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Keywords:
21-aminosteroids, Lazaroids, cholesterol, methylprednisolone, antioxidant, brain microvessel endothelium, blood-brain barrier, lipid packing, fluorescence

Abstract:
Studies were conducted to demonstrate 21-aminosteroid distribution into the hydrophobic or lipid domains of biological membranes, a presumed site at which these compounds inhibit lipid peroxidation. Bovine brain microvessel endothelial cells (BMECs) were labeled with diphenylhexatriene fluorophores and interactions with cell membranes characterized with fluorescence anisotropy and lifetimes. Two 21-aminosteroids (U-74500A and U74006F) were shown to preferentially alter the fluorescence anisotropy and lifetime parameters of the diphenylhexatriene probe distributing into membranes throughout the BMECs. Little or no effect of the compounds on the fluorescence parameters of the diphenylhexatriene probe the localized on the surface of BMEC plasma membranes. By contrast, cholesterol used as a positive control altered substantially the fluorescence parameters of BMECs labeled with either diphenylhexatriene probe. Results suggest 21-aminosteroid-induced changes in the molecular packing order and drug:fluorescent probe interactions in membrane hydrophobic (or lipid) domains throughout the BMEC. Concentrations of 21-aminosteroids altering the fluorescence parameters of diphenylhexatriene labeled BMECs correspond to those concentrations of 21-aminosteroids effective in vitro in inhibition lipid peroxidation.

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Text of paper:

EVIDENCE FOR 21-AMINOSTEROID ASSOCIATION WITH THE HYDROPHOBIC DOMAINS OF BRAIN MICROVESSEL ENDOTHELIAL CELLS

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Running Title: 21-Aminosteroids in Membranes

INTRODUCTION

The focus of many recent investigations on central nervous system (CNS) tissue injury processes involved in trauma and stroke has been directed at oxygen radicals and their role in lipid peroxidation. The current state of knowledge for the precise contributions and the chemistry of oxygen radicals in lipid peroxidation, however, remains relatively limited. Despite the absence of a clear understanding of their significance in tissue trauma or injury, observations in neuroscience and pharmacologically based literature strongly implicate an important function for oxygen radicals in trauma and stroke (1,2). Accordingly, pharmacological approaches to protecting and/or treating CNS tissue injury due to trauma or stroke includes effective antioxidants (2,3).

The 21-aminosteroids ("Lazaroids") are a new series of compounds developed to treat CNS tissue injuries originating from trauma or stroke (Figure 1). These compound were designed to distribute into cell membranes for the purpose of inhibiting lipid peroxidation (4). To date, cell membrane activity has been implied from 21-aminosteroid-induced changes in membrane permeability and inhibition of arachidonate release from injured cells (5,6). The purpose of this study was to provide further evidence in support of the interactions of the 21-aminosteroids with lipid or hydrophobic domains of cells, the presumed site of action for these agents. Brain microvessel endothelial cells (BMECs) were labeled with well-known fluorophores, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethyammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH), and fluorescence anisotropy and lifetime measures of these probes used to examine the interactions of 21-aminosteroids with cellular lipid domains (7-11). BMECs represented an appropriate experimental system since the microvasculature of the central nervous system has been suggested as a major target for oxygen radicals in trauma and stroke (1-3).

MATERIALS AND METHODS

Chemicals:

21-Aminosteroids (U-74500A, U-74006F), methylprednisolone sodium succinate (U-9088), and 6-à-methylprednisolone (U-7532) were gifts from The Upjohn Company, Kalamazoo, MI. Fluorophores, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethyammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH) were purchased from Molecular Probes, Eugene, OR. Cholesterol, heparin sulfate, ascorbic acid, fluorescein sodium, 1,4-bis[4-methyl-5-phenyl-2-oxazoyl]benzene (dimethyl POPOP), Percoll, trypsin/EDTA mixture, and antibiotics were purchased from Sigma Chemical Co., St. Louis, MO. Tetrohydrofuran and dimethylformamide were purchased from Fisher Scientific, St. Louis, MO. Equine serum was purchased from HyClone, Logan, UT. Culture mediums were purchased from Dutchland-Hazelton, Lenexa, KS. All other agents used in this study were of the highest grade commercially available. Polycarbonate filters (13 mm, 3 µm pore) for transcellular permeability studies were purchased from Nucleopore Corporation, Pleasanton, CA.

Cell Isolation and Culture Methods:

Bovine brain microvessel endothelial cells (BMECs) were isolated from gray matter of the cerebral cortices and were seeded into primary culture as specifically detailed elsewhere (12,13). Briefly, BMECs were isolated by a two-step, dispase and collagenase-dispase enzymatic, and centrifugation method whereby a homogenous population of endothelial cells was isolated over a Percoll gradient. The morphological, functional assymmetric, and immunohistochemical, biochemical, transport, and metabolic characteristics of these BMECs as monolayers in primary culture has also been described previously (12-19). The purity of monolayers of BMECs isolated and grown in primary culture by this method has been estimated to be greater than 95% (16).

Cell Harvesting and Fluorophore Labeling:

BMEC monolayers were harvested by a modified method of Kuhry et al. (20,21). The modified method required a milder trypsin exposure to isolate viable cells grown up in monolayers for fluorescence studies. Briefly, BMECs were grown to confluent monolayers (10-14 days post-seeding) in 100 mm culture dishes and washed three times with 0.01 M phosphate buffered saline (PBS), pH 7.4. The BMECs were

exposed to a solution of 0.5% trypsin and 0.2% EDTA in PBS for 2 min at 37ºC. Excess trypsin and EDTA solution was aspirated off and the incubation was continued for an additional 2 min at 37ºC. Following the second incubation the cells were suspended by gentle agitation in PBS containing 1% bovine serum albumin to inactivate the trypsin. The suspended BMECs were then washed three times by centrifugation at 200 x g for 10 min in fresh PBS. Following the centrifugation, the BMECs were counted and viability (>90%) determined by trypan blue exclusion. Cells were diluted to a final concentration of 2 x 10^5 cells/ml in PBS and used immediately for fluorescence experiments.

BMECs were labeled with DPH by adding 2.5 µl of a 1 mM freshly prepared stock in tetrohydrofuran to 2.5 ml of 2 x 10^5 cells/ml PBS and incubated at 37ºC for 30 min before fluorescence measurements were made. Alternatively, BMECs were labeled with TMA-DPH by adding 2.5 µl of a 0.5 mM freshly prepared stock in dimethylformamide to 2.5 ml of 2 x 105 cells/ml PBS and incubated for 37ºC for 2 min before fluorescence studies were performed. These labeling conditions have been found to allow maximal fluorescent probe incorporation and allow stable measurements over the experimental period (22).

Fluorescence Anisotropy and Lifetime Measurements:

The fluorescence anisotropy was measured with an SLM-AMINCO Subnanosecond Lifetime Fluorometer, Model 4800c, equipped with a Zenith 158 computer for data analysis essentially as described by Audus and Gordon (23) and Audus et al. (22). Briefly, photomultiplier tubes were placed to the right and left of the dual chamber sample cell with Glans-Thompson polarizers inserted in emission and excitation beams. A Schott KV-389 filter was inserted into the horizontally polarized emission beam and an SLM MC320 monochromator set at 430 nm was placed in the vertically polarized emission beam. Fluorescence intensity was first measured with the sample excited (i.e., source was 450 W Xenon lamp) at 360 nm (set with an SLM MC320 monochromator) with a horizontally polarized beam of light, and then measured a second time with the sample excited with a vertically polarized beam of light. Corrected fluorescence anisotrophy data were calculated from the relationships:

$P = (A/B - 1)/(A/B + 1)$

$r = 2P/3-P$

where P was the fluorescence polarization, A was the ratio of fluorescence intensities parallel and perpendicular to the plane of vertically polarized excitation light, B was the ratio of fluorescence intensities parallel and perpendicular to the plane of horizontally polarized excitation light, and r was the fluorescence anisotropy. Light scattering due to turbidity has been found to be negligible at the low concentration of cells (<0.2 absorbance) used in this study both in our laboratory (22) and others (20,21). Light scattering from the light source was reduced to very low levels with a cutoff filter and a monochromator (22). The temperature of the dual chamber was controlled by an external water bath maintained at indicated temperatures and the BMECs were maintained in suspension by stirring, for both labeling and fluorescence experiments, with a magnetic stirrer. Two samples cells (i.e., quartz cuvets) containing BMECs suspended in PBS were always used. One cuvet received indicated drugs and the second, the control cuvet, received the equivalent amounts of solvent in which the drug was dissolved, alone (i.e., in PBS or 95% ethanol). U-74500 was soluble in PBS. All other drugs were dissolved in 95% ethanol. Drugs were prepared fresh before each experiment in a stock concentrations of 10-12 mM. The BMECs were allowed to incubate 5 min after each treatment before an anisotropy measurement was made.

In experiments where the effect of temperature on fluorescence anisotropy was examined, BMEC suspensions were labeled with the appropriate fluorophore as described above and exposed to a single concentration of a 21-aminosteroid. After incubation at 37ºC for 5 min, the suspension was cooled to 5ºC and the fluorescence anisotropy recorded. Where the temperature was varied, the temperature was generally increased by 5ºC and the anisotropy recorded again after a 5 min incubation.

Fluorescence lifetimes were calculated from phase and modulation measurements made with the SLM 4800 fluorometer described above (23). The phase shift and modulation change of each sample relative to a reference was measured alternately 5 times, with the interval between each of about 5 sec. Data was averaged and the lifetimes calculated from the following relationships:

$$\tau_{\varphi} = 1/\omega \tan \theta$$

$$\tau_m = 1/\omega\ (1/M^2 - 1)^{½}$$

where τφwas the phase lifetime in nsec, θ the phase shift in degrees caused by a sinusoidally modulated emission from a fluorophore of a lifetime τ, ω was the angular frequency of excitation which was 2π. modulation freqency (18 or 30 MHz), τ_m the modulation lifetime, and M the demodulation factor of sinusoidally modulated emission from a fluorophore of a lifetime τ (24).

The sample was excited with 360 nm light and emission was monitored through a KV-389 Schott filter on one photomultiplier tube. A rhodamine quantum counter solution was placed in the reference chamber and emission monitored through an RG630 filter on the second multiplier tube. Lifetimes were determined relative to a reference with of known lifetime, dimethyl-POPOP (~ 1.45 nsec) diluted in 95% ethanol to provide appropriate intensities (24). The contents of the sample cuvets were stirred continuously with a magnetic stirrer and the temperature of the sample chamber controlled with an external circulating water bath at 37ºC.

The viability of the BMECs remained >90% as assessed by trypan blue exclusion following exposure to the drugs under the conditions of the studies as described above.

Transcellular Permeability Assay:

Primary cultures of BMECs were grown to confluence on permeable, translucent PC filters. After formation of a monolayer on the PC filter, the PC filters were carefully lifted out of the culture dishes, rinsed gently in 10 ml of fresh PBS by immersion in a second culture dish, and placed in a horizontal side-by-side diffusion cell (Crown Glass Co.) as detailed previously (13,14,18,22). Once placed in the diffusion apparatus, 3 ml of a supplemented buffer (PBSA; 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.74 $MgSO_4$, 5.3 mM glucose, and 0.1 mM ascorbic acid, pH 7.4), was added to each chamber. The donor chamber of the diffusion cell was designated as the chamber facing the side of the PC filter on which the confluent BMEC monolayer was located and the receptor chamber as the opposite side. The diffusion

cells were thermostated with an external water bath to 37ºC and both chambers stirred continously at 600 rpm with magnetic stir bars (22). A 10 µl

aliquot of 21-aminosteroid (40 µM) or equivalent 10 µl amount of the drug's solvent (PBS or 95% ethanol) was added to the donor chamber and allowed to incubate for 5 min. After the incubation, an aliquot of fluorescein was added to the donor chamber to a final concentration of 10 µM. Sample aliquots of 100 µl were removed from the receptor chamber at various subsequent times up to one hour. Fresh buffer was added back to the receptor chamber to maintain constant volumes across the monolayers. Samples were diluted to 1 ml with PBSA in a microcuvet and fluorscence measured with the SLM fluorometer described above with an excitation of 490 nm and emission recorded at 520 nm (22). The viability of the BMEC monolayers remained >90% following the transcellular assay as determined by trypan blue exclusion. In other experiments, 21-aminosteroid effects on the transcellular diffusion of fluorescein across PC filters alone (without BMEC monolayers) were performed as additional controls.

RESULTS

The effect of solvents used for the 21-aminosteroids, either 95% ethanol or PBS, were initially examined for effects on the steady-state fluorescence anisotropy of both TMA-DPH and DPH labeled BMECs. In control experiments, neither PBS nor ethanol significantly altered the steady-state fluorescence anisotropy of the probes (not shown). The average steady-state fluorescence anisotropy remained at 0.162 ± 0.003 and 0.247 ± 0.004 at 37ºC for DPH and TMA-DPH in BMECs, respectively, over the approximately one hour time period required for the experiments (with or without additions of either PBS or 95% ethanol). Ethanol concentrations used in this study were well below those concentrations that decreased the fluorescence anisotropy of DPH in synaptosomal membranes as reported by Harris and Bruno (25). The consistency of the anisotropy measurements and intensity measurements (not shown) over the entire experimental period also suggested stability in the fluorophore-labeling.

Addition of 21-aminosteroids, U-74006F and U-74500A, to labeled BMECs resulted in concentration-dependent increases in the steady-state anisotropy of both DPH and TMA-DPH as shown in Figure 2A and 2B. However, the magnitude of the effects of the 21-aminosteroids were much greater in the DPH-labeled BMECs. By contrast, in Figure 2A and 2B, neither of the methylprednisolone drugs, U-9088 and U-7532, had significant effects on the steady-state anisotropy of either DPH or TMA-DPH. Also shown in Figure 2A and 2B, cholesterol produced changes of similar magnitude in the steady-state anisotropy of both DPH and TMA-DPH. Therefore, cholesterol, as a positive control, confirmed the sensitivity of both DPH and TMA-DPH labeled domains to perturbations under the conditions of this study. Over a similar concentration range, ascorbic acid, an extracellular antioxidant, did not alter the fluorescence anisotropy of either diphenylhexatriene probe (data not shown). Onset of the maximal effects of the 21-aminosteroids and cholesterol on steady-state fluorescence anisotropy of these probes occurred within two to three minutes (data not shown).

The fluorescence anisotropy measurement for DPH reflects a contribution of a kinetic (r_f) component which depends upon the rate of motion and fluorescence lifetime, and a dominant static or structural component (r_s) which reflects an angular constraint on motion (26-29). The structural component was estimated for controls and for a 40 µM 21-aminosteroid treatment of cells labeled with DPH by the relationship $r_s = 1.33r - 0.1$ (9,10). An order parameter, S, analogous to the electron spin resonance (ESR) order parameter was then estimated from $S^2 = r_s/r_o$ (26). The value used for the limiting anisotropy, ro, was 0.362 (27). Exposure of DPH labeled BMECs to 40 µM of either U-74500A and U-74006F increased both the calculated rs and S estimates from control measures as shown in Table I. The greatest 21-aminosteroid-induced changes (46-60%) were associated with the dominant component, r_s, of fluorescence anisotropy. The calculated values suggested either an increased structural order (average molecular packing order) around the probe or alternatively, 21-aminosteroid-induced quenching of the fluorophore which would also increase the fluorescence anisotropy.

Table II summarizes the calculated τ_φ and τ_m lifetimes (24) in the presence and absence of 40 µM of each 21-aminosteroid. The calculated phase fluorescence lifetimes at 30 MHz were most sensitive to U-74006F and U-74500A with observed reductions of 36% and 25%, respectively. These changes suggested the possibility of fluorescence quenching (direct drug:DPH interactions).

Except at high concentrations of U-74006F, changes in the fluorescence anisotropy of DPH-labeled domains were mirrored in smaller magnitude in TMA-DPH-labeled domains of BMECs following 21-aminosteroid exposure. The τ_φ and τ_m of TMA-DPH labeled BMECs was not sensitive to the presence of 40 µM 21-aminosteroids, as shown in Table III. The latter results suggested the absence of significant drug:TMA-DPH interactions.

The steady-state anisotropy of DPH labeled BMECs exposed to either PBS, in Figure 3A, or 95% ethanol, in Figure 3B, was temperature-dependent. As expected, a distinct phase transition (for anisotropy versus temperature) was not observed for BMECs due to the heterogeneity of the fluorophore-labeled cellular domains. Exposure to U-74500A produced a relatively parallel upward shift in the phase transition curve for DPH in BMECs as shown in Figure 3A. In contrast, the effect of U-74006F on the phase transition curve for DPH-labeled BMECs was reduced at lower temperatures, as indicated in Figure 3B. These observations were more obvious and consistent in the concentration-dependent profiles of the 21-aminosteroids at 37º, 30º and 25ºC in Figures 4A and 4B. As summarized in Figure 5A and 5B, U-74006F, reduced the fluorescence anisotropy of TMA-DPH and U-74500 had little or no effect on TMA-DPH fluorescence anisotropy with increasing concentration and decreasing temperature. From these results, U-74006F seemed to produce a disordering (decreased anisotropy) of the superficial cell domains more effectively at lower temperatures.

The transcellular permeability of BMEC monolayers to a membrane impermeant marker, fluorescein, were not altered by 21-aminosteroids (not shown). The 40 µM concentration of the drug used in these studies was chosen from the DPH steady-state anisotropy studies where significant changes in the labeled cell domains were observed. As for the fluorescence anisotropy studies above, the concentrations of ethanol

used here were well below those observed to alter the transcellular permeability of BMEC monolayers (unpublished observations, P.M. Reardon and K.L. Audus) and the BBB in vivo (30). The viability of cell preparations in both the fluorescence and transcellular studies assessed by trypan blue exclusion was not altered by 21-aminosteroid exposure (data not shown).

DISCUSSION

The basis for development of the 21-aminosteroids, or Lazaroids, was that they specifically localize in hydrophobic domains of cell membranes and inhibit lipid peroxidation reactions (4). Membrane level activity has been implied by a few studies. Agents such as arachidonate that produce vasogenic edema subsequent to disruption of the blood-brain barrier, can be blocked by pretreatment with U-74006F in vivo suggesting that this compound modulates permeability (5). Activity at the membrane level was further supported by evidence that 21-aminosteroids block release of arachidonate from injured cell membranes (6).

Brain microvessel endothelial cells, potential cellular sites of action for these compounds (3), were labeled with two well characterized fluorescent membrane probes to provide further support for the activity of 21-aminosteroid either on or within the cell membrane systems. The cationized probe, TMA-DPH, localizes at the lipid-water interface and can be used to measure the lipid order (average molecular packing) changes at the surface of cells or in the glycerol side-chain domains of membranes (7,8,11). This probe remains on the surface of living endothelial cells for up to 4 h (31). The second probe, DPH, is used to measure the average molecular packing order of the deeper lipid or hydrophobic regions of membranes throughout the cell (9-11). In this study we observed the apparent selectivity of 21-aminosteroids for interactions with a DPH labeled domain over a TMA-DPH labeled domain within the BMECs at 37ºC. This observation was not unusual. Harris and Bruno (25), for instance, have observed that ethanol's effects were greatest in DPH-labeled domains of synaptosomes. Like the 21-aminosteroids, ethanol (25) had only minor effects on TMA-DPH labeled domains in the synaptosomes.

Evidence for the interactions of the 21-aminosteroids with lipid or hydrophobic domains of BMECs was supported by the collective fluorescence anisotropy, lifetime, and temperature-dependence data of DPH and TMA-DPH. If the fluorescence lifetime of DPH remains reasonably constant, an increased fluorescence anisotropy is reflective of a more highly constrained motion and lowered fluidity (29). Fluorescence anisotropy measures for DPH are generally interpreted as the average molecular packing order of membrane lipid or hydrophobic domains. For example, an increase in steady-state fluorescence anisotropy is consistent with an increase in lipid packing order (i.e., a decrease in what is often mistakenly referred to as "membrane fluidity") (9,10,26,29). The 21-aminosteroid-induced increase in steady-state anisotropy was reflected in substantial increases in the static component of anisotropy (46 to 60%) and the order parameter, *S* (21-26%). The dynamic component of anisotropy, the parameter sensitive to probe rate of motion and lifetime, which becomes less significant as the anisotropy increases, was observed to decrease by about 28 to 35%. However, to state that the increased anisotropy here definitively represents an increase in the average lipid packing order of the BMEC membrane requires additional information on the lifetime and motional changes of DPH.

The magnitude and heterogeneity of our lifetime measurements for DPH and TMA-DPH, in the absence of 21-aminosteroids, were in good agreement with those reported for endothelia and model membrane systems (11,28,31). We observed 25-36% decreases in the τ_{φ} for DPH at 30 MHz. The magnitude of these changes may or may not be large enough to contribute to the increase in the fluorescence anisotropy through 21-aminosteroid:DPH interactions which quench probe fluorescence (29). In order for the fluorescence lifetime to significantly affect the anisotropy of DPH, presuming there were no motional changes, the lifetime would have to be reduced almost by one half (29). Since the lifetime was reduced information on the motional changes of DPH in BMECs following exposure to 21-aminosteroids would be important in appropriately analyzing the anisotropy changes. Unfortunately, a more rigorous analysis of 21-aminosteroid effects on the motional changes of DPH in complex biomembranes based on fluorescence lifetime heterogeneity with only two modulating frequencies (18 and 30 MHz) may not be valid. Accordingly,

we have interpreted the changes in the respective fluorescence anisotropy and lifetime information recorded here as reflecting potentially both a 21-aminosteroid-induced ordering of hydrophobic domains labeled with DPH and direct 21-aminosteroid:DPH interactions. Agents such as tricyclic antidepressants (23,32) and ethanol (25) are capable of altering anisotropy without affecting the fluorescence lifetime significantly. Thus, the altered fluorescence anisotropy of DPH in model and biological membrane systems in those studies reflected lipid packing order changes. Since, the lifetime of the other probe in this study, TMA-DPH, was not affected by the 21-aminosteroids, the fluorescence anisotropy of TMA-DPH reflected a minor ordering of the superficial domains of the BMEC in the presence of the 21-aminosteroids (with exception of high concentrations of U-74006F) at 37øC.

The interactions of the 21-aminosteroids with membranes would not be expected to be similar due to the differing chemical nature of the two agents. As a result of these different chemical properties, the U-74500A is much more water-soluble than U-74006F. Although the fluorescence anisotropy and lifetime data suggest a similarity in drug interactions with DPH labeled domains at 37ºC, differences in interactions with the BMECs were obvious in the temperature-dependence studies. Particularly notable was the reversal in U-74006F effects on the fluorescence anisotropy of DPH at low temperatures and an increased disordering effect of the drug on BMEC lipid order (i.e., the decreased fluorescence anisotropy of TMA-DPH) at lower temperatures. Drawing on observations for lindane, cholesterol (33), and tricyclic antidepressants (32) in model membranes, the interactions of U-74006F could indicate either an affinity of the drug for labeled membrane domain depending on the phase transition state of the lipids or a temperature-dependent displacement of the drug from the inner (DPH-labeled) to outer (TMA-DPH-labeled) membrane domains depending on the phase transition of surrounding lipids. By contrast, U-74500A exposure produced changes in fluorescence anisotropy in inner (DPH-labeled) membranes remained rather constant in magnitude with decreasing temperature. The phase transition of lipids was apparently not important in U-74500A interactions with deeper membrane domains. The minor interactions of U-74500A with outer (TMA-DPH-labeled) membranes were eliminated with decreasing temperature, perhaps an indication that phase

transition of superficial BMEC lipid domains may be important in small degree in determing the cellular distribution of U-74500A. Identifying those specific intracellular domains that may be affected by 21-aminosteroids and subsequent drug-induced changes will require more specific labeling and measures. However, these studies will form the basis for considering the complex interactions of 21-aminosteroids within specific hydrophobic or lipid cell domains. For instance, the more widespread distribution of a 21-aminosteroid into inner and outer portions of cell membranes might help explain the agent's antioxidant efficiency. The effect of the agent on lipid packing order may not be an important focal point.

The concentrations at which 21-aminosteroid-induced changes in the steady-state fluorescence anisotropy of DPH occur were consistent with their 2-60 μM IC_{50}s for effective inhibition of iron-dependent lipid peroxidation in vitro (4). Methylprednisolone, for which 21-aminosteroids were developed to mimic, are effective at higher concentrations in inhibiting lipid peroxidations (4). The methylprednisolones used in this study did not alter steady-state anisotropy measures over the concentration range examined. Cholesterol, also a weak inhibitor of lipid peroxidation, was examined in this study as a positive control. As opposed to the methylprednisolones, cholesterol has strong interactions at lower concentrations with biomembranes. Cholesterol:membrane interactions include a molecular ordering effect within lipid or hydrophobic domains (9,34). Moreover, van Ginkel et al. (34) has demonstrated that cholesterol has similar effects on fluorescence anisotropy measures of both DPH and TMA-DPH in different model membranes. In those model membranes, cholesterol was shown to increase both the average molecular order and the motional parameters for DPH (34). Cholesterol behaved similarly in this study with respect to the increased steady-state anisotropy measures for both DPH and TMA-DPH in BMECs. These findings suggest that the TMA-DPH probe was responsive to molecular ordering perturbations of the superficial domains of BMECs. Our results suggest that 21-aminosteroids do interact with TMA-DPH labeled superficial hydrophobic regions of BMECs but perhaps not to the same degree as cholesterol. An extracellular antioxidant, ascorbic acid, was used as a negative control and had no effect on the fluorescence parameters of the membrane associated fluorophores in this study.

Alterations in the average packing order of membranes by ethanol in DPH labeled synaptosomes has been correlated with changes in membrane ion permeability (25). Agents that interact with BMEC monolayer membrane systems such as aluminum, ethanol, angiotensin, bradykinin, and phorbol esters, have also been shown to alter permeability pathways in this in vitro system much like they influence the corresponding in vivo functions of the cells (18,22). Fluorescein is a membrane-impermeant marker that would indicate changes in monolayer integrity due to alteration of nonspecific transcellular pathways or possibly toxicity to cells. Despite having interations with hydrophobic domains of BMECs, 21-aminosteroids did not alter the transcellular permeability of the BMECs under the conditions of this study. However, this finding does not preclude the possibility that specific transport functions in the BMECs such as ion carriers may be influenced by these 21-aminosteroids in the absence of lipid peroxidation. Consistent with the retention of monolayer integrity was the lack of viability changes as judged by trypan blue exclusion following the transcellular experiments. Future studies will consider the role of 21-aminosteroids in modulating BMEC monolayer permeability following insults that stimulate lipid peroxidation. Establishing such a model may permit the characterization other membrane or biochemical level events modulated by these compounds under pathophysiological conditions.

In summary, we conclude that 21-aminosteroids do localize in or very near lipid or hydrophobic domains of BMECs as suggested by the fluorescence anisotropy and lifetime measures of DPH and to a lesser degree, TMA-DPH. Since DPH diffuses throughout the cell, we can not speculate on the precise membrane localization of the 21-aminosteroids in the intact cell at this time. However, the more efficient inhibitor of lipid peroxidation, U-74006F, was determined to be localized in or near both inner and outer membrane domains depending on concentration and the temperature. Intracellular lipid or hydrophobic domains throughout the cell presumably represent potential sites for lipid peroxidation and the region to which the 21-aminosteroids were specifically designed to target. The potential role of selective drug:membrane association in relation to 21-aminosteroid effectiveness in inhibiting lipid peroxidation remains to be established.

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ABBREVIATIONS

BMEC brain microvessel endothelial cell

CNS central nervous system

Dimethyl POPOP 1,4-bis[4-methyl-5-phenyl-2-oxazoyl]benzene

DPH 1,6-diphenyl-1,3,5-hexatriene

PBS phosphate buffered saline

TMA-DPH 1-(4-trimethylammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate

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TABLE I. Effects of 21-Aminosteroids on Static and Dynamic Components of the Steady-State Fluorescence Anisotropy of 1,6-Diphenyl-1,3,5-hexatriene (DPH) in Bovine Brain Microvessel Endothelial Cells.

Parameter*	Control	40 μM U-74006F	40 μM U-74500A
r	0.162 ± 0.003	0.213 ± 0.001	0.202 ± 0.005
r_s	0.116	0.183	0.169
r_f	0.046	0.030	0.033
S	0.566	0.711	0.683

* r is the steady-state fluorescence anisotropy; r_s is the structural or static component of anisotropy; r_f is the kinetic or dynamic component of anisotropy; and S is the order parameter for DPH as defined by Heyn (26) and van Blitterswijk et al. (9).

Data represent the means ± standard deviation for three to five membrane preparations.

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TABLE II. Effects of 21-Aminosteroids of the Fluorescence Lifetimes of 1,6-Diphenyl-1,3,5-hexatriene (DPH) Labeled Bovine Brain Microvessel Endothelial Cells (BMECs) at 37ºC.

	Calculated Fluorescence Lifetimes*			
	30 MHz		18 MHz	
Treatment	τ_φ nsec	τ_m nsec	τ_φ nsec	τ_m nsec
10 µl PBS	7.5 ± 0.1	8.8 ± 0.1	7.9 ± 0.1	9.1 ± 0.2
10 µl 95% Ethanol	7.5 ± 0.2	8.8 ± 0.1	8.0 ± 0.1	9.1 ± 0.2
40 µM U-74006F	4.8 ± 0.1	8.1 ± 0.2	6.1 ± 0.1	9.2 ± 0.2
40 µM U-74500A	5.6 ± 0.2	7.7 ± 0.2	7.1 ± 0.l	8.7 ± 0.1

* τ_φ is the phase lifetime and τ_m is the modulation lifetime of DPH in BMECs (24).

Data represent the means ± standard deviation for three to five membrane preparations.

Audus, K.L., Guillot, F.L., and Braughler, J.M. (1991) Evidence for 21-aminosteroid association with the hydrophobic domains in brain microvessel endothelial cells. Free Rad. Biol. Med. 11, 361-371. PMID: 1797623. Publisher's official version: http://dx.doi.org/10.1016/0891-5849(95)00049-4. Open Access version: http://kuscholarworks.ku.edu/dspace/.

TABLE III. Effects of 21-Aminosteroids of the Fluorescence Lifetime of 1-(4-Trimethyammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH) Labeled Bovine Brain Microvessel Endothelial Cells (BMECs) at 37ºC.

	Calculated Fluorescence Lifetimes*			
	30 MHz		18 MHz	
Treatment	τ_φ nsec	τ_m nsec	τ_φ nsec	τ_m nsec
10 µl PBS	4.7 ± 0.1	5.5 ± 0.1	4.9 ± 0.4	5.9 ± 0.3
10 µl 95% Ethanol	4.6 ± 0.1	5.3 ± 0.1	5.1 ± 0.4	5.9 ± 0.2
40 µM U-74006F	4.7 ± 0.1	5.4 ± 0.3	5.0 ± 0.4	5.6 ± 0.3
40 µM U-74500A	3.9 ± 0.2	4.8 ± 0.2	4.5 ± 0.2	5.0 ± 0.3

* τ_φ is the phase lifetime and τ_m is the modulation lifetime for TMA-DPH in BMECs (24).

Data represent the means ± standard deviation for three to five membrane preparations.

FIGURE LEGENDS

FIGURE 1. Structures of 21-Aminosteroids (4).

FIGURE 2. Concentration-Dependent Effects of Selected Drugs on the Steady-State Fluorescence Anisotropy of A) 1,6-Diphenyl-1,3,5-hexatriene (DPH) and B) 1-(4-Trimethyammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH) in Bovine Brain Microvessel Endothelial Cells at 37ºC. Data represent the means for three to five membrane preparations. The standard deviations of data points were 1 to 4% of the means.

FIGURE 3. Temperature-Dependent Effects of 21-Aminosteroids, A) U-74500A and B) U-74006F, on the Steady-State Fluorescence Anisotropy of 1,6-Diphenyl-1,3,5-hexatriene (DPH) in Labeled Bovine Brain Microvessel Endothelial Cells. Data represent the means for three to five membrane preparations. The standard deviations of data points were 1 to 4% of the means.

FIGURE 4. Concentration-Dependent Effects of A) 21-Aminosteroid U-74500A and B) U-74006F Addition on the Steady-State Fluorescence Anisotropy of 1,6-Diphenyl-1,3,5-hexatriene (DPH) in Labeled Bovine Brain Microvessel Endothelial Cells at 25, 30 and 37ºC. Data represent the means for three to five membrane preparations. The standard deviations of data points were 1 to 4% of the means.

FIGURE 5. Concentration-Dependent Effects of A) 21-Aminosteroid U-74500A and B) U-74006F Addition on the Steady-State Fluorescence Anisotropy of 1-(4-Trimethyammoniumphenyl)-6-diphenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH) in Labeled Bovine Brain Microvessel Endothelial Cells at 25, 30 and 37ºC. Data represent the means for three to five membrane preparations. The standard deviations of data points were 1 to 4% of the means.

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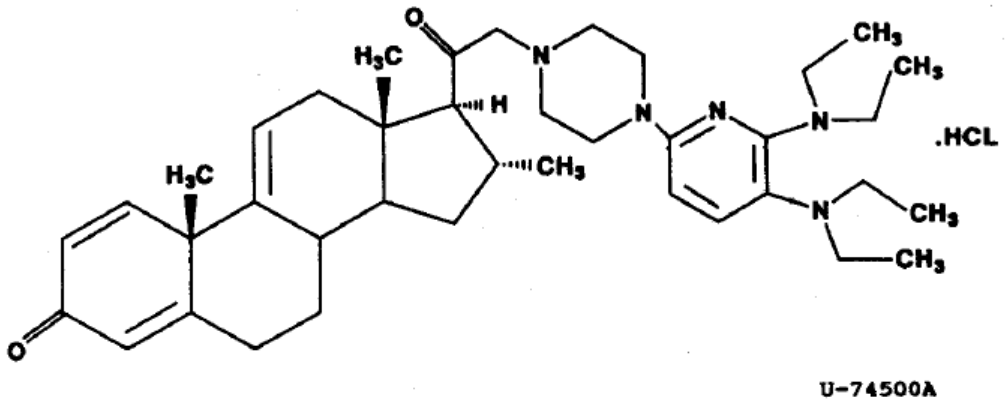
Figure 1



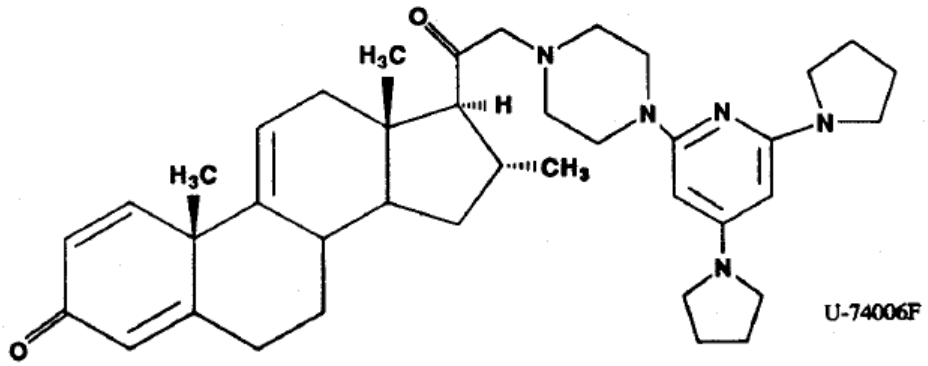


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Figure 2

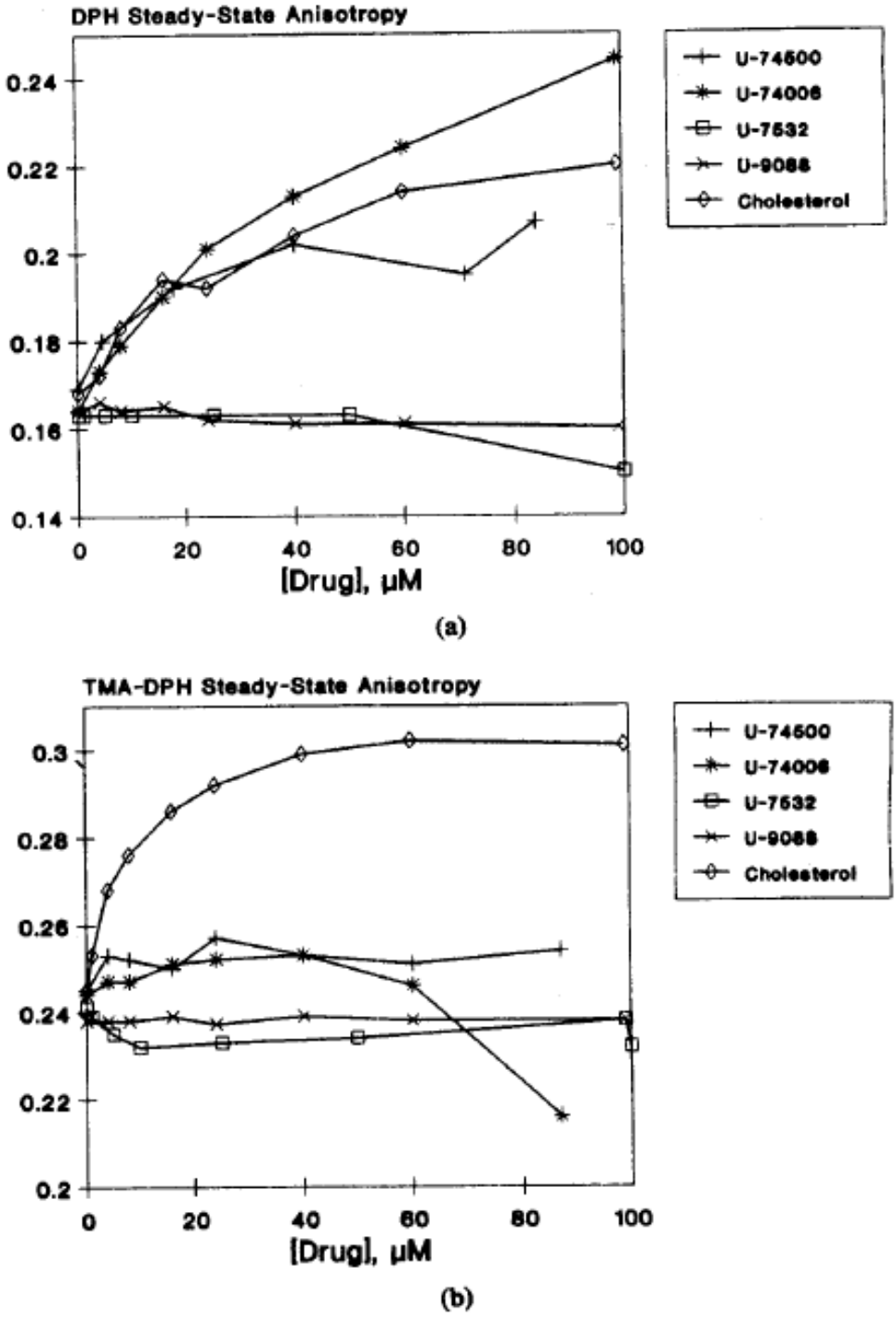

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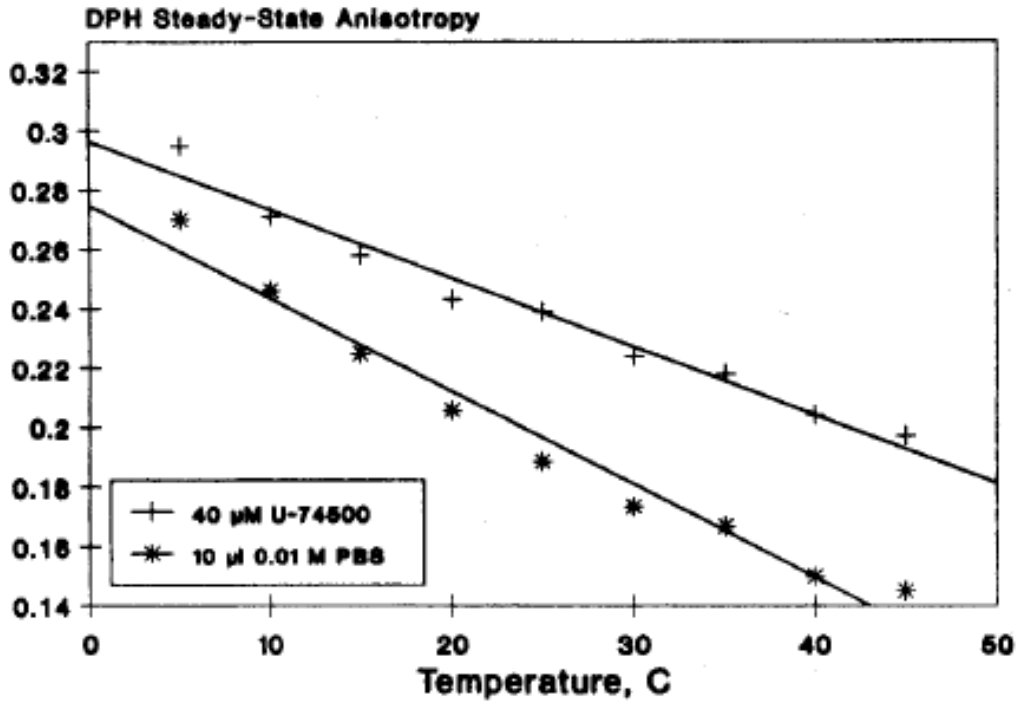
Figure 3



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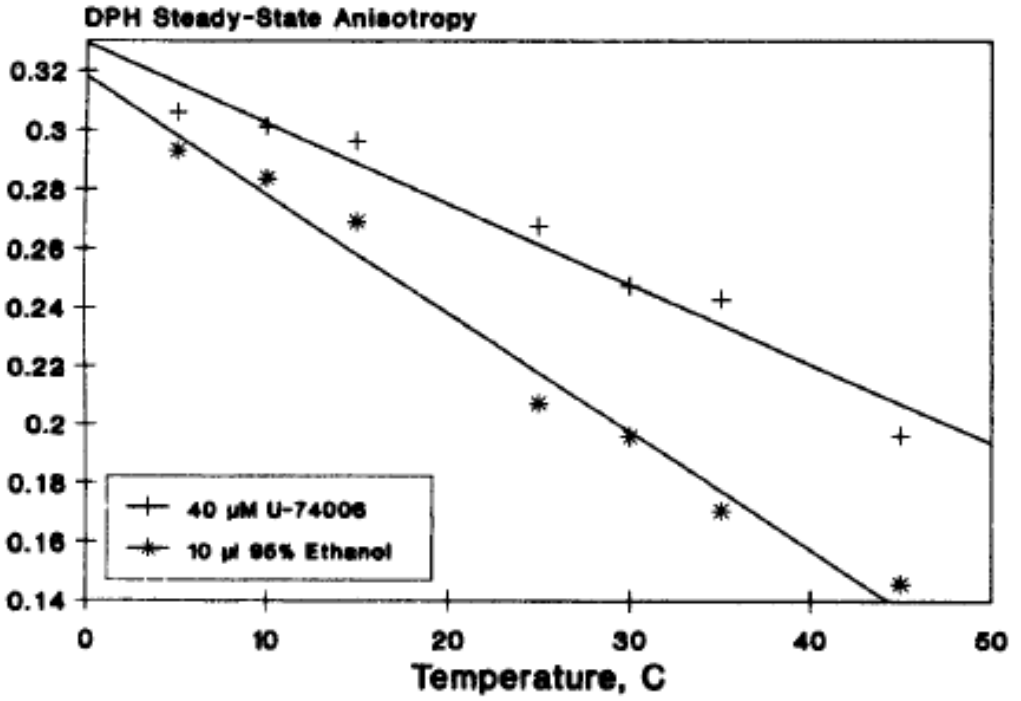

(b)

Audus, K.L., Guillot, F.L., and Braughler, J.M. (1991) Evidence for 21-aminosteroid association with the hydrophobic domains in brain microvessel endothelial cells. Free Rad. Biol. Med. 11, 361-371. PMID: 1797623. Publisher's official version: http://dx.doi.org/10.1016/0891-5849(95)00049-4. Open Access version: http://kuscholarworks.ku.edu/dspace/.

Figure 4

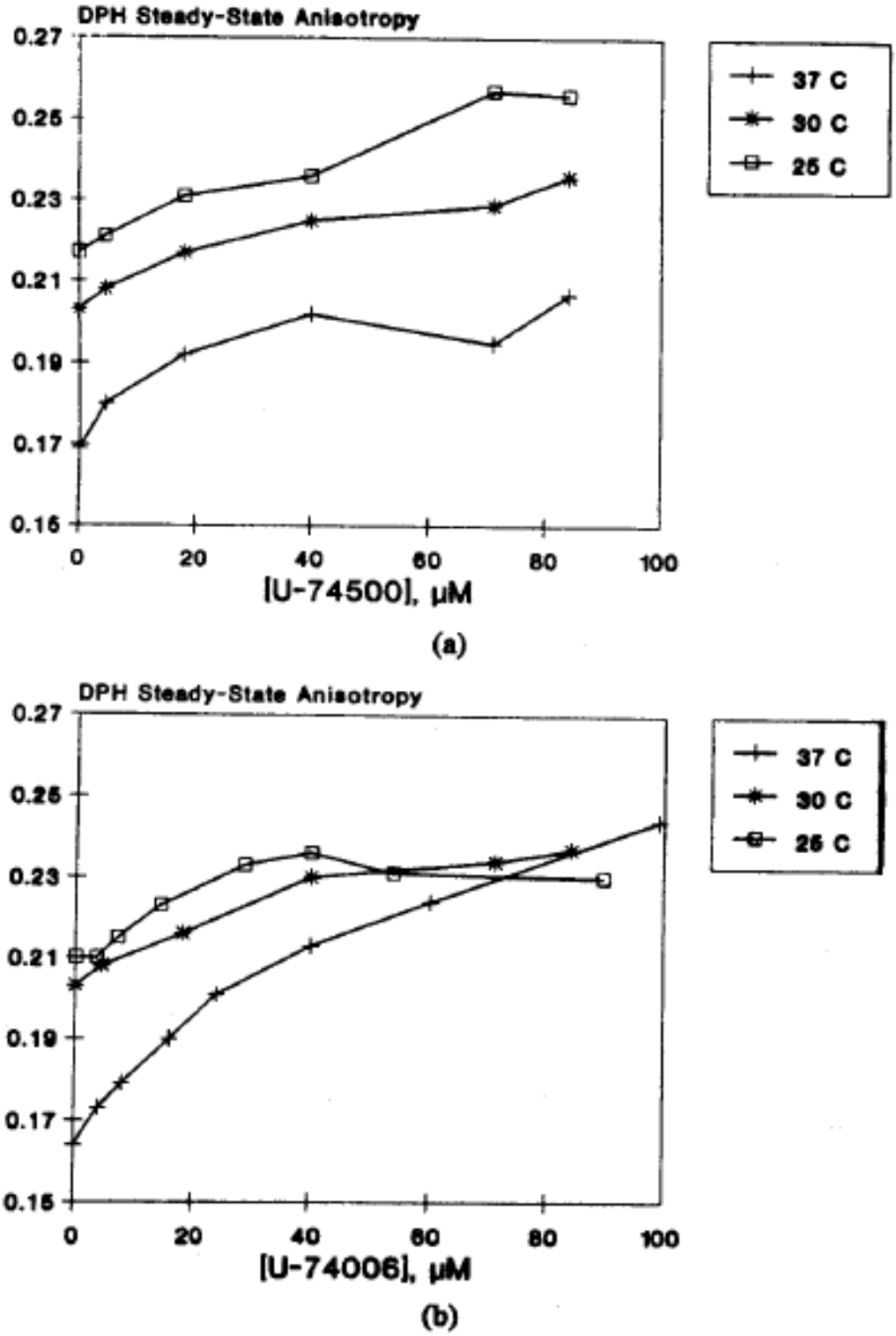

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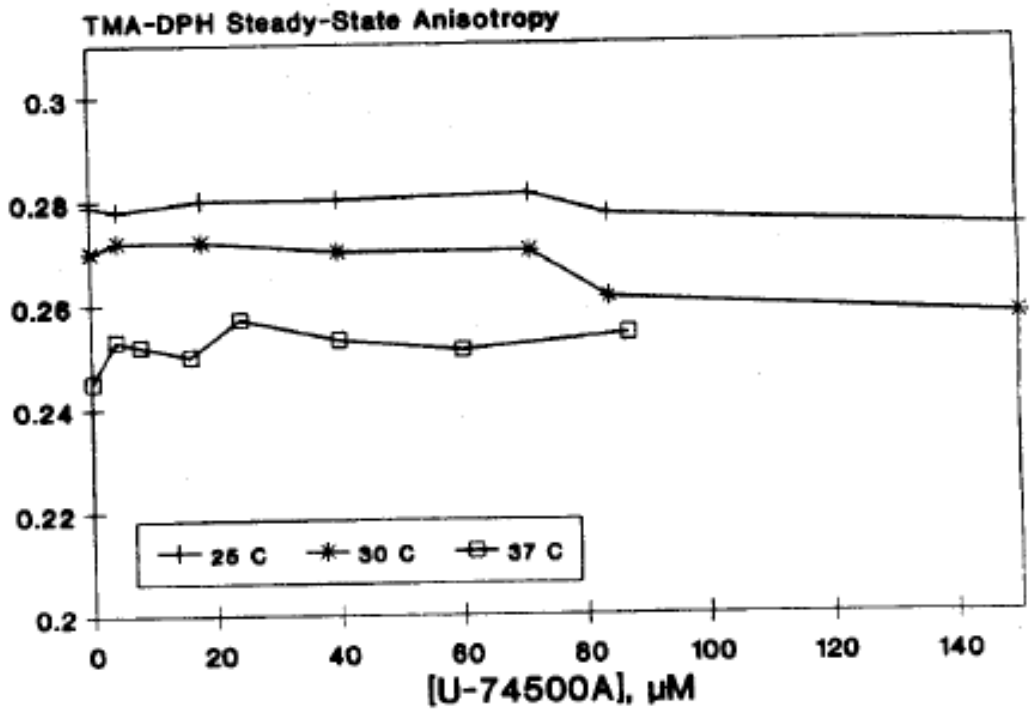
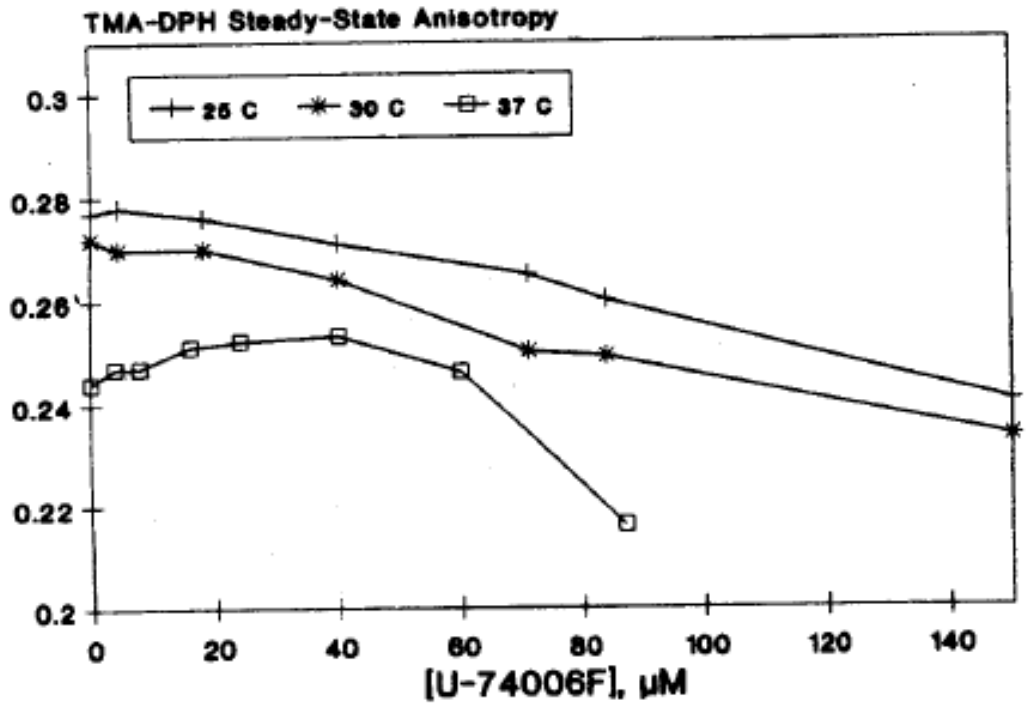
Figure 5



(a)



(b)