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3-Isopropyloxy-6-morpholino-2-phenylphenalen-1-one as Lipophilic Fluorescent Probe for Lymphocyte Investigations

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Abstract Fluorescent properties of two naphthalimides and a phenalenone derivative in organic solvents and when they bind to human peripheral blood lymphocytes were investigated. Different spectral characteristics were observed using lymphocytes of healthy donors and patients with nonmalignant (chronic myeloid leukemia) and malignant (B-cell lymphoid leukemia) diseases. It was found that spectral properties of the used fluorophores in cell suspension qualitatively characterize its structural and functional alterations during pathological phenomena. The intensity of fluorescence increased in samples from patients with B-cell lymphoid leukemia, and the fluorescence maximum shifted to the long-wavelength region by 20 nm compared with normal lymphocytes. It is concluded that 3-isopropyloxy-6-morpholino-2-phenylphenalen-1-one as most promising probe may be applied to the study of malignant diseases.

Keywords Lipophilic fluorescent probe · Phenalenone derivative · Naphthalimide · Lymphocytes · Malignant diseases

Introduction

Today, fluorescence spectroscopy became a valuable tool in the study of living cells due to its great sensitivity [1]. In such investigations, researchers use emission properties of native fluorophoric species of cells or more often employ covalent or noncovalent labeling of biological structures by fluorescent dyes.

The considerable amount of the used fluorescent labels is usually bound covalently to the biomolecules under investigation. One of the main types here is fluorescein derivatives with various reactive functional groups, which have been found to be particularly effective in monitoring lymphocyte proliferation and differentiation study [2–4].

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The recent trend is the use of noncovalent labeling of bioobjects in clinical analysis [5]. The noncovalent labeling can involve ionic, electrostatic, hydrophobic, and hydrogen bonding interactions. Although these interactions are substantially less stable than the covalent linkages, they occur at a faster rate and at a physiological pH range.

Mainly, there are lipophilic fluorescent probes designed to localize in a hydrophobic region within a biological specimen, for example, binding with lipids, which play an essential role in multiple processes important to cells, including cell structure, cell signaling, maintaining a membrane potential, etc. It is known that cellular mechanisms involved in malignant transformation of normal cells are associated with structural, functional, and dynamic changes in the cell surface membrane [6, 7]. These changes 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 the cell membrane structure [8]. It is very important for clinics to receive information about the properties of immune competent cells by an express method. The fluorescent probe proves to be an excellent, independent model for such studies [9].

In the past, various hydrophobic fluorescent dyes have been developed mostly for biological membranes studies. For example, derivatives of naphthylamine (8-anilino-1-naphthalenesulfonic acid (ANS), TNS, NPN, etc.) are widely used to detect lymphocyte activation [10–12]; derivatives of benzanthrone (3-methoxybenzanthrone (MBA), ABM) were exploited to reveal lymphocyte population heterogeneity [13] and to estimate immune state during pathologies [14, 15]. One more promising class of hydrophobic fluorescent probe comprises dyes derived from naphthalic acid such as naphthalimides and phenalenones. These dyes having electron-donor and electron-acceptor substituents display a high sensitivity to the polarity of the local environment, and their spectroscopic behavior is dependent on the physicochemical properties of surrounding environment [16]. Therefore, they have found application in a number of areas including fluorescent probes and markers for cells [17, 18].

In the present work, we investigated spectral properties of three derivatives [4-(1-phenyl-5-(*p*-methoxyphenyl-2-pirazolyl-3)-naphthalic acid *N*-(5-hydroxypenthyl)imide (I), benzo [*k*,*l*]thioxanthen-3,4-dicarboxylic acid *N*-octadecylimide (II), 3-isopropyloxy-6-morpholino-2-phenylphenalen-1-one (III)] earlier synthesized from naphthalic acid, which were chosen from various analogous derivatives due to their more intense emission. A possibility of their use for detection of structural and functional alterations of lymphocytes was also evaluated in this work.

Methods and Materials

Chemicals

Organic solvents (benzene, chloroform, and other liquids) were purchased from Reanal (Hungary). The target fluorophores were synthesized from naphthalic acid and purified according to methods reported previously [19, 20]. Figure 1 shows the chemical structure of these probes.

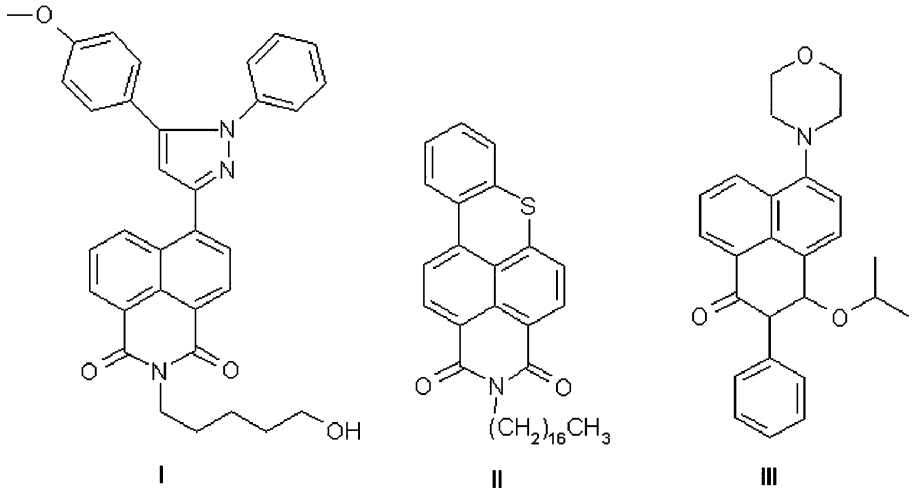

Fig. 1 The chemical structure of studied probes: 4-(1-phenyl-5-(*p*-methoxyphenyl-2-pirazolyl-3) naphthalic acid *N*-(5-hydroxypenthyl)imide (*I*), benzo[*k*,*l*]thioxanthen-3,4-dicarboxylic acid *N*-octadecylimide (*II*), and 3-isopropyloxy-6-morpholino-2-phenylphenalen-1-one (*III*)

Study Subjects

The study subjects were:

1. Healthy donors (n=16)
2. Chronic myeloid leukemia patients (n=15)
3. Chronic B-cell lymphoid leukemia patients (n=17)

The mean age was 48.5±0.9 years, age range 21–76 years. Duration of the disease is 2 to 14 years. Individuals who have been under treatment for acute infections diseases etc. were excluded. In all patients, the diagnosis of lymphoproliferative disease was established by studying cell morphology, immunological parameters, and laboratory and clinical characteristics of patients.

Blood Collection, Preparation of Lymphocytes, and Liposomes

For each individual, peripheral blood samples were collected from a vein into disposable vacuum tubes containing preservative-free heparin 30 IU/ml.

Lymphocytes were isolated from the freshly withdrawn heparinized venous blood by a standard verographine method. Three milliliters of verographine were layered on 3 ml of blood and centrifuged at 1,700×g for 30 min (60% verographine, Spofa: aqua pro inj., 2:5). The ring of cells formed on the density interface was aspirated and washed three times with Na/K phosphate buffered saline (pH 7.3, osmolarity 290 mOsm) by centrifugation at 1,400×g for 10 min and resuspended in amino acid solution (RPMI 1640). The resulting 0.5×10^6 cells/ml of cell suspension was subjected to the fluorescence measurements.

Liposomes were prepared from phosphatidylcholine (Lipid Products England) using the method described in [21]. Liposomes were suspended in isotonic Na/K phosphate buffer (0.01 M phosphate buffer; pH 7.0), concentration 0.5 mg/ml.

Fluorescent Measurements

The "blank" sample for each experiment contained a probe of the same concentration (10^{-5} M) but without cells. In binding experiments, 1 ml of cell suspension was incubated with 0.01 ml of ethanol solution of dye at room temperature for 20 min and was added to 1.0 ml of lymphocyte suspension and incubated at room temperature for 20 min (the final concentration of probe in the sample is 46 μmol/l). The time interval between the cell isolation and fluorescence measurement was constant, 3 h for all samples. Fluorescence parameters were registered at room temperature in 10 mm quartz cuvettes on the spectrofluorimeter Spectrofluo JY3 (ISA Jobin Yvon Instruments) at the excitation wavelengths of 250–500 nm and emission wavelength in 300–700 nm region with spectral resolution 0.5 nm. Excitation slit width was set at 5 nm. Fluorescence intensity was measured in arbitrary units (a.u.).

A degree of fluorescence polarization is determined from the measurements of fluorescence intensities parallel and perpendicular to the plane of linearly polarized excitation light [22].

Statistical Analysis

Statistical differences and correlation of independent variables were determined using the Student's *t* test and Mann–Whitney *U* test [23].

Results and Discussion

Spectral Characteristics of the Studied Fluorophores in Solvents

In order to study solvent sensitivity of the excitation and emission spectral characteristics of the studied dyes (I–III), these spectra were measured in several polar and nonpolar media. The main photophysical properties are presented in Table 1, where solvents are listed according to the increase of orientation polarity (*f*) defined by the dielectric constant of the solvent and the refraction coefficient [1].

Table 1 Spectral-fluorescent properties of compounds I, II, and III in various organic solvents.

Organic solvent	*f*	I			II			III (λ_{ex}=480 nm)	
		λ_{ex} (nm)	λ_{em} (nm)	*F* (a.u.)	λ_{ex} (nm)	λ_{em} (nm)	*F* (a.u.)	λ_{em} (nm)	*F* (a.u.)
$CHCl_3$	0.0014							577	32.3
C_6H_6	0.0016	500	575	260.3	494	509	15.2	580	32.9
Et_2O	0.16							586	37.8
THF	0.2096	500	630	88.7	502	520	20.9	590	50.5
DMF	0.2744	500	650	18.7	497	528	15.2	615	66.1
Acetone	0.2841	495	665	23.7	494	520	14.4	605	65.3
EtOH	0.2887	495	665	15.4	500	528	19.3	615	54.9

The studied derivatives display an orange or red intense fluorescence in the solutions, which strongly depends on the nature of the solvent, especially on its polarity. An observed bathochromic shift on passing from benzene to ethanol for these compounds is 90 nm (dye I), 19 nm (dye II), and 35 nm (dye III). These results indicate that fluorescence of these fluorophores is sensitive to polarity changes in the microenvironment as expected for such class of donor–acceptor π-conjugated chromorophores.

A substantial difference in fluorescence intensity was found, with the fluorescence intensity changing from a nonpolar environment to a polar medium, i.e., compound I shows an intense fluorescence in benzene and a significant 17-fold decrease of emission in ethanol solutions; dye II has similar intensities of emission both in polar and nonpolar surroundings; in contrast, dye III displays a 1.7-fold increase of fluorescence intensity in ethanol solutions in comparison with benzene solutions.

It is known that suitability of a fluorescent probe for the investigation of bioobjects depends on their photophysical characteristics such as quantum yields, excitation, and emission wavelength. Long-wavelength fluorophores, which emit in the red region of the electromagnetic spectrum, are preferred for biological applications because cells excited at wavelength below 500 nm produce considerable autofluorescence from flavoproteins, nicotine nucleotides, etc., which can swamp the used probe fluorescence.

Fluorescent probes most commonly used in lymphocyte investigations (ANS, AS, MBA) have high fluorescence quantum yields but emit at short wavelength (300–500 nm), where the biological background fluorescence can cause interference. Therefore, the development of new probes with strong fluorescence in long-wavelength region plays crucial role in advancing techniques for bioobjects investigations.

Binding of the Fluorophores with Lymphocytes and Liposomes

For lymphocyte and model membrane binding studies, dye III was used due to its promising spectral data such as an intensive long-wavelength emission and expressed solvatochromic properties. The spectral characteristics of the fluorophore III have been examined using model membranes composed of egg yolk phosphatidylcholine and its mixture with cholesterol. The emission spectra in liposomes enriched with cholesterol exhibit a shift towards shorter wavelengths as compared to the spectrum of this dye in phosphatidylcholine liposomes. The shift of emission spectra came along with decrease in fluorescence intensity (see Table 2). These results are in agreement with observations of Shinitzky and Inbar concerning the effect of cholesterol on liposomes and cell fluidity [6, 7]. It is evident that a higher fluorescence intensity of compound III is associated with a lower level of membrane cholesterol, due to its ability to increase the dipole potential and the hydration of interfacial bilayer [24]. Therefore, a high cholesterol level may cause a bathochromic shift in emission spectra of lipophilic fluorophore.

The binding of the phenalenone derivative to the lymphocytes of healthy donors and patients with leukemic diseases was investigated. The maximum of dye I fluorescence in the lymphocyte suspension of the observed group of patients is situated at 595 nm, which corresponds to the fluorescence seen in healthy people. Various subgroups of the observed patients showed differences only in the fluorescence intensity of cell suspensions. Fluorescence intensity was higher in B-cell lymphoid leukemia patients in comparison with chronic myeloid leukemia patients and healthy donors.

In chronic B-cell lymphoid leukemia patient's lymphocyte suspension, the compound III fluorescence zone is shifted to the long-wave region of the spectrum by 20 nm (λ_{max}

Table 2 Spectral characteristics of dye III in liposomes and lymphocyte suspensions of patients with leukemic diseases.

Group under study	λ_{ex} (nm)	λ_{em} (nm)	F (a.u.)
Liposomes (egg lecithin)	480	630	42.9
Liposomes (egg lecithin + cholesterol)	480	650	18.6
p_1			>0.001
Healthy donors (n=15)	480	595	18.3±0.05
Chronic myeloleukemia patients (n=15)	480	595	20.4±0.04
p_1			>0.05
B-cell lymphoid leukemia patients (n=17)	480	615	37.6±0.05
p_1			<0.002
p_2			<0.005
Isotonic phosphate buffer	503	628	2.5

p_1 level of significance (between the leukemia patients and healthy donors); p_2 level of significance (between observed groups of patients)

615 nm). The maximum of the compound III fluorescence spectrum in chronic myeloid leukemia patients (λ_{max} 595 nm) was not changed compared with the data obtained from practically healthy donors.

Fluorescence intensity of dye III, binding to the lymphocytes, significantly increased in B-cell lymphoid leukemia patients and did not change in chronic myeloid leukemia patients as compared to healthy donors. There is a direct correlation between spectral parameters of compound III and malignantly transformed state of cells from hematopoietic system.

Significant structural, metabolic, and functional deviations of lymphocytes play a leading role in the pathogenesis of different human pathologies [6–8]. Physical and chemical alterations in membrane structure may contribute to the changed membrane microviscosity. Changes in membrane microviscosity of human lymphocytes have been shown to correlate not only with changes in plasma lipid levels but also with the decline in in vitro mitogen responsiveness [25]. The above-mentioned alterations in cell membrane obviously influence the incorporation of fluorescent probe into the cell [8].

The different spectral characteristic probe III binding with lymphocytes of the observed subgroups of patients can be explained by specific and qualitatively different changes of membrane properties in patients with nonmalignant and malignant diseases. Cytoskeleton of tumor cells undergoes structural and functional alterations that are associated with an increase in fluidity of surface membrane lipid layers [8].

It is also confirmed by the data related to different histogenesis of lymphoproliferative diseases [25, 26]. The morphology of cells in chronic myeloid leukemia patients does not significantly differ from the norm. Previous studies of other authors stated morphological and immunological variety of lymphocytes in B-cell lymphoid leukemia patients. They present large amounts of pathological clones with a lower stability of lipoprotein complexes [8]. These various types of structural alterations of tumor cells can be associated with functional activity and increase in fluidity of the surface membrane lipid layers [6, 7].

Today, analytical methods based on polarization of fluorescence are used in many fields of molecular analysis [1]. Using emission polarization measurements makes it possible to obtain information on molecular orientation and mobility and on the processes that modulate the phenomenon [9, 27]. Thus, in the work [28], the degree of lipid fluidity in normal and leukemic lymphocytes was quantitatively monitored by fluorescence polariza-

tion analysis of a fluorescent probe that is embedded in lipid regions of cellular membranes. In the present work, the study of fluorescence polarization of normal and leukemic human lymphocytes did not reveal any differences that could be attributed to the leukemic transformation.

Previous authors [2, 3] who studied normal and leukemic populations from both experimental animals and humans have observed that the surface membrane of the leukemic cells have a more fluid lipid core than that of normal lymphocytes. The increase in fluidity in leukemic cells is predominantly caused by a decrease of cholesterol phospholipids in the cell surface membrane and in human leukemia; it correlates with the acuteness of the disease.

Moreover, it was shown that the reduction of membrane fluidity induced by the introduction of exogenous cholesterol into the surface membrane of intact lymphoma cells from mice resulted in a marked inhibition of their tumorigenicity. Conversely, a controlled increase in membrane fluidity of normal lymphocytes induced by a reduction of membrane cholesterol resulted in a significant increase in the activation of the normal lymphocytes by plant mitogens [29].

Suggestions of the above-mentioned authors and results of our experiments have confirmed that the difference between compound III spectral parameters in nonmalignant and malignant lymphocyte populations mainly originated from the difference in the cholesterol/phosphorlipids ratio in the cell membrane of two cell types. Spectral parameters of probe III can serve as a qualitative tool for distinguishing between normal and leukemic cells. Such preliminary procedure for malignant lymphocyte detection can propose:

Lymphocytes Assay by Fluorescent Probe III

After lymphocyte isolation by standard verographine method, 1 ml of cell suspension with concentration 0.5×10^{6} cells/ml was incubated with 0.01 ml of ethanol solution (concentration 10^{-5}M) of 3-isopropyloxy-6-morpholino-2-phenylphenalen-1-one at room temperature for 20 min. Then, fluorescence spectra were recorded in cuvettes on spectrofluorimeter or using microplate reader. Obtained results of fluorescence parameters (intensity and maximum) of studied lymphocytes were compared with data from healthy donor lymphocytes. If red shift (10–30 nm) was observed, it can be concluded that lymphocytes undergo structural alterations which are induced by malignant diseases.

Conclusion

The present study evaluated applicability of three fluorescent probes (two naphthalimides and the phenalenone derivative) in lymphocyte investigations. For this purpose, the use of the red emitting dye, such as 3-isopropyloxy-6-morpholino-2-phenylphenalen-1-one, reduces the background interference that is typical of biological matrix. Distinctions were observed in spectral characteristics of the investigated dye in various solvents and when bound to lymphocytes of patients with nonmalignant and malignant diseases. The investigated lipophilic probe can provide an effective approach to the study of basic differences between normal and malignant cells. Such an analysis has a great potential for determining the control mechanisms associated with the induction and development of the malignant transformed state in the hematopoietic system as well as in other mammalian tissue.

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