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INHIBITION OF *IN VITRO* LIPID PEROXIDATION BY 21-AMINOSTEROIDS

EVIDENCE FOR DIFFERENTIAL MECHANISMS

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Abstract—In a previous report (Ryan and Petry, *Arch Biochem Biophys* **300**: 699–704, 1993), the effects of two 21-aminosteroids (U-74500A and U-74006F) on the oxidation and reduction of iron in a buffer/organic solvent system were investigated. In those studies, U-74500A was found to be an efficient iron reductant and potential iron chelator, whereas U-74006F had little effect on iron redox chemistry. As an extension of those studies, we now report the effects of U-74006F and U-74500A on lipid peroxidation in systems that are dependent upon iron oxidation/reduction. In liposomes, U-74500A inhibited ADP:Fe(II)-dependent lipid peroxidation in a concentration-dependent manner, whereas U-74006F was minimally effective in this system. The mechanism of U-74500A-dependent inhibition probably involved interactions with iron, as iron oxidation was inhibited in the presence of this compound. No effects on iron oxidation were observed in the presence of U-74006F. Addition of Ferrozine® to liposomal incubation mixtures indicated that at least two iron pools were present in samples containing U-74500A, one immediately bound by Ferrozine®, and another that was bound more slowly. Furthermore, ADP:Fe(III)/ascorbate-dependent lipid peroxidation was blocked completely by U-74500A, presumably by formation of a redox inert complex upon reduction of the iron. U-74500A partially protected ADP:Fe(II) from oxidation by H_2O_2 and lipid hydroperoxides, indicating that the U-74500A:iron complex was stable in the presence of biologically relevant oxidants. U-74006F did not markedly affect iron oxidation or reduction when incorporated into phospholipid liposomes. In microsomal lipid peroxidation systems containing ADP:Fe(III) and NADPH, both U-74500A and U-74006F inhibited lipid peroxidation. U-74006F-dependent inhibition of microsomal lipid peroxidation was dependent on both NADPH and Fe(III). Further, it was enhanced when U-74006F was allowed to preincubate in this system prior to iron addition. Preincubation of U-74006F with microsomes, NADPH, and ADP:Fe(III) produced several metabolites detectable by HPLC. These results suggest that U-74500A inhibits lipid peroxidation by directly affecting iron redox chemistry, whereas U-74006F-mediated inhibition is enhanced by preincubation with a metabolically competent microsomal system.

Alterations in membrane integrity resulting from the oxidative modification of membrane phospholipids have been implicated in the pathogenesis of a variety of diseases, sequelae to ischemic events, and exposure to redox active xenobiotics [1–3]. Although lipid peroxidation is not always the causative agent in cellular damage induced by free radical-mediated oxidations, it has been implicated in many pathological conditions in which a role for oxidative stress has been suggested [1, 4, 5]. Lipid peroxidation is a free radical process that requires an electron-deficient species capable of abstracting a labile hydrogen atom from a polyunsaturated fatty acid for initiation. Radicals capable of initiating lipid peroxidation can be derived from the combination of oxygen and redox active metals, and the initiator concentration need not be very large to cause widespread damage to cellular membranes [6, 7]. The transition metal iron is of particular interest as a catalyst of lipid peroxidation because of its biological relevance and its proven ability to react with oxygen (either molecular [O_2] or partially reduced [$O_2^{\cdot}$, H_2O_2]) to form species capable of initiating peroxidative events [3, 6–9]. Because only catalytic amounts of iron are required to promote oxidation of cellular components, sequestration of catalytic iron from biological systems would seem to be a rational approach for prevention and/or treatment of free radical-mediated toxicities. In accord with this hypothesis, several metal chelating antioxidants have been developed, which are effective inhibitors of *in vitro* lipid peroxidation [10–12] and at least partially effective versus *in vivo* pathologies [12–15].

The 21-aminosteroids U-74500A and U-74006F (tirilazad mesylate, Freedox®) were designed to serve as membrane-specific antioxidants [16, 17]. While U-74500A has consistently been shown to be a potent inhibitor of iron-dependent lipid peroxidation in all *in vitro* model systems tested, U-74006F has proven only minimally effective in certain *in vitro* model systems [16–21]. In contrast, U-74006F has been shown to be an excellent inhibitor of *in vivo* toxicities associated with oxidative injury, most notably the sequelae associated with central nervous system trauma [22–25]. Accordingly, U-74006F has been chosen as a clinical candidate and

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is currently undergoing trials for the treatment of head injury, ischemic stroke, subarachnoid hemorrhage and spinal cord injury.

Previously, we have shown that U-74500A and U-74006F are different with respect to their abilities to affect the redox activity of iron in the absence of lipid. In those studies, U-74500A was an effective iron reductant and a potential iron chelator, whereas U-74006F was much less active in this regard [26]. The present studies further explore the interactions of U-74500A and U-74006F with iron, assessing mechanistic aspects of lipid peroxidation inhibition by these two compounds in liposomal and microsomal systems.

MATERIALS AND METHODS

The 21-aminosteroids U-74500A (21-[4-[5,6-bis-(diethylamino) - 2 - pyridinyl] - 1 - piperazinyl] - 16α-methyl-pregna-1,4,9(11)-triene-3,20 dione, hydrochloride), and U-74006F (21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl] -16α-methyl - pregna-1,4,9(11)-triene-3,20 dione, monomethane sulfonate; tirilazad mesylate, Freedox®) were synthesized within The Upjohn Co. Ferrozine® (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine), $FeCl_2$, $FeCl_3$, and ADP (Grade (III) from yeast) were obtained from the Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (30%) was purchased from Mallinckrodt (Paris, KY). Buffers used throughout these studies were chromatographed over Chelex 100 ion exchange resin (Bio-Rad, Cincinnati, OH) to minimize the effects of adventitious metals.

Liver microsomes were isolated from 250–300 g male Sprague–Dawley rats (Charles River, Portage, MI) using differential centrifugation as described by Pederson and Aust [27], stored at −70°, and used within a month of preparation. Microsomal phospholipids were extracted from freshly isolated microsomes as per Folch *et al.* [28]. All solvents were argon purged and all steps were carried out at 4°. Lipid was quantitated by phosphate analysis according to Bartlett [29], stored at −20° in argon-purged chloroform/methanol (2:1), and used within a month of preparation. Lipid peroxidation (thiobarbituric acid-reactive substances; TBARS) was assayed as per Buege and Aust [30], using 25-mL beakers containing either 2 or 3 mL incubation volumes with gentle shaking at 37°. Specific incubation conditions are indicated in the figure legends. TBARS were measured by removing 0.5-mL aliquots at given time points and combining them with 2.0 mL TBA reagent. Iron oxidation experiments were performed by removing aliquots from the lipid peroxidation incubations described above and combining with the Fe(II) chelator Ferrozine® as described previously [31]. Test compounds were dissolved in ethanol and added to aqueous incubation mixtures so that the ethanol concentration in test incubations or vehicle controls was 1% (v/v).

HPLC analysis of microsomal incubations containing U-74006F was performed using a PE410 pump (Perkin Elmer, Norwalk, CT), a Perkin Elmer LC 235 UV detector, and a PE ISS-100 autosampler.

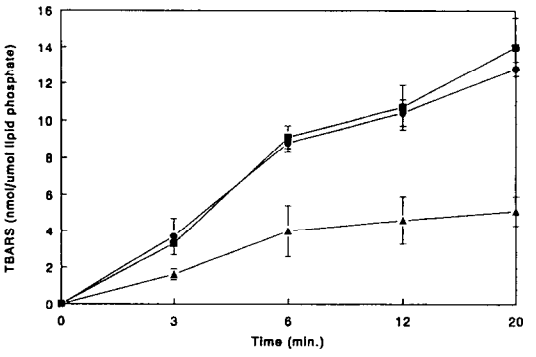

Fig. 1. Time-dependent effects of U-74500A and U-74006F on ADP:Fe(II)-dependent liposomal lipid peroxidation. Incubations contained ADP:Fe(II) (250 μM:50 μM) and phospholipid liposomes (1 μmol lipid phosphate/mL), in 0.2 M sodium acetate buffer, pH 7.0. Additions were as follows: control (1% ethanol vehicle) (■), 50 μM U-74500A (▲), or 50 μM U-74006F (●). Aliquots were removed at the indicated times and assayed for TBARS as described in Materials and Methods. Values are the means ± SD of three separate experiments.

Analytical separations were performed using a Kromasil 5 μM C18 column (250 × 4.6 mm; Bodman, Aston, PA). The mobile phase consisted of solvent A (100 mM ammonium acetate) and solvent B (100% acetonitrile). A 60-min linear gradient (0.5 mL/min) from 20 to 100% B was used, followed by 40 min at 100% B. UV absorbance was monitored at 230 nm. Detection of ^{14}C-labeled peaks (for confirmatory studies) was accomplished using a Radiomatic model 200 flow counter equipped with a 0.5-mL homogenous flow cell. Flow scint II scintillation fluid (Radiomatic, Meriden, CT) was introduced post column at a rate of 1.5 mL/min. Incubations were prepared for HPLC by dilution of microsomal incubations with equal volumes of mobile phase A and subsequent injection of 150 μL on column.

RESULTS

When added to a liposomal lipid peroxidation system, U-74500A, at a concentration equal to that of exogenous ADP:Fe(II), effectively inhibited TBARS formation (Fig. 1). In this system, U-74006F had no significant inhibitory effect. The effects of U-74500A were concentration dependent, with a substantial decrease in TBARS production at concentrations as low as 1 μM (Table 1).

Addition of the colorimetric Fe(II) indicator, Ferrozine®, to these mixtures after 20 min of incubation indicated that all of the added Fe(II) had been oxidized in the absence of U-74500A (Fig. 2). Note that times indicated on the abscissa are hours after quenching the 20-min incubation. U-74006F had no effect on this rate of Fe(II) auto-oxidation, as no difference between U-74006F and control experiments was observed at time = 0. U-74500A had marked effects on iron oxidation during this phase, however, as approximately 50 nmol of the original 150 nmol Fe(II) added was detected as

Table 1. Concentration-dependent effects of U-74500A and U-74006F on ADP:Fe(II)-dependent liposomal lipid peroxidation

	TBARS (nmol/μmol lipid P)
Control	30.8 ± 3.6
U-74500A (50 μM)	7.3 ± 1.0
U-74500A (10 μM)	13.0 ± 1.6
U-74500A (1 μM)	22.5 ± 2.3
U-74006F (50 μM)	26.1 ± 1.0
U-74006F (10 μM)	28.8 ± 1.3
U-74006F (1 μM)	34.3 ± 4.6

Incubations contained ADP:Fe(II) (250 μM:50 μM), phospholipid liposomes (1 μmol lipid phosphate/mL) and the indicated amount of drug or vehicle (1% ethanol) in 0.2 M sodium acetate buffer, pH 7.0. TBARS were measured as described in Materials and Methods after 20 min of incubation. Values are the means ± SD of three independent samples.

Table 2. Effects of U-74500A and U-74006F on ADP:Fe(III)-dependent liposomal lipid peroxidation

Additions	TBARS (nmol/μmol lipid P)	Fe(II) (nmol)
None	0.8 ± 0.3	0 ± 0
Fe(III)	1.3 ± 0.5	0 ± 0
Fe(III)/Asc.	10.2 ± 0.0	0 ± 0
Fe(III)/U-74500A	0.7 ± 0.3	9.3 ± 4.4
Fe(III)/U-74006F	0.7 ± 0.0	10.9 ± 2.6
Fe(III)/U-74500A/Asc.	2.0 ± 0.2	91.8 ± 13.8
Fe(III)/U-74006F/Asc.	8.4 ± 0.2	2.1 ± 1.3

Incubations were performed using phospholipid liposomes (1 μmol lipid phosphate/mL) in 0.2 M sodium acetate buffer (pH 7.0) with or without the following additions: ADP:Fe(III) (250 μM:50 μM), ascorbate (25 μM), and/or 21-aminosteroid (50 μM) or vehicle (1% ethanol). TBARS and Fe(II) concentrations were measured as described in Materials and Methods following 20 min of incubation. Values are the means ± SD of three independent samples.

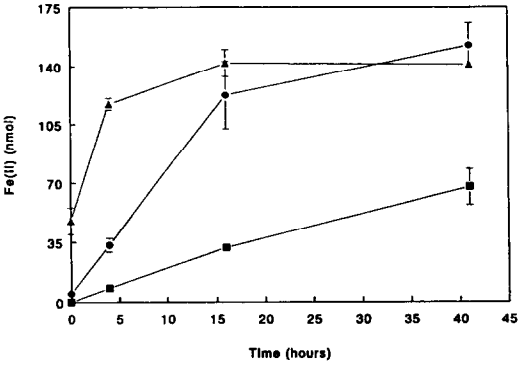

Fig. 2. Effects of U-74500A and U-74006F on ADP:Fe(II) oxidation in the presence of liposomes. Incubation conditions were as described in the legend of Fig. 1 [3 mL, 15 nmol (50 μM) Fe(II)]. After a 20-min incubation, 0.5-mL aliquots were removed and assayed for Fe(II) (time = 0) as described in Materials and Methods. Absorbance measurements were then recorded at the indicated times post quench. Key: control (1% ethanol vehicle) (■), 50 μM U-74500A (▲), and 50 μM U-74006F (●). Values are the means ± SD of three independent experiments.

Table 3. Effects of U-74500A and U-74006F on H_2O_2-mediated iron oxidation in solution

Additions	Fe(II) (nmol)
Control time = 0 min (quench)	55.3 ± 1.7
Control time = 5 min	3.2 ± 0.6
25 μM U-74500A	2.4 ± 0.6
50 μM U-74500A	1.4 ± 0.4
100 μM U-74500A	18.9 ± 2.5
25 μM U-74006F	1.9 ± 0.3
50 μM U-74006F	1.2 ± 0.7
100 μM U-74006F	1.5 ± 0.8

Incubations contained ferrous ammonium sulfate (50 μM, 50 nmol), H_2O_2 (50 μM), and the indicated concentration of test compound in 0.2 M sodium acetate buffer, pH 7.0, containing 30% ethanol. Reactions were terminated by the addition of 0.5 mL Ferrozine® (15 mM) after 5 min of incubation (except in time zero quench control). Values are the means ± SD of three independent experiments.

Fe(II) upon addition of Ferrozine®. Furthermore, reduction of any oxidized iron was approximately 85% complete 4 hr post Ferrozine® addition in the presence of U-74500A (Fig. 2, time = 4 hr), whereas in the absence of drug, re-reduction was not complete, even after several days (data not shown). Although U-74006F had no effect on iron oxidation during the lipid peroxidation experiment, reduction of the oxidized iron post Ferrozine® quench was stimulated markedly.

The effects of U-74500A on liposomal lipid peroxidation and the oxidation state of iron were investigated in experiments employing ADP:Fe(III) and ascorbate (Table 2). In this system, 50 μM U-74500A inhibited lipid peroxidation by more than 80% while facilitating Fe(III) reduction. Iron reduction occurred rapidly, as essentially all iron was detectable in the reduced form after 20 min of incubation. In this system, a small, but reproducible amount of iron reduction was observed with U-74006F, and lipid peroxidation was inhibited slightly. Although a small amount of iron reduction was observed in the absence of ascorbate with both U-74500A and U-74006F, lipid peroxidation was not evident in either case, demonstrating that neither of these compounds have the pro-oxidant activities observed with ascorbate and other reductants in similar lipid peroxidation systems [32]. One mechanism that would allow an iron reductant to

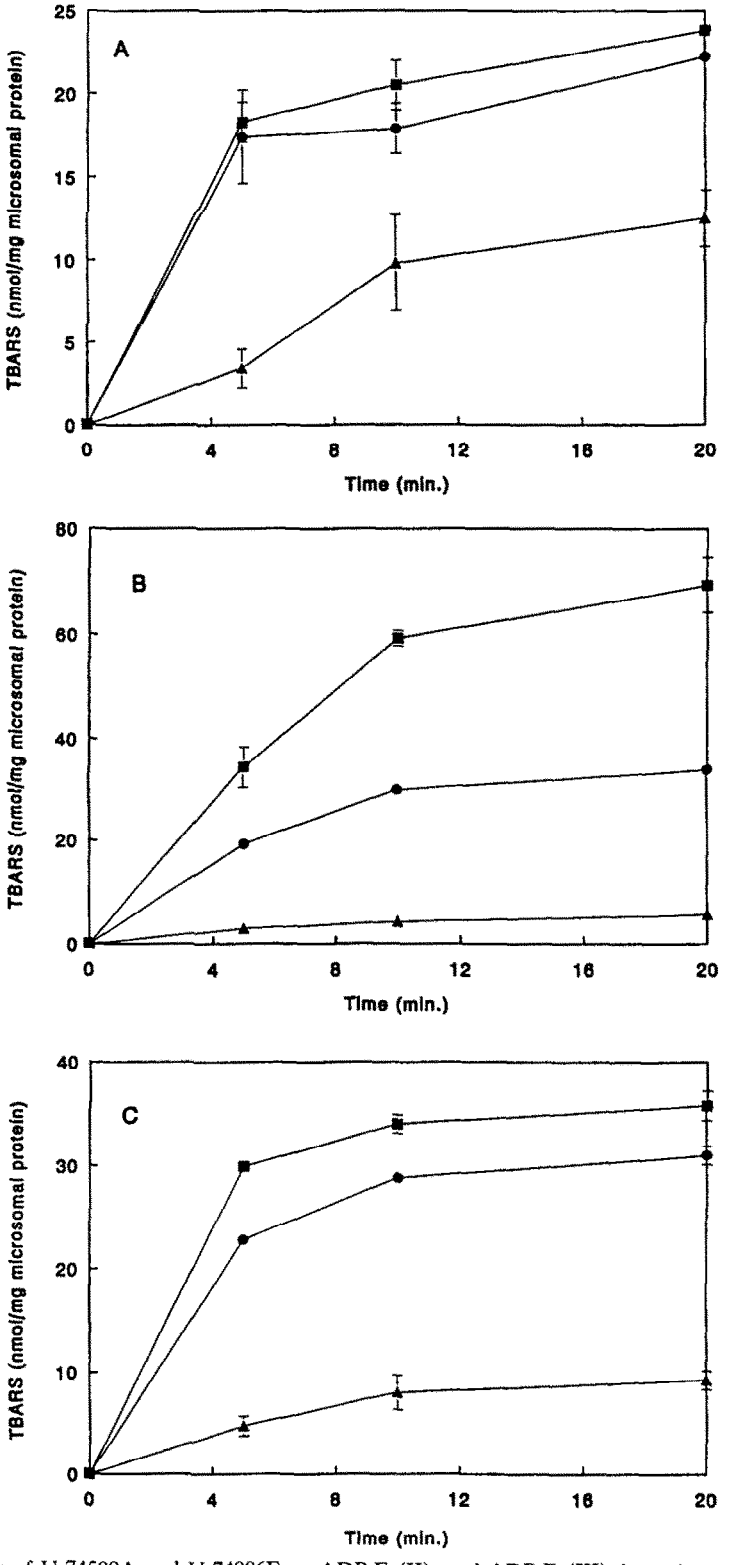

Fig. 3. Effects of U-74500A and U-74006F on ADP:Fe(II)- and ADP:Fe(III)-dependent microsomal lipid peroxidation. Incubations contained microsomes (0.5 mg/mL) in 0.2 M sodium acetate buffer, pH 7.0. Additions were as follows: (A) ADP:Fe(II) (250 μM : 50 μM); (B) ADP:Fe(III) (250 μM : 50 μM) and NADPH (100 μM); or (C) ADP:Fe(III) (250 μM : 50 μM) and ascorbate (50 μM). Aliquots were removed at the indicated time points and assayed for TBARS as described in Materials and Methods. Key: vehicle control (■), 50 μM U-74500A (▲), and 50 μM U-74006F (●). Values are the means ± SD of three independent experiments.

Table 4. Effects of U-74500A and U-74006F on microsomal lipid peroxidation and iron oxidation in acetate vs phosphate buffer

	TBARS (nmol/mg protein)	Fe(II) remaining (nmol)
Control (phosphate)	8.5 ± 1.2	0
+U-74006F (phosphate)	4.8 ± 0.6	0
+U-74500A (phosphate)	0.6 ± 0.2	0
Control (acetate)	50.3 ± 0.7	8.4 ± 3.9
+U-74006F (acetate)	46.3 ± 4.3	1.3 ± 10.3
+U-74500A (acetate)	2.8 ± 0.6	438.6 ± 12.2

Incubations contained microsomes, ferrous ammonium sulfate (600 nmol), with or without the indicated 21-aminosteroid (50 μM) or vehicle (1% ethanol) in the indicated buffer. TBARS were measured as described in Materials and Methods following 20 min of incubation. Values are the means ± SD of three independent experiments.

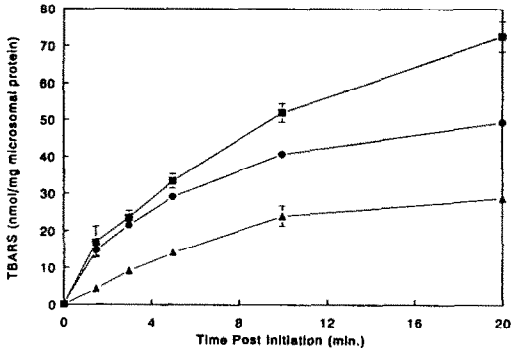

Fig. 4. Effects of preincubation of U-74006F with microsomes on microsomal lipid peroxidation. Incubations contained microsomes (0.5 mg/mL), and NADPH (100 μM, 50 μM upon preincubation and 50 μM at initiation) preincubated for 20 min at 37° in sodium acetate buffer, pH 7.0, with or without U-74006F. Reactions were initiated by the addition of ADP:Fe(III) (250 μM:50 μM). Additions were as follows: vehicle control (■), U-74006F added with iron (no preincubation) (●), or U-74006F added prior to preincubation period (▲). Aliquots were removed at the indicated time points and assayed for TBARS formation as described in Materials and Methods. Values are the means ± SD of three independent experiments.

act as a potent inhibitor of Fe(III)-dependent lipid peroxidation would involve reduction accompanied by formation of a redox inert Fe(II) chelate. As can be seen in Table 3, U-74500A was able to prevent iron oxidation by hydrogen peroxide, whereas U-74006F was without effect.

In contrast to results obtained in liposomal systems, U-74006F inhibited microsomal lipid peroxidation under the appropriate experimental conditions. This became apparent when U-74006F was examined in a microsomal system comparing the effects of ADP:Fe(II) with those of ADP:Fe(III)/NADPH or ADP:Fe(III)/ascorbate (Fig. 3). U-74006F did not inhibit microsomal lipid peroxidation initiated with ADP:Fe(II) alone (Fig. 3A), whereas partial inhibition was observed in microsomal experiments employing ADP:Fe(III) and NADPH (Fig. 3B). NADPH was required for maximal inhibition of lipid peroxidation by U-74006F in microsomes. Inhibition when ascorbate was substituted for NADPH was small, but reproducible (Fig. 3C), consistent with the effects observed with U-74006F on ADP:Fe(III)/ascorbate-dependent lipid peroxidation in liposomes. U-74500A inhibited lipid peroxidation in all microsomal systems examined, although the extent of inhibition was greatest in the ADP:Fe(III)/NADPH system. Inhibition of lipid peroxidation by U-74006F was highly system dependent. For example, U-74006F effectively inhibited Fe(II)-dependent microsomal lipid peroxidation in phosphate buffer but not in acetate buffer (Table 4). This difference may have been a function of the rate of iron auto-oxidation, which differed markedly in these two systems. Iron was oxidized completely within a matter of seconds in phosphate buffer, while complete oxidation in acetate buffer required the entire 20-min incubation period.

When microsomes were preincubated with U-74006F, inhibition of microsomal lipid peroxidation (about 50%) was observed at all time points examined (Fig. 4). Without preincubation, no inhibition was observed at early time points, although inhibition did become apparent approximately 10 min into the incubation. Preincubation of U-74006F with microsomes resulted in the formation of multiple metabolites, which were not characterized further. Metabolite formation was NADPH dependent, and the metabolite profile was markedly different upon inclusion of ADP:Fe(III) in the incubation mixture (Fig. 5).

DISCUSSION

The 21-aminosteroids U-74500A and U-74006F have been studied in a variety of *in vitro* and *in vivo* models in which lipid peroxidation has been implicated [16–25]. However, the effects of these compounds on liposomal lipid peroxidation (a system which lacks the biotransforming enzymes present in microsomes, homogenates, and whole cells), have not been reported. When U-74500A and U-74006F were examined in Fe(II)-dependent liposomal lipid peroxidation systems, it was found that U-74500A inhibited peroxidation whereas U-74006F was without effect (Fig. 1, Table 1). The effects of U-74500A on TBARS formation were concentration dependent, with 1 μM U-74500A substantially inhibiting lipid peroxidation in a system employing 50 μM iron (Table 1). In this system, TBARS formation is dependent upon oxidation of Fe(II) to Fe(III) (reviewed in Ref. 3). The effects of U-74500A on lipid peroxidation involved either chelation and/or maintenance of iron in a non-catalytic, reduced form, as approximately 30% of the iron was detectable as Fe(II) upon termination of lipid peroxidation experiments in the presence of U-74500A. No Fe(II) was detectable at the end of a 20-min incubation in the presence of U-74006F or

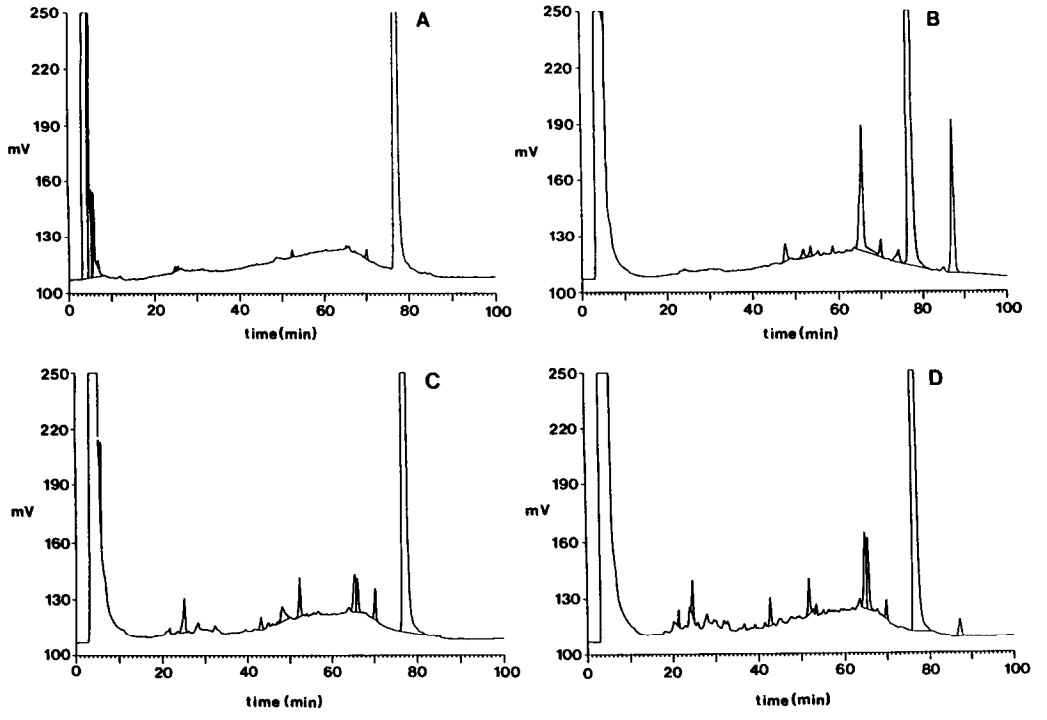

Fig. 5. HPLC profiles of U-74006F following incubation with microsomes. Incubation conditions and times were as described in the legend of Fig. 4 with sample preparation as described in Materials and Methods. Additions were as follows: (A) microsomes plus U-74006F; (B) A plus NADPH; (C) A plus ADP:Fe(III); and (D) A plus NADPH and ADP:Fe(III). Chromatograms (UV, 260 nm) are representative of three independent experiments. The U-74006F peak eluted at approximately 77 min.

when no 21-aminosteroid was present (Fig. 2). The effects of U-74500A and U-74006F on iron oxidation in these liposomal lipid peroxidation experiments paralleled those previously reported for these two compounds in a buffer/organic solvent system [26]. In both systems, U-74500A prevented Fe(II) oxidation and effectively reduced Fe(III). However, the absolute rate of U-74500A-dependent iron reduction was slower in the presence of phospholipid liposomes. The effects of U-74006F on Fe(II) oxidation in both systems were subtle compared with those of U-74500A, although in the liposomal preparations substantial iron reduction occurred after the lipid peroxidation phase of the experiments had been terminated. The mechanistic significance of the effects of U-74500A on Fe(II) oxidation deserves further comment, as this compound prevented iron oxidation in the presence of lipid hydroperoxide. As lipid hydroperoxides react rapidly with Fe(II) [33], this result suggests that U-74500A forms a relatively stable Fe(II) complex. To further test the stability of Fe(II) in the presence of U-74500A, Fe(II) was incubated in the presence of H_2O_2, a potent and biologically relevant iron oxidant. As can be seen in Table 3, U-74500A partially prevented H_2O_2-mediated Fe(II) oxidation whereas U-74006F had no effect. These results, taken together with the ability of U-74500A to prevent iron oxidation in the presence of lipid hydroperoxides, suggest that Fe(II) in the presence of U-74500A exists as a complex resistant to biological oxidations.

The effects of U-74500A and U-74006F on ADP:Fe(III)/ascorbate-dependent lipid peroxidation are shown in Table 2. Lipid peroxidation in this system was ascorbate dependent, and the iron reduced by ascorbate was quickly re-oxidized either through initiation or propagation of peroxidation, as none was detectable as Fe(II) at the end of 20 min. The stoichiometry of ascorbate and iron in this model system has been described previously by Miller and Aust [32], and allowed reduction of iron so that TBARS production was maximal while any Fe(II) produced in the experiment was oxidized quickly in the peroxidative process. Interestingly, although U-74500A and U-74006F alone were capable of reducing iron in the absence of ascorbate, neither compound exhibited the pro-oxidant effects associated with ascorbate and other iron-reducing systems [32]. In fact, both compounds actually inhibited lipid peroxidation when compared to Fe(III) alone, further demonstrating that the iron reduced by U-74500A must be inert in the presence of biologically relevant oxidants. In the ADP:Fe(III)/ascorbate-dependent lipid peroxidation system, U-74500A completely inhibited lipid peroxidation while U-74006F had only a slight, but consistent inhibitory effect. Accordingly, U-74500A maintained all of the added iron in the reduced

form, whereas U-74006F only slightly retarded iron re-oxidation. The significance of partial inhibition of Fe(III)-, but not Fe(II)-dependent liposomal lipid peroxidation by U-74006F is not known, but this same trend was also apparent in microsomal systems (*vide infra*).

When assayed in microsomal systems, U-74500A inhibited lipid peroxidation under all conditions examined. However, U-74006F, which was a poor inhibitor of liposomal lipid peroxidation, did inhibit microsomal lipid peroxidation under certain conditions (Fig. 3). When U-74006F was assayed in a Fe(II)-dependent microsomal lipid peroxidation experiment, no effect on lipid peroxidation was observed (Fig. 3A). When assayed in an ADP:Fe(III)-dependent system, however, U-74006F was inhibitory (Fig. 3B). These effects appeared to be NADPH dependent, however, as inhibition of lipid peroxidation was minimal, although detectable (consistent with similar liposomal systems), when ascorbate was substituted for NADPH as the supplier of reducing equivalents (Fig. 3C). The effects of U-74006F on microsomal lipid peroxidation were highly system dependent (Table 4). For example, U-74006F did not inhibit lipid peroxidation using ferrous ammonium sulfate in acetate buffer. However, when this same experiment was performed in phosphate buffer, U-74006F effectively inhibited lipid peroxidation. One difference in the latter two systems is that in acetate buffer, ferrous ammonium sulfate oxidized slowly over the course of the 20-min incubation period, while in phosphate buffer, the ferrous ammonium sulfate oxidized in seconds, essentially making it a Fe(III)-dependent system. These data, taken with the results in liposomal and microsomal systems described above, suggest that U-74006F can inhibit Fe(III)-dependent lipid peroxidation, although the presence of NADPH enhanced its efficiency.

Princubation of U-74006F with microsomes and NADPH for 20 min resulted in substantially greater inhibition of lipid peroxidation (Fig. 4). The kinetics of inhibition suggest that activation of U-74006F may have occurred. When U-74006F was preincubated with microsomes and NADPH, inhibition of peroxidation was apparent initially and throughout the incubation. In contrast, when U-74006F was added immediately prior to initiation, inhibition was minimal for the first several minutes of the incubation. These results are consistent with a time-dependent conversion of U-74006F to a species capable of inhibiting lipid peroxidation, although a contribution of membrane partitioning cannot be excluded. When identical incubations were subjected to HPLC analysis, several metabolites were formed during the preincubation period. In addition, the presence of Fe(III) in the microsomal preincubation mixture altered the HPLC profile, strengthening the argument that a metabolite of U-74006F may be responsible for the observed antioxidant activity of this compound.

Several lines of evidence suggest the importance of lipid peroxidation in conditions toward which these compounds are targeted [1–5]. The fact that U-74500A was a potent inhibitor of lipid peroxidation, probably via its ability to render iron non-reactive, suggests that lipid peroxidation is indeed an important factor in several of the models in which it has shown efficacy [34, 35]. It is tempting to speculate that a metabolic product(s) of U-74006F might have similar properties, but until such a product can be isolated and characterized, we have no data to support such a claim. In fact, other mechanisms, i.e. effects on membrane order/fluidity ([36]; Ryan TP and Petry TW, unpublished data), have been postulated and cannot be excluded based on our current data. Additionally, effects not directly related to inhibition of lipid peroxidation, i.e. effects on leukocyte infiltration [37] or cytokines [38], discussion of which is beyond the scope of this manuscript, could also contribute to the *in vivo* efficacy of these compounds.

In summary, these studies indicate that U-74500A inhibits both microsomal and liposomal lipid peroxidation, and appears to do so by affecting the redox status of iron and therefore its activity as a catalyst in the peroxidative process. In contrast, U-74500A did not inhibit lipid peroxidation in liposomal systems, but did inhibit it in a metabolically competent hepatic microsomal system. The implications of this disparity with regard to the mechanism(s) of U-74006F activity *in vivo* are currently being pursued.

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