclear cells (lymphocytes; ly) were determined. The fluorescence was found to be sensitive polarity changes to the environment. Distinctions were observed in the spectral characteristics of the investigated compound when bounded to liposomes. It was established that spectral characteristics of ABM in cell suspension qualitatively characterize the structural and functional alterations of ly during pathological phenomena and correlate directly with the clinical view of disease. The ABM is shown to be a perspective in the screening for various pathologies.

KEY WORDS: Fluorescent probe; lymphocytes; diagnostics.

INTRODUCTION

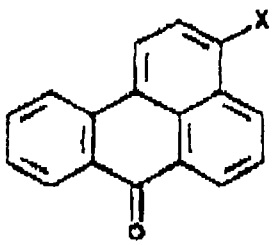
New synthesis methods for heteroaromatic compounds with marked luminescent properties were worked out as research on them started in the middle 1980s at the Department of Organic Chemistry, Faculty of Chemistry Technology, Technical University of Riga. Many of these compounds have been successively approved as fluorescent probes [1]. As known, pathological cellular function can be caused by or result from changes in cellular membrane structures. The molecular structure and mechanism of action of membranes pose challenging problems. Changes in composition and molecular organization are the principal determinants of the alterations of membrane fluidity observed in many human diseases. Recent studies of structure and function relationships in biological membranes have shown that membrane lipids play an important role in the regulation of cellular function. Many immunological functions may be heavily dependent on cell membrane structure [2,3].

It is very important for clinics to receive information on the properties of immune competent lymphocytes (ly) by an express method. The fluorescent probe proved to be an excellent, independent model for studying cell membranes [4].

In our work we have investigated the possibility of using the fluorescent probe ABM for detection of ly structural and functional alterations.

MATERIALS AND METHODS

ABM (conditional name) was synthesized at the Technical University of Riga, Department of Organic Chemistry.



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Synthesis was performed by means of substituting the bromide atom in 3-bromobenzanthrone with an appropriate amine.

Benzene, chloroform, dimethylsulfide, and ethanol are used as solvents for spectral and luminescence studies. In binding experiments, ethanol solutions of compounds were added to biological materials (resulting ethanol content in samples was 0.01%). Liposomes were prepared from phosphatidyl choline using the method described in [4,5]. Liposomes were suspended in isotonic phosphate (pH 7.0) buffer (concentration, 0.5 mg/ml). Samples were prepared by adding compounds to a solution or matrix suspension by means of a micropipette. Blood was drawn from humans at 8–10 am. Donors and patients ranged from 20 to 75 years of age.

Peripheral blood ly were separated accordingly [6]. The resulting suspension contained $0.5 \cdot 10^6$ cells/ml. The suspensions were incubated with ABM at room temperature for 2 min. Fluorescence characteristics were registered on a Sigma 4M spectrofluorimeter at an excitation wavelength of 470 nm and an emission wavelength of 630 nm.

Anisotropy index "*r*" was calculated according to Shinitzky [7]. To measure proliferate activity a radioisotope method was used [8]. Flow cytometric experiments were performed with a flow cytometer (Becton–Dickinson III).

Statistical differences and correlation of independent variables were determined using programs for the Student *t* test and Whitney–Mann *U* test [9, 10].

SPECTRAL CHARASTERISTICS OF ABM IN SOLVENTS

Spectral characteristics of compound ABM dissolved in various organic solvents are shown in Table I. Solvents are listed in order of orientation polarity increase defined by the dielectric constant of the solvent and refraction coefficient [11].

Table I. Spectral Characteristics of the Benzanthrone Derivative ABM in Different Organic Solvents[a]

Organic solvent	λ_{max} (nm)		*F*
	Excitation	Emission	(rel. u.)
Benzene	452	583	7.89
Chloroform	470	625	9.13
Dimethylsulfoxide	476	649	4.6
Ethanol	483	650	2.49
Methanol	483	653	2.07

[a] Concentration of probe in organic solvents, 10 μ*M*.

These results indicate that the fluorescence of this compound is sensitive to polarity changes in the microenvironment. The maximum wavelength of fluorescence for ABM increases in correspondence with Δf. Maximum λ_{max} change on passing from benzene to ethanol for a compound is 67 nm.

Many pathological cases are characterized by structural and functional property changes of cell membranes resulting from unfavorable conditions outside the cell or disturbed intracellular metabolism. Therefore, it is important that the binding of the fluorescent probe to the membrane "feels" the above-mentioned changes.

DISTRIBUTION OF ABM IN CELLS

Fluorescent microscopy revealed the distribution of ABM in such membranes of ly as plasma, mitochondrial, and nuclear, but there were no signs of localization of ABM inside the nucleus (Fig. 1).

Flow cytometric experiments revealed a strong bimodal distribution, with high and low ABM fluorescence intensity (F), respectively. Nearly 90% of ly showed high F, and the other 10% low F (Fig. 2).

The results might be explained by certain differences in properties of the membranes of T and B cells. There are data that ly can be divided into two groups according to the fluorescence intensity of 3-methoxybenzanthrone (MBA), "bright" and "dim." The proportion of bright ly corresponded to the B cell content, but the proportion of dim corresponded to the content of T cells. The differences which distinguish T and B cells revealed by fluorescent probe are based on certain differences in properties of the membranes [12].

The chemical structure of the synthesized compound ABM bears a resemblance to the structure of the fluorescent probe MBA. But despite is extensive spectrum of possibilities, the use of MBA is limited by two properties of this probe: first, it destroys cells after a short period of time; in luminescent microscopes it fades rapidly, being influenced by beams (approx. 80% in 6–8 min). Our data witness that ABM is nontoxic for cells and is photostable [4].

The addition of Triton X-100 did not lead to any changes in ABM F, while incubation of ly at 37°C resulted in increased ABM F. The above facts suggest localization of ABM deep in the phospholipid bilayer. The emission maximum of ABM in phosphatidyl choline liposomes is at 650 nm, which corresponds closely to that of the compound in methanol. Thus the environment of ABM in the phosphatidyl choline bilayer is quite polar, similar to that of methanol [13].

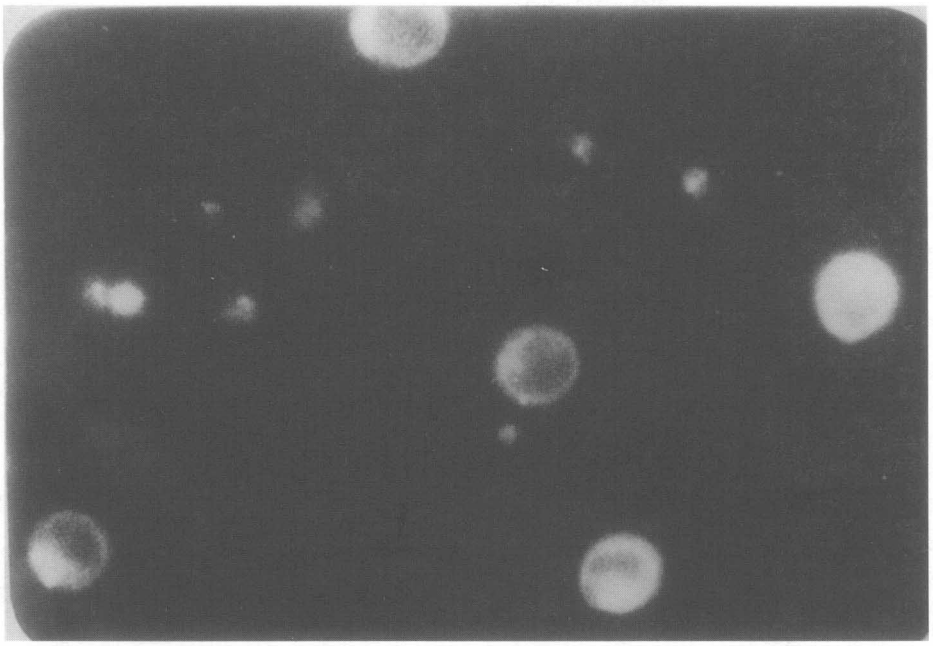

Fig. 1. Localization of ABM in lymphocytes (luminescent microscopy).

ABM FLUORESCENCE IN CELLS AND CHANGES OF IT WITH MITOGENES AND IN TUBERCULOSIS

Liposomes comprised of phosphatidyl choline alone (egg lecithin) and enriched with cholesterol and human peripheral ly showed different emission spectra maxima and fluorescence intensity (Fig. 3).

In comparison with phosphatidyl choline liposomes, a blue shift of emission spectra was noted in both liposomes enriched with cholesterol (molar ratio, 1:2) and human peripheral blood ly. The blue shift of emission spectra comes along with decreased F. ABM F in ly suspension is in inverse correlation with membrane microviscosity (Fig. 4).

The results obtained are in agreement with previous theoretical considerations that a rather high cholesterol level may cause a blue shift of emission spectra [2],

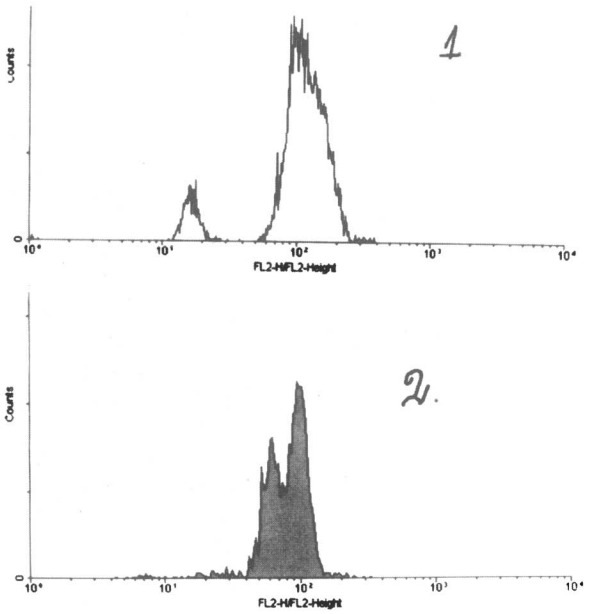

Fig. 2. ABM fluorescence in lymphocytes recorded by FACScan. (1) Healthy donors; (2) tuberculosis (TB) patients.

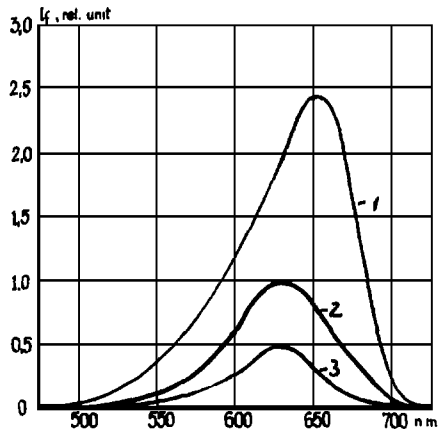

Fig. 3. Fluorescence emission spectra of ABM in phosphatidylcholine liposomes (1), phosphatidylcholine liposomes enriched with cholesterol (molar ration 1:2)(2), and human peripheral blood lymphocytes (3).

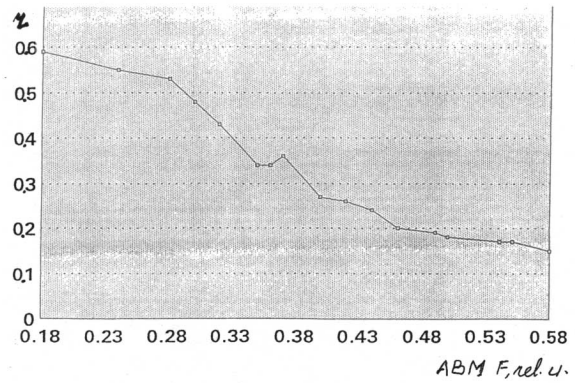

Fig. 4. Correlation between ABM fluorescence intensity in the cell suspension and membrane microviscosity. Correlation coefficient r = 0.97.

The mitogenic assay plays a very important role in clinical immunology. The proliferation potential of a cellular population is determined not only by the number of proliferating cells, but also by their respective proliferation rates.

The possibility of using ABM to determine functional properties of ly was examined. We measured spectral characteristics of native and mitogene-stimulated ly taken from healthy test donors. Isolated cells were stimulated *in vitro* by mitogens, such as phytohemagglutinin (PHA-P) and concanavalin A (Con A) (mostly T cells) and PWM (mostly T-dependent B cells). At the same time, characteristics of the ly blast transformation reaction (LBTR) (DNA synthesis activity) in these ly were measured. It was ascertained that the fluorescence maximum of ABM in the stimulated ly suspension is λ 630_{nm} (λ 470_{nm}), no different from that of the intact cells (Fig. 5).

The method is more sensitive when using PWM at a lower concentration of ABM, 9.9 μm not 19.6 μm

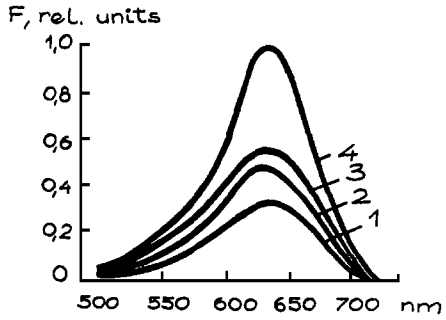

Fig. 5. Emission spectra of ABM fluorescence (λ_{ex}, 470 nm) in lymphocyte suspensions. (1) Intact cells; (2) activated by PWM (0.1 μg/ml); (3) activated by Con A (0.5 μg/ml); (4) activated by PHA (0.5 μg/ml). ABM concentration in sample: 19.6 μM (1, 3, 4) and 9.9 μM (2).

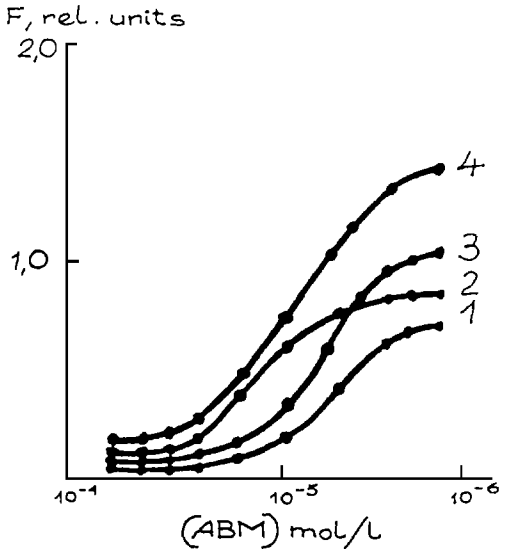

Fig. 6. Dependence of ABM fluorescence intensity on its concentration in mitogene-activated lymphocyte suspensions. Symbols as in Fig. 5.

as with PHA and Con A (Fig. 6). The subpopulation stimulated by PWM (mostly T dependent B cells) is more sensitive to the compound ABM.

Results testify that both ABM and LBTR characteristics are increased in ly suspensions activated by T mitogenes and B mitogenes in comparison with control cells (Table II). The above-mentioned characteristics are higher in the presence of PHA and Con A than in the case of PWM. It must be mentioned that the averaged values of these characteristics for the observed donor groups are

Table II. Lymphocyte Blast Transformation Reaction (LBTR) Level and ABM Fluorescence Intensity in the Lymphocyte Suspension of Healthy Donor Cells Activated by Different Doses of Mitogenes[a]

Mitogene	Dose (μg/ml)	Spectral characteristic I_f (rel. u.)	LBTR level (cpm)
K		0.51 + 0.02	280 + 40
PHA (n = 15)	1	0.99 + 0.04	68280 + 13911
	5	1.41 + 0.07	93870 + 6458
	10	1.66 + 0.10	97984 + 8747
K		0.29 + 0.02	140 + 11
Con A (n = 15)	1	0.49 + 0.05	2068 + 376
	5	0.54 + 0.05	8596 + 2324
	10	0.50 + 0.05	5271 + 810
K		0.22 + 0.1	216 + 15
PWM (n = 15)	0.05	0.47 + 0.1	30981 + 5568
	0.1	0.53 + 0.2	30633 + 5115
	1	0.61 + 0.3	36431 + 5780

[a] Cell count = $0.5 \cdot 10^6$ cell/ml. Concentration of probe in cell suspension activated by PHA and Con A, 19.6 μM; in that activated by PWM, 9.9 μM. K, control.

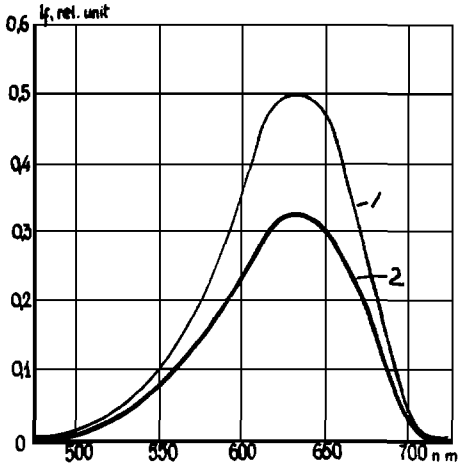

Fig. 7. Fluorescence emission spectra of ABM in healthy donors (1) and in patients with lung tuberculosis (2).

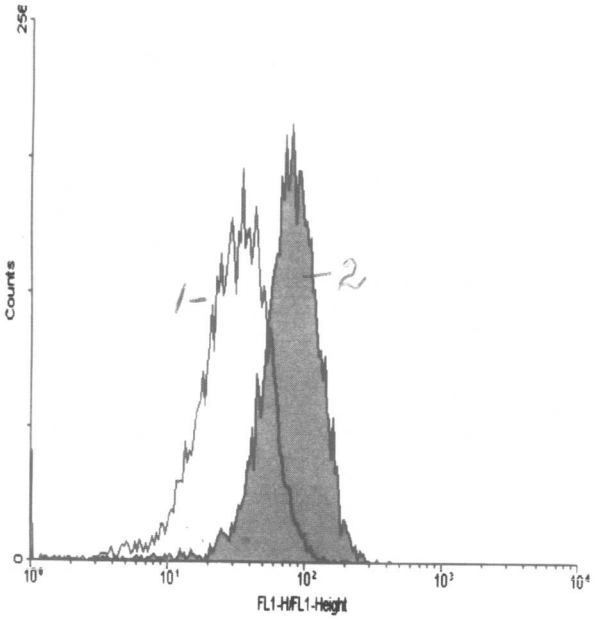

Fig. 8. Oxidative metabolism evaluated by mean of 2′, 7′-dichlorofluorescein diacetate (DFH-DA)(an indicator of intracellular peroxides level). (1) Healthy donors; (2) lung tuberculosis patients.

increased in correlation with the doses of applied mitogene. It was established that spectral parameters of ABM in the suspension of cells depend on the mitogene used for the blast transformation reaction (PHA, Con A, PWM), its dose, and which a subpopulation of ly is stimulated. (Table II). ABM was shown to be useful to determine the proliferate activity of ly [14].

In patients with lung tuberculosis (TB), the ABM emission spectrum maximum was not changed in comparison with the same parameter in the healthy control group (Fig. 7). Therefore absence of blue shift emission of spectra indicates no significant cholesterol increase in ly membrane of patients with lung TB.

ABM F and stimulation of the DNA synthesis in the ly of TB patients decrease, but membrane fluidity increases, in comparison with indices of healthy donors (Table III). There is a good correlation between ABM F, all observed indices, and the clinical view of the disease [15–18]. Results obtained provide support that significant structural, metabolic, and functional disorders of mononuclear cells play a leading role in the pathogenesis of acute tuberculosis. Structural disturbances of cells are a consequence of their lowered functional activity [19]. Blastic transformation of ly is a complex process of growth and differentiation in which cells undergo pronounced morphological, biochemical, and functional changes resulting in mature blast cells [20,21]. There is information on the changes in fatty acid composition of human ly during the entire period of blastic transformation. Studies on lymphoid cells have demonstrated an enrichment of polyunsaturated fatty acids in the early steps of activation [22–25]. This fact has a significant positive influence on membrane microviscosity. Changes in membrane microviscosity of human ly have been shown to correlate not only with changes in plasma lipid levels, but also with a decline in *in vitro* mitogen responsiveness [21]. The functional activity of patients' ly was found to depend on the nature and dynamics of the tuberculosis process and the predominance of the oxidative or productive inflammation phase. A considerable reduction

Table III. Spectral Characteristics of ABM and Anisotropy Index in Suspensions of Lymphocytes of Patients with Advanced Lung Tuberculosis

Diagnosis	Native cells		Activated cells (PHA: 10 μg/ml)		LBTR level (cpm)
	F (rel. U)	*p*	*F* (rel. U)	ρ	
Disseminated tuberculosis	0.31 ± 0.04	0.30	0.47 ± 0.05	0.26	19,025 ± 2,015
Infiltrative tuberculosis(spread of infiltrative lessions with destruction)	0.22 ± 0.03	0.36	0.30 ± 0.04	0.32	8,365 ± 967
Healthy donors(control)	0.51 ± 0.02	0.25	1.66 ± 0.10	0.15	97,984 ± 8,747

of ly function activity was found in the presence of dissemination in pulmonary tissue [26].

The degree of fatty acid unsaturation is influenced by intracellular peroxides and circulating free radical products. Both of them lessen the degree of unsaturation by impairing double bonding of polyunsaturated fatty acids and end up arising the membrane microviscosity; In the case of TB an increase in ABM F in 2',7'-dichlorofluorescein diacetate (an indicator for intracellular peroxides level) was noted (Fig. 8). These results are in agreement with a report that increased circulating levels of free radical activity are found in active lung tuberculosis and hence may play a role in the resultant fibrosis [27].

In the case of investigated pathology we have revealed significantly decreased ABM F among highly fluorescent ly (Fig. 2). Moreover, the strong bimodal distribution disappeared due to ly with intermediate F occurrence (Fig. 2). Active lung tuberculosis comes along with both impairment of membrane microviscosity of ly and disordered oxidative metabolism. Structural changes in cell membranes have an influence on the incorporation of the fluorescent probe ABM. Our results support the conclusion that observed changes in patents with advanced lung tuberculosis are in good correlation with the severity of the disease [26].

The results testify that ABM offers perspective for screening structural and functional alterations of cell membranes during pathological phenomena.

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