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PROBING MEMBRANE ALTERATIONS ASSOCIATED WITH ANTHRACYCLINE RESISTANCE USING FLUORESCENT DYES

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Abstract—Properties of membranes of intact P388 murine lymphoblastic leukemia and a sub-line selected for resistance to adriamycin (P388/ADR) were examined using two fluorescent probes: diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (T-DPH). Time-dependent changes in dye accummulation, fluorescence anisotropy, and lifetimes were measured. Accumulation of DPH, which eventually labels all cellular lipids, increased with time. Uptake of T-DPH, a more membrane-specific probe, reached a maximum in less than 1 min. No alteration could be detected in fluorescence anisotropy or lifetime of either dye associated with anthracycline resistance or anthracycline treatment. The rate of uptake of the T-DPH was not different in P388 versus P388/ADR cell lines, suggesting no differences in membrane "traffic". Calcium-channel antagonists could partially reverse anthracycline resistance in P388/ADR, but this was not accompanied by alterations in fluorescence parameters.

The use of fluorescence probes for measuring the "fluidity" of membrane lipids has been useful when the probes have sufficient specificity to provide meaningful signals. One such probe is DPH†, which has several favorable photophysical properties [1]. This dye binds so as to be oriented normal to the membrane plane, and relatively small displacements of the axis of symmetry result in a change in the degree of anisotropy of fluorescence emission. Unfortunately, DPH eventually partitions to all lipid-rich cellular regions, and a composite signal is obtained [2–4].

The DPH analog T-DPH [5] has been suggested as a more specific membrane probe. This agent is initially bound at surface membrane loci and can report on outer membrane lipid mobility [6]. This binding property makes T-DPH a useful probe for membrane exocytosis, since the bringing of new membrane material to the outer surface will result in an increase in total cellular fluorescence [7, 8]. Differences in membrane "traffic" have been proposed as a determinant of anthracycline resistance [9].

Both DPH and T-DPH were used to assess membrane properties of P388 murine lymphoblastic leukemia cells and of a sub-line selected for anthracycline resistance (P388/ADR). Measurements of fluorescence anisotropy of these dyes have been used previously to estimate the relationship between membrane fluidity and drug resistance [10]. Similar studies involving another probe, *trans*-parinaric acid, have also been reported [11]. The present experiments extend the former study by examining fluorescence intensity, anisotropy and lifetimes as a function of the time of exposure of cells to the dyes. Effects of anthracyclines and a calcium channel antagonist on fluorescence were also determined.

MATERIALS AND METHODS

The P388 murine leukemia and an anthracycline-resistant sub-line, P388/ADR, have been described before [12–14]. P388/ADR is 100-fold resistant to DNR and shows a 6-fold impaired ability to accumulate the latter agent; drug resistance is partially reversed by the calcium channel antagonist verapamil [14]. Conditions for culture of these cell lines are described in Ref. 14.

All incubations were carried out in Earle's salts at pH 7.4 containing 1 mg/ml glucose. Fluorescence studies were carried out in 3 ml cuvettes which were fitted with magnetic stirring bars. P388 and P388/ADR cells (10^5/ml) were exposed tro 1 μM DPH or T-DPH at 37° for various intervals, and dye uptake was measured via fluorescence intensity (excitation at 350 nm, emission at 422 nm) as a function of time. In other studies, cells were incubated with DPH or T-DPH for 30 min at 37°, then washed and suspended in fresh medium for the measurement of cellular fluorescence. The effect of varying the number of cells from 5×10^4 to 10^6/ml on fluorescence data was also examined.

Fluorescence anisotropy was measured at 37° in stirred cuvettes, using an SLM 4800S instrument in the "T" configuration. The excitation wavelength was 350 nm (bandwidth = 2 nm), and Schott KV410 filters limited light reaching the PMTs to wavelengths >420 nm. The voltage to the PMTs was adjusted so

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† Abbreviations: ADR, adriamycin; DNR, daunorubicin; DPH, diphenylhexatriene; PMT, photomultiplier tube; T-DPH, trimethylammonium diphenylhexatriene; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

that excitation with 90° polarized light resulted in an equal current from the PMT monitoring vertically-polarized and the PMT monitoring horizontally-polarized emissions. When the excitation polarizer was set at 0°, the ratio of output from the PMTs could then be used to calculate anisotropy as a function of time. All measurements were corrected for light-scattering [15].

In some studies, cells were treated with 0.1 to 10 μM ADR or DNR at 37°. Where specified, 10 μM verapamil was also present. After 30 min, DPH or T-DPH was added (final concentration = 1 μM), and dye fluorescence was measured 5 min later.

Fluorescence lifetimes were measured by the phase-modulation technique [16], using POPOP as the reference standard ($\tau = 1.35$ nsec). Both phase and modulation were measured at 6, 18 and 30 mHz frequencies, and lifetimes were calculated from these data [16].

Fluorescence data shown in Figs. 1–3 were taken directly from the SLM fluorometer output and transformed to hard copy using the Mirage computer program provided by Zenographics, Inc., Irvine, CA.

RESULTS

Kinetics of DPH and T-DPH accumulation were determined at 37° by measurement of 420 nm fluorescence emission (excitation = 350 nm). Uptake of DPH was a slow process (Fig. 1, top), with a steady-state not reached until approximately 30 min. In contrast, T-DPH accumulation was very rapid, and little additional uptake was observed after 30 sec (Fig. 1, bottom). The slight variation in T-DPH fluorescence with time is unlikely to represent photobleaching, since the total cuvette volume was 3 ml and this was constantly stirred. When a steady-state was reached, there was judged to be no significant difference in DPH or T-DPH fluorescence between P388 versus P388/ADR cells. Data shown in Figs. 1 and 2 represent results of typical experiments; the variation in fluorescence intensity from replicate experiments was ± 5% of values shown here. Treatment with DNR or ADR (with or without additional varapamil) did not affect fluorescence data obtained with either dye.

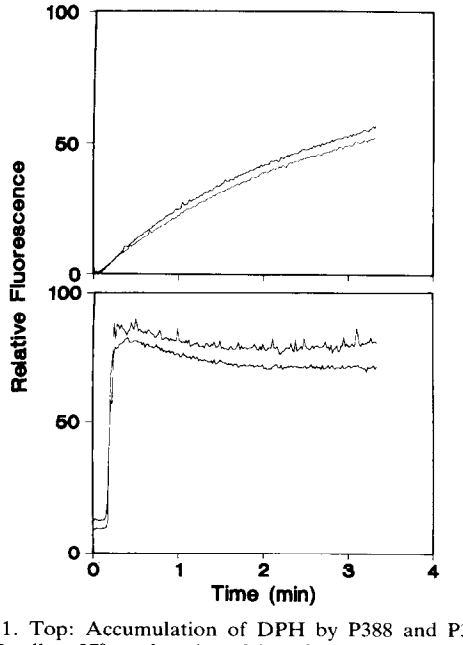

Fig. 1. Top: Accumulation of DPH by P388 and P388/ADR cells at 37° as a function of time. Data are expressed as percent of the steady-state value which was not significantly different for the two cell lines. Upper tracing = P388, lower tracing = P388/ADR cells. Bottom: Accumulation of T-DPH by P388 and P388/ADR cells at 37° as a function of time, as indicated by fluorescence emission intensity. Upper tracing = P388, lower tracing = P388/ADR cells. These results represent data from typical experiments; in replicates involving the same cell density, a variation of ±5% was observed.

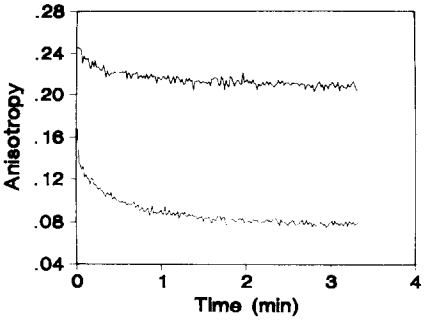

Fig. 2. Anisotropy of DPH (bottom tracing) and T-DPH (top tracing) fluorescence in P388 cells as a function of time of exposure of cells to the dye at 37°.

T-DPH anisotropy decreased slightly with incubation time (Figs. 2 and 3, top tracing). In contrast, the fluorescence anisotropy of DPH was affected significantly by the time of incubation (Figs. 2 and 3, bottom tracing). This reflects movement of the probe from the plasma membrane to intracellular sites where rotational motion was less restricted. We could not detect any significant difference in fluorescence anisotropy using either dye when values of P388 versus P388/ADR were compared (Figs. 2 and 3). Results shown in Figs. 2 and 3 were not affected by varying the cell density from 5×10^4 to 10^6/ml.

A 30-min exposure to 0.1 to 10 μM DNR or ADR did not affect fluorescence anisotropy of P388 or

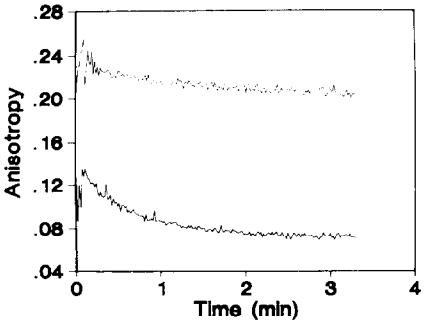

Fig. 3. Fluorescence anisotropy of DPH (bottom tracing) and T-DPH (top tracing) in P388/ADR cells as a function of time of exposure to the dye at 37°.

Table 1. Fluorescence anisotropy and drug resistance

	Fluorescence anisotropy			
	DPH		T-DPH	
Cell line	Control	DNR-treated	Control	DNR-treated
P388	0.082	0.082	0.214	0.211
P388/ADR	0.081	0.078	0.214	0.212

Cells were incubated for 30 min at 37°, with 1 μM DNR present if specified. DPH or T-DPH (1 μM) was then added, and fluorescence anistropy was measured after 5 min. These values are corrected for the contribution of light scattering. All numbers shown are subject to an error of ±3%.

P388/ADR cells (data for 1 μM DNR are shown in Table 1), nor did addition of 10 μM verapamil alter these results.

Information on fluorescence lifetimes was also obtained, to aid in the interpretation of anisotropy data. With both probes, we could fit the data to a 2-lifetime model, 3–4 and 8–10 nsec. The results did not differ significantly when P388 versus P388/ADR cells were compared; anisotropy data, therefore, can be interpreted in terms of probe mobility without errors which occur when lifetime values differ.

DISCUSSION

In the present study, fluorescence intensity, anisotropy, lifetime and localization of two probes in P388 and P388/ADR cells at 37° as a function of incubation time were examined. Results from P388 murine leukemia cells and a sub-line selected for DNR resistance are shown. The latter cell line exhibits impaired DNR accumulation which can be reversed by a calcium channel antagonist; these results are typical of cell lines which demonstrate the multidrug-resistance phenotype [14, 17].

DPH is known to penetrate to all cellular lipids [2–4], whereas T-DPH is, at least initially, associated with the outer membrane [6]. The kinetic data obtained with DPH are consistent with these reports; dye accumulation was a gradual process, and fluorescence anisotropy fell with time as the probe became associated with intracellular loci where rotational mobility was greater. A similar finding has been obtained with another cell line [18].

In contrast, T-DPH was initially confined to the outer membrane, although penetration to the cellular interior does eventually occur [6, 17]. There was no consistent difference in fluorescence anisotropy of either probe associated with anthracycline resistance, nor with short-term exposure to anthracyclines. Moreover, the partial reversal of drug resistance in P388/ADR by verapamil [14] did not affect any fluorescence parameter described here.

Increased "membrane traffic" has been proposed as one determinant of anthracycline resistance [9]. Since T-DPH has been used as a probe for such a phenomenon [7], it is interesting to note that accumulation of this dye was not significantly different in P388 versus P388/ADR (Fig. 1, bottom panel).

Differences in membrane "fluidity" have been reported in conjunction with anthracycline resistance [19–22]. But such findings have been questioned recently [9, 10]. Since fluorescence anisotropy of DPH and T-DPH can vary with time of exposure of cells to the probes, misleading values may be obtained if incubation times are not controlled carefully. But drug resistance, in different systems, may be mediated by other factors and, in any event, the single example shown here cannot be said to necessarily apply to all cases. With regard to the P388 and P388/ADR cell lines, no major differences in lipid composition have been detected [23], and this, together with data shown here, indicate that alterations in cellular lipid order cannot be implicated in this example of anthracycline resistance.

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