MODIFICATION OF ALVEOLAR MACROPHAGE FUNCTION WITH BIS-BASIC ETHERS OF FLUORENE AND FLUOREN-9-SUBSTITUTED DERIVATIVES

J. Y. C. Ma, Y. H. Cheng, M. W. Barger, W. F. Xin, D. M. Lewis, V. Castranova, and J. K. H. Ma □ School of Pharmacy, West Virginia University, and Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

□ *Bis-basic ethers of fluorene and fluoren-9-substituted derivatives such as tilorone have been reported to inhibit silica-induced fibrosis in rats. The potential antifibrotic potency of 2,7-bis(diethylamino)ethoxy fluorene (F-9-H,H), fluorenone (F-9-one), fluorenoxime (F-9-oxime), and fluorenol (F-9-ol) was F-9-oxime > F-9-one ~ F-9-H,H ≫ F-9-ol. Since the release of reactive oxygen species and growth factors from alveolar macrophages (AM) in response to silica exposure has been linked to the development of pulmonary fibrosis, the present study was carried out to determine the inhibitory effects of these compounds on rat AM activity* in vitro. *The following parameters were monitored: (1) cellular viability; (2) zymosan-induced respiratory burst activity (superoxide and hydrogen peroxide release, chemiluminescence, and oxygen consumption) of AM; (3) drug binding to AM; and (4) lipopolysaccharide (LPS)-stimulated interleukin-1 (IL-1) release from AM. The bis-basic ethers, at 40 μM, did not affect cell viability when incubated with AM for 30 min, but significantly inhibited zymosan-induced macrophage respiratory burst activity. The inhibitory effect of these agents was F-9-oxime > F-9-one ~ F-9-H,H ≫ F-9-ol. Binding of these drugs to AM was time and dose dependent, and exhibited the following binding affinity: F-9-oxime > F-9-one > F-9-H,H > F-9-ol. F-9-oxime was shown to inhibit LPS-stimulated IL-1 release by AM in a dose-dependent manner. This inhibition of IL-1 release by AM cannot be explained as a decrease in viability. In addition, these drugs were also shown to impair human fibroblast proliferation in response to serum stimuli without impairing cell viability. These results indicate a positive correlation between drug binding to AM or other cell types and their inhibitory effects on cellular activities including oxygen consumption, superoxide release, hydrogen peroxide secretion, chemiluminescence, IL-1 release, and proliferation. The ability of these bis-basic ethers to modify AM and fibroblast functions* in vitro *suggests that further investigation of their reported antifibrotic potency* in vivo *is warranted.*

Keywords *alveolar macrophages, silicosis, bis-basic ethers of fluorene derivatives, antifibrotic agents*

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Address correspondence to Dr. Joseph K. H. Ma, School of Pharmacy, WVU Robert C. Byrd Health Sciences Center, Morgantown, WV 26506, USA.

Silicosis is a fibrotic lung disease resulting from chronic occupational exposure to crystalline silica [1]. Fibrosis has been regarded as an irreversible process [2]. However, depending on the extent of disease development, therapy for silicosis and coal miner's pneumoconiosis (CWP) may be targeted toward altering the natural history of these diseases [3]. This may be done by preventing the development of associated diseases such as mycobacterial infection, by pharmaceutically controlling and theoretically reversing or halting the progression of fibrosis, or by removing the fibrogenic stimuli of dust, activated macrophages and other stimulated effector cells, and cytokines which perpetuate the fibrotic process. Over the past several decades, there have been a number of clinical trials of potential therapeutic agents for the treatment of silicosis and CWP. These treatment strategies are summarized in a review article [4]. While the therapeutic outcome remains largely inconclusive, animal studies have suggested that compounds such as tetrandrine and certain bis-basic ethers of polycyclic aromatic compounds may reduce silica-mediated fibrosis by controlling the fibro-proliferative activity in the lungs [5–7]. Aside from their therapeutic potential, agents such as tetrandrine have been proven useful in helping to probe the biochemical mechanisms of fibrosis [8, 9].

Bis-basic ethers of fluorene derivatives, including 2,7-bis(diethylaminoethoxy)-9H-fluorene (F-9-H,H), tilorone or 2,7-bis(diethylaminoethoxy)fluoren-9-one (F-9-one), and 2,7-bis(diethylaminoethoxy)fluoren-9-oxime (F-9-oxime), have been reported to exhibit antiviral activity, stimulate interferon production, and inhibit cell-mediated immunologic reactions [10–12]. These compounds have also been tested for their potential antifibrotic effect. In experimental animals exposed to silica, F-9-oxime, a synthetic analog of tilorone, reportedly exhibits antifibrotic potency (as indicated by measurements of dry lung weight and lung histology) slightly greater than that of tilorone and F-9-H,H with significantly low systemic toxicity [7, 13], which may be attributed to its pharmacokinetic behavior which favors rapid body clearance [14]. Intravenous delivery of F-9-oxime in rats showed a plasma half-life of 43–55 min with a short peak time of 3.6–7.2 min.

The antifibrotic potential of F-9-oxime is shown to be comparable to that of tetrandrine, a drug that has been used in a clinical trial for the treatment of silicosis [15]. However, F-9-oxime is shown to be less toxic than tetrandrine for use in chronic therapy [14, 16]. These studies suggest that the bis-basic ethers of fluorene derivatives such as F-9-oxime warrant further investigation.

The cellular action and effects of the bis-basic ethers of fluorene

derivatives on AM activity have not been investigated. Previously, we have shown that the response of alveolar macrophages to silica exposure is manifested by excessive release of reactive oxygen species and inflammatory cytokines [17, 18]. Such activation of phagocytes has been linked to the fibrotic process and its measurement may provide in vitro screening of the antifibrogenic activity of drugs [9, 17]. The present study was carried out to examine the cellular interaction and effects of F-9-H,H, F-9-oxime, and tilorone (F-9-one) on AM function upregulated by known stimuli. In addition, the corresponding fluorenol, 2,7-bis(diethylaminoethoxy)-9H-fluoren-9-ol (F-9-ol), which is relatively less lipophilic and reportedly only marginally antifibrotic [7], was also studied and used as a negative control. The objective of this study was to provide a correlation between drug effect on cellular activity in vitro and their reported antifibrotic potency in vivo.

METHODS

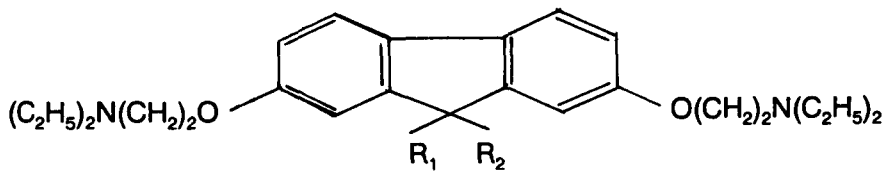
Materials

2,7-Bis(diethylaminoethoxy)-9H-fluorene (F-9-H,H), structurally related fluorene derivatives varying only at the central aromatic nucleus, including F-9-oxime, F-9-one, and F-9-ol, and tetrandrine were obtained as gifts from the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Beijing, China. The structures of the bis-basic ethers are given in Figure 1. Crystalline silica (Min-U-Sil: 98.5% purity; 80% of the particles having a diameter of less than 5 μm, and a specific surface area of 3.97 m^2/g), was obtained from Pennsylvania Sand and Glass Corporation (Pittsburgh, PA, USA).

Isolation of Alveolar Macrophages

Male Sprague–Dawley rats (175–225 g), obtained from Charles River Laboratories (Wilmington, MA, USA), were anesthetized with sodium pentobarbital (0.2 g/kg) and exsanguinated by cutting the renal artery. Alveolar macrophages were obtained by bronchoalveolar lavage with a Ca^{2+}- and Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.0 mM KCl, and 5.5 mM glucose; pH 7.4). Cells were centrifuged at 500*g* for 5 min, washed, and resuspended in Hepes-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5.5 mM glucose, and 1.0 mM $CaCl_2$; pH 7.4). Cell counts and purity were measured using an electronic cell counter equipped with a

BIS-BASIC ETHOXYFLUORENE DERIVATIVES



COMPOUND	R_1 , R_2
F-9-H,H (FLUORENE)	H, H
F-9-ONE (FLUORENONE)	=O
F-9-OXIME (FLUORENOXIME)	=N-OH
F-9-OL (FLUORENOL)	H, OH

Figure 1. Structural variations of 2,7-bis(diethylaminoethoxy) ethers of fluorenol, fluorenone, fluorene, and f

cell sizing attachment. Cell viability was determined microscopically by monitoring the exclusion of trypan blue dye [19].

Binding of Drugs to Alveolar Macrophages

Binding of F-9-H,H, F-9-one, F-9-oxime, and F-9-ol to alveolar macrophages was carried out in Hepes-buffered medium at 2×10^6 cells/mL. Cells were preincubated at 37°C for 10 min. Drug at a desired concentration was added to the cell suspension, and aliquots of the sample were removed at various time intervals and centrifuged to separate the cells from the supernates. Drug concentrations in the supernates were determined using a reverse-phase high-performance liquid chromatographic (HPLC) method, and the results were analyzed for the time-dependency of the binding. For the study of dose-dependent binding, varying concentrations of the drug ranging from 5 to 40 μM were added to AM suspensions and incubated for 20 min. The supernates were then prepared and analyzed for drug content. The HPLC assay for drug analysis was developed using a Waters HPLC system equipped with a model 440 UV detector (Waters Associates, Milford, MA, USA). Separation of the bis-basic ethoxyfluorene derivatives was achieved using a μBondapak C_{18} column and a mobile phase of CH_3CN/Hepes buffer (pH 4.5)/*n*-butanol (25:72:3). Drug detection and analysis was monitored by UV at 254 nm. Note that drug remaining in the supernate indicates unbound drug. In the absence of cell binding, recovery of free drug approached 100%.

Measurement of Respiratory Burst Activity

Oxygen consumption was measured with a Gilson K-IC oxygraph equipped with a Clark electrode (Gilson Medical Instruments, Middletown, WI, USA). AM (3.5×10^6/1.55 mL) were preincubated at 37°C for 10 min in Hepes buffer and oxygen consumption of the cells was determined at rest. Zymosan (2 mg/mL) was then added to cell suspension and oxygen consumption was measured to indicate zymosan's stimulatory effect. To determine the effect of drugs on zymosan stimulation, 40 μM of each drug was added to the cell suspension prior to the addition of zymosan and oxygen consumption of the cells measured before and after zymosan treatment.

Superoxide anion release was determined spectrophotometrically at 550 nm as the reduction of cytochrome C [20]. Briefly, AM (3×10^6/mL) were preincubated at 37°C for 10 min in Hepes-buffered medium containing 0.12 mM cytochrome C and treated with drug (40 μM) and/or zymosan. Superoxide release over the next 30 min was measured. Ab-

sorbance changes were converted to nanomoles of reduced cytochrome C using an extinction coefficient of 18.7 $mM^{-1}cm^{-1}$ [20].

Hydrogen peroxide secretion was determined fluorometrically at the excitation and emission wavelengths of 350 and 460 nm, respectively, as the oxidation of scopoletin in the presence of horseradish peroxidase [21]. AM (3×10^6/3mL) were preincubated at 37°C for 10 min in Hepes-buffered medium. Scopoletin (1.2 μM) and type IX horseradish peroxidase (6.6 units/mL) were added and fluorescence of the cell suspension was monitered in the absence or presence of the bis-basic ethers and/or zymosan (2 mg/mL) for 10 min. The fluorescence changes were converted to nanomoles of H_2O_2 using predetermined standard curves.

Chemiluminescence (CL) generated by AM was determined using an automated luminometer (Berthold Autolumat LB 953, Wallac Inc., Gaithersburg, MD, USA). Rat AM (1×10^6/mL) were preincubated at 37°C for 5 min in Hepes-buffered medium containing 0.0067% luminol. Silica (0.5 mg/mL) or zymosan (2 mg/mL) was added to the sample and CL was immediately measured for 30 min. To determine the effect of each bis-basic ether on silica- or zymosan-mediated macrophage generation of CL, 40 μM of each drug was added to the cells and incubated for 5 min prior to the addition of silica or zymosan and subsequent measurement of chemiluminescence. Data were collected as a plot of cpm vs. time for 30 min, and the area under the curve was integrated to represent the total chemiluminescence.

The effect of drugs on the viability of AM was determined by monitoring the ability of the cells to exclude trypan blue dye [19]. AM (1×10^6/mL) were incubated at 37°C for 30 min in the absence or presence of 40 μM drug. Trypan blue was then added and membrane integrity was determined by light microscopy.

Alveolar Macrophage Cultures and Thymocyte Proliferation Assay

The thymocyte proliferation assay for monokines [22] was carried out using a procedure previously described [18]. In brief, AM were obtained from rats by bronchoalveolar lavage with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution. Lavage cells were washed with 50 mL of the same buffer solution and resuspended in RPMI-1640 media (Whittaker) containing 2 mM glutamine, 100 units/mL mycostatin, and 10% heat inactivated fetal calf serum (FCS). Cell number, purity, and mean cell volume were determined. Aliquots of 1×10^6 cells in 1 mL were added to 24-well plates (Costar, Cambridge, MA, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 2 h. The nonadherent cells were then

removed with two 1-mL washes of the RPMI media. The adherent cells were further incubated in 1 mL of the RPMI media containing 10 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* (Difco, Detroit, MI, USA) or in LPS-free media with or without F-9-oxime in the concentration range of 4 to 20 μM. After incubating the cell cultures for 20 h, the supernates were collected, filtered through a 0.22-μm Miller filter (Millipore, Deford, MA, USA), analyzed for possible drug content by HPLC, and frozen at −20°C until time for thymocyte proliferation assay. Potential effect of drug on AM cell viability was monitored via trypan blue exclusion after the cells were exposed to various concentrations of the drug for 20 h.

For the determination of IL-1-like activity, thymocytes were obtained from male CD-1 mice (6–10 weeks of age) and suspended in RPMI-1640 media with 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 units/mL mycostatin, 10% heat inactivated FCS, and 2×10^{-5} M mercaptoethanol. Cells were counted and adjusted to a concentration of 1×10^7 cells/mL. An aliquot of 100 μL of the macrophage-conditioned supernate was added in quadruplicate to 96-well microculture plates, and 100 μL of thymocyte syspension was place in each well. After 48 h incubation at 37°C in 5% CO_2, the cultures were pulsed for 4–6 h with [^{3}H]thymidine (1.0 μCi/well; activity: 2.0 Ci/mmol, Dupont NEN Products, Boston, MA, USA) and harvested using a PHD cell harvester (Cambridge Technology, Inc., ME, USA). The radioactivity in the collecting glass filter disks was measured using a liquid scintillation counter (1214 Rackbeta, Wallac, Finland). The levels of IL-1-like activity in the tested macrophage supernates were expressed as counts per minute and then calculated as a percentage of the unstimulated control sample response.

Fibroblast Proliferation Assay

Human fetal lung fibroblast cell line (American Type Culture Collection HFL1-#CCL 153, Rockville, MD, USA) from a 1-mL ampule containing 3.5×10^6 fibroblasts was plated in a single 75-cm^2 tissue culture flask containing 15 mL of MEM plus 10% fetal calf serum. Cultures were incubated at 37°C until confluent, with media changed three times weekly according to a previously developed procedure [23]. Pulmonary fibroblasts were harvested from cultures by trypsinization using 0.25% trypsin in phosphate-buffered saline at 22°C. For the proliferation studies, cultures of 10th through 18th passage were used. Fibroblasts, 5×10^4 cells/mL, were plated in 25-cm^2 culture plates with a density of 1×10^4 cells/cm^2 in MEM with 10% fetal calf serum, incubated for 48 h,

and further incubated in serum-free medium for 48 h. The cultures were then treated with drug and 10% human serum for 6 h, and with [^{3}H]thymidine (1 μCi/mL) for 24 h in serum-free media with or without drug. The medium alone and medium with 10% human serum were used as negative and positive controls, respectively. Mitogenicity was measured and expressed as DPM/cell. The effect of drug on fibroblast viability was monitored by trypan blue exclusion after cells were exposed to various drugs at 10 μM for 30 h.

Statistics

Data presented are means ± standard errors of at least four experiments. Statistical significance was determined using a Student's t test with significance set at $p < .05$.

RESULTS

Previously, we have demonstrated that the respiratory burst activity of AM and its production of IL-1-like cytokines are useful parameters for the in vitro screening of potentially useful antifibrotic agents. Tetrandrine, for example, is among the most potent bisbenzylisoquinoline alkaloids in antifibrotic potential and in the inhibition of macrophage activity [3, 8, 9, 18]. Owing to their bis-basic functional groups, tilorone and structurally related compounds may also interact with cellular plasma membranes. Hence, their reported in vivo antifibrogenic activity may be associated with their ability to interact with alveolar macrophages and to inhibit the cell's inflammatory response to fibrotic stimulation.

The potential cellular toxicity of selected bis-basic ethers toward AM was tested. Table 1 shows the measured cell viability of AM following incubation with a high concentration (40 μM) of F-9-H,H, F-9-one, F-9-oxime, and F-9-ol for 30 min. These results indicate that the bis-basic ethers have no effect on macrophage membrane integrity over a time period sufficient to measure inhibition of macrophage respiratory burst activity. Table 2 shows the effect of drugs on the respiratory burst activity of AM stimulated by unopsonized zymosan. Studies have shown that these drugs have no effect on the resting level of macrophage activity (data not shown). The bis-basic ethers of the fluorene derivatives are weak inhibitors of macrophage release of superoxide anion, but show significant effect on zymosan-stimulated oxygen consumption and secretion of hydrogen peroxide by AM. Among the bis-basic ethers tested, F-9-oxime was the most potent inhibitor of the macrophage respiratory burst, F-9-one and F-9-H,H exhibit intermediate effect, whereas F-9-ol, a

Table 1 Effect of 2,7-bis(diethylaminoethoxy) derivatives of fluorenol, fluorenone, fluorenoxime, and fluorene on the membrane integrity of alveolar macrophages

Drug (40 μM)	Cell viability (%)
Control	82.6 ± 1.1
F-9-ol (fluorenol)	78.8 ± 1.4
F-9-H,H (fluorene)	79.2 ± 1.1
F-9-one (fluorenone)	80.3 ± 0.9
F-9-oxime (fluorenoxime)	81.8 ± 0.4

Note. AM (1×10^6/mL) in the absence and presence of 40 μM drug were incubated at 37°C for 30 min. Cell viability studies were carried out by measurement of trypan blue exclusion. Values are means ± standard errors from five experiments.

compound with relatively low lipophilicity and little or no antifibrotic potency, shows only a slight effect on macrophage oxygen consumption. For comparison, effects of tetrandrine on macrophage activity were also determined and included in Table 2. Data show that the bis-basic ethers are less effective inhibitors of macrophage respiratory burst in vitro than tetrandrine. The results shown in Table 2 are further substantiated by measurement of drug effects on chemiluminescence generated by AM after challenge with silica (Figure 2). Here, F-9-oxime was consistently more effective than F-9-H,H and F-9-one, and all these compounds were substantially more potent than F-9-ol in their ability to inhibit macrophage activity.

The effect of the bis-basic ethers of fluorene and substituted fluorene

Table 2 Effects of 2,7-bis(diethylaminoethoxy) ethers of fluorenol, fluorenone, fluorene, and fluorenoxime on zymosan-induced respiratory burst activity by alveolar macrophages

Drug (40 μM)	Oxygen consumption (%)	Superoxide release (%)	Hydrogen peroxide (%)
Control	100.0	100.0	100.0
F-9-ol	74.1 ± 6.0	92.8 ± 4.6	103.1 ± 2.0
F-9-H,H	57.6 ± 3.9	80.3 ± 2.5	81.8 ± 2.3
F-9-one	66.4 ± 7.2	94.0 ± 2.7	75.6 ± 0.8
F-9-oxime	52.3 ± 3.4	82.3 ± 4.7	59.5 ± 2.6
Tetrandrine	29.8 ± 5.5	60.2 ± 8.7	32.7 ± 2.6

Note. The control values of zymosan-stimulated oxygen consumption, superoxide anion release, and hydrogen peroxide secretion are 3.81 ± 0.52 (n = 5), 4.46 ± 0.22 (n = 4), and 1.61 ± 0.11 (n = 4) nmol/10^6 cells/min, respectively. Values measured for tetrandrine are included to provide a comparison for these two classes of compounds.

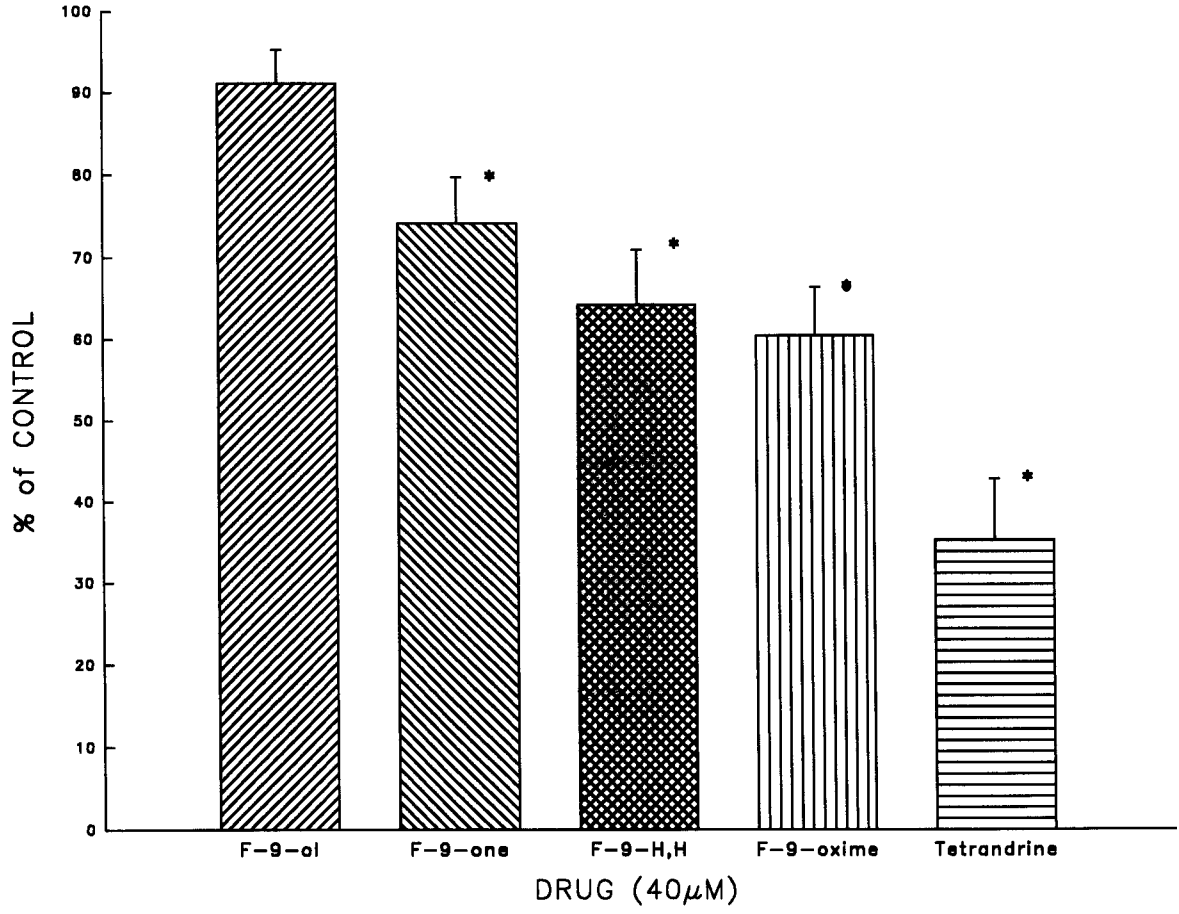


Figure 2. Effects of F-9-ol, F-9-one, F-9-H,H, and F-9-oxime (all 40 μM) on silica (0.5 mg/mL)-induced generation of chemiluminescenc
Data are expressed as the % of control (silica-stimulated CL), which has an integrated area under the curve of $3.75 \pm 0.64 \times 10^4$ cps/10
min ($n = 4$). Data obtained for tetrandrine are included for comparison of activity between these two classes of compounds.

on macrophage function may be attributed to the drug's cellular binding affinity. Figure 3 shows the time-dependent binding of F-9-oxime, F-9-one, and F-9-ol to AM at 37°C. Drug binding to the cells was indicated by a decrease in drug concentration in the medium when cells were removed by centrifugation. At each time point, the binding affinity of F-9-oxime is slightly greater than that of F-9-one and substantially greater than that of F-9-ol. Figure 4 shows the comparison of binding affinity of various drugs to AM measured following 20-min incubation. These results indicate that the order of binding affinity is F-9-oxime > F-9-one > F-9-H,H > F-9-ol, which is consistent with the order of drug potency observed in the respiratory burst measurement. It is noteworthy that these drugs do not bind to metabolically dead cells (treated with cyanide and iodoacetic acid) or sonicated AM preparations. Therefore, drug–cell interaction is not merely nonspecific binding to membrane lipids but rather specific binding to structures requiring viable cell preparations. Indeed, previous studies have indicated that the binding of tetrandrine to viable macrophages requires an intact cytoskeletal system [8]. Direct comparison of the binding affinity between F-9-oxime and F-9-ol at various concentrations is shown in Figure 5. Here, the results clearly indicate that the ability of the drug to inhibit celluar activity is related to its cellular binding capacity. The binding data are also consistent with the reported antifibrotic effect for these agents. As reported by Cheng et al. [7, 24], F-9-oxime exhibits slightly greater antifibrotic activity than F-9-one and F-9-H,H, and substantially greater effect than F-9-ol in the inhibition of silica-induced collagen formation.

Table 3 shows the effect of F-9-oxime on the macrophage production of IL-1-like activity at rest and after stimulation by LPS. In the absence of LPS, the supernates of AM treated with varying concentrations of drug appeared to have a moderate, but positive stimulatory effect on thymocyte proliferation. HPLC analysis of the drug content showed that after 20 h incubation, the AM supernates did not contain F-9-oxime, indicating that the thymocyte proliferation assay was not influenced by the presence of drug. Studies also indicated that F-9-oxime, in the concentration range used, had little or no effect on AM viability when incubated for 20 h, i.e., AM viability was decreased by 4% with 16 μM drug and 13% with 20 μM F-9-oxime. The moderate drug effect associated with unstimulated AM supernates was also observed for tetrandrine [18]. This may be attributed to drug binding to cellular membrane, resulting in a modification of the release of IL-1-like cytokines by nonstimulated AM. In contrast, F-9-oxime inhibited LPS-stimulated release of IL-1-like cytokines by AM in a dose-dependent manner, with 68% inhibition at 16 μM and 84% inhibition at 20 μM of the drug (Table 3). That is, the

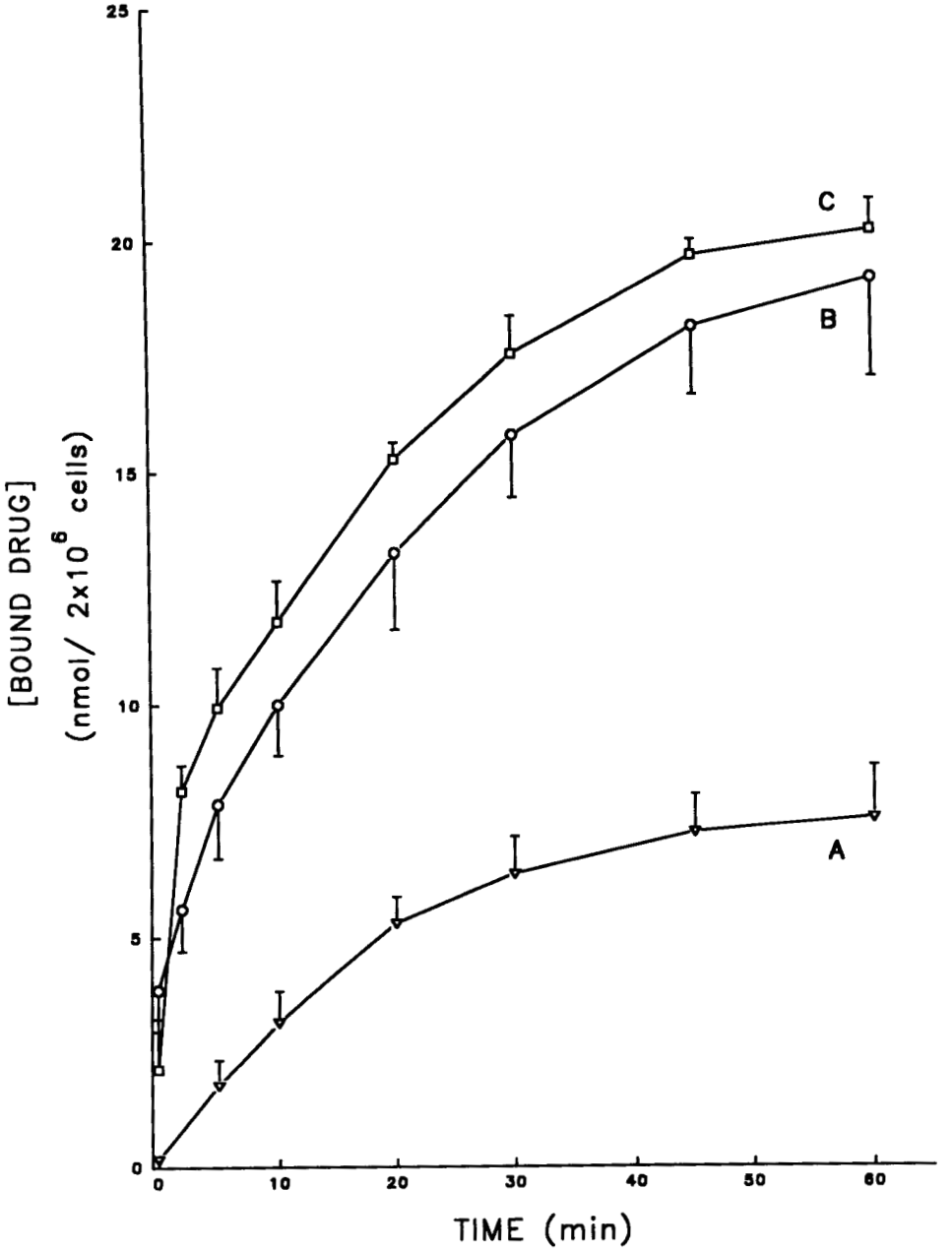


Figure 3. Time courses for the binding of F-9-ol (A), F-9-one (B), and F-9-oxime (C) to alveolar macrophages. AM (2×10^6/mL) were incubated with 25 μM of drug at 37°C and supernates analyzed by reverse phase HPLC at various time periods. Values are means ± standard errors from four experiments.

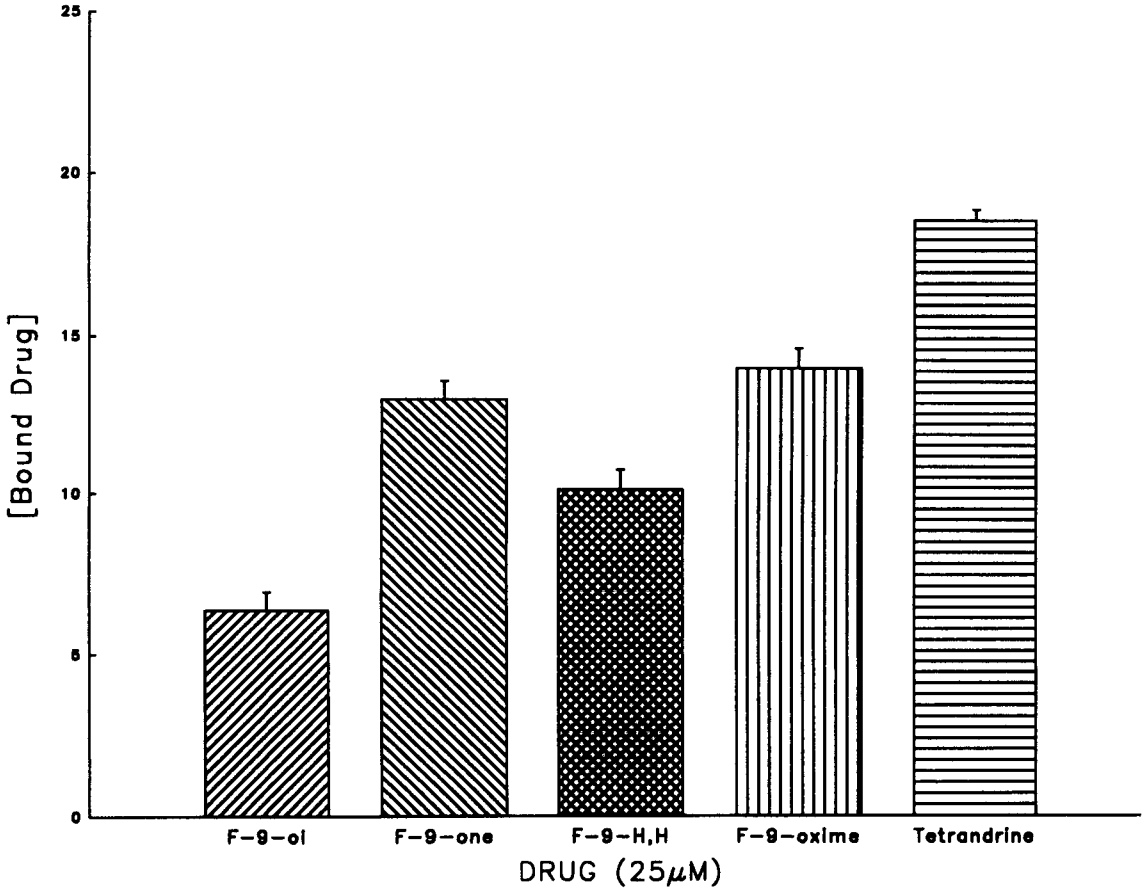
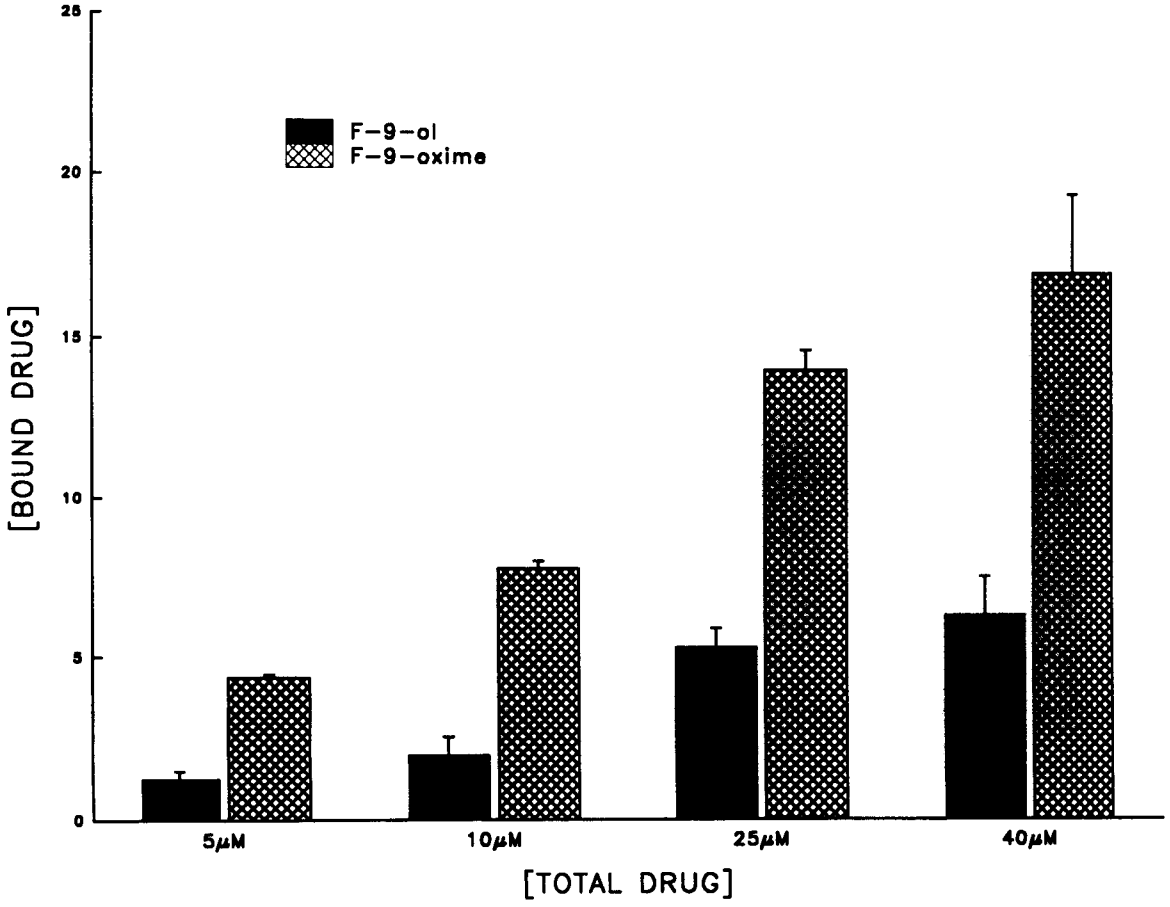


Figure 4. Relative binding affinities of the bis-basic ethers to alveolar macrophages. AM (2×10^6/mL) were incubated with various d at 37°C for 20 min and binding was analyzed by reverse-phase HPLC. Data obtained for tetrandrine are included to provide comparis for these two classes of drugs ($n = 4$).



igure 5. Comparative binding affinities of F-9-ol (dark bars) and F-9-oxime (light bars) to alveolar macrophages at various drug concentration
2×10^6/mL) were incubated with varying concentrations of drug for 20 min and binding was analyzed by reverse-phase HPLC. Data represent
standard errors from four experiments.

Table 3 Effect of F-9-oxime on LPS-stimulated release of IL-1-like cytokines by alveolar macrophages as measured via stimulation of thymocyte proliferation by AM supernates

Drug concentration (μM)	Thymocyte proliferation (as ratio to control)	
	Nonstimulated supernate	*LPS-stimulated supernate*
Control	1.00	6.50 ± 2.42*
4	2.32 ± 0.87	4.80 ± 1.68*
8	1.73 ± 0.64	3.89 ± 1.74*
12	2.25 ± 1.02	3.83 ± 1.26*
16	3.30 ± 0.52*	2.13 ± 0.97**
20	0.72 ± 0.31	1.06 ± 0.50**

Note. AM (1×10^6/well) were incubated with varying concentrations of drug in the absence and presence of LPS (10 μg/mL) for 20 h. Supernates were collected for thymocyte proliferation assay. Cell proliferation is expressed as a ratio to the nonstimulated control. Values are means ± standard errors of six experiments. Asterisk and double asterisk indicate significant difference from nonstimulated and stimulated controls, respectively. Previous studies showed that the LPS-stimulated release of IL-1-like cytokines corresponds to 0.097 ng/mL of IL-1 activity [18].

supernate of LPS-treated AM showed marked stimulation of thymocyte proliferation due to the presence of IL-1-like cytokines released from AM [18, 22], while supernates from AM treated with LPS and varying concentrations of F-9-oxime showed reduced stimulation of thymocyte proliferation. Similar inhibition of IL-1 release has been reported previously with tetrandrine [18]. These results indicate that F-9-oxime was a potent inhibitor of macrophage release of IL-1-like cytokines, which has been shown to play an important role in the fibrogenic stimulation of fibroblast growth and collagen synthesis [25, 26].

Since F-9-oxime shows binding affinity toward AM, it may also interact with other cell types in the lungs. In pulmonary fibrosis, abnormal growth of fibroblast leads to increased collagen synthesis and secretion. It is possible that the potential antifibrotic mechanism of the bis-basic ethers may involve interaction of drug with fibroblasts, resulting in an inhibition of stimulated cell proliferation. Indeed, Table 4 shows the dose-dependent inhibition of human fibroblast proliferation by the bis-basic ethers of fluorene derivatives. Here, F-9-oxime and F-9-one were potent inhibitors, F-9-H,H showed intermediate activity, and F-9-ol was least active in the inhibition of fibroblast proliferation. Via cell viability measurement, Table 5 shows that the inhibition of proliferative capacity by these compounds was not the result of a decrease in fibroblast viability. These results show an order of drug potency that is very consistent with that obtained in the macrophage studies using the rat model, suggesting that the pharmacological action of the bis-basic ethoxyfluorene derivatives may be derived from their cellular binding activity.

Table 4 Dose-dependent inhibitory effect of bis-basic ethers of fluorene derivatives and tetrandrine on human fibroblast proliferation

Concentration (μM)	Fibroblast proliferation (% of control)				
	F-9-ol	F-9-H,H	F-9-one	F-9-oxime	Tetrandrine
0 (Control)	100	100	100	100	100
2	105.2 ± 3.9	91.1 ± 6.2	114.9 ± 8.4	105.2 ± 8.3	78.2 ± 3.3*
4	93.1 ± 5.1	86.2 ± 14.9	97.0 ± 7.5	87.6 ± 5.7*	54.0 ± 5.6*
6	75.9 ± 1.0*	58.1 ± 3.0*	50.6 ± 3.3*	51.1 ± 1.1*	16.1 ± 0.6*
8	70.7 ± 3.6*	46.9 ± 7.4*	40.2 ± 5.4*	32.1 ± 2.5*	13.8 ± 1.1*
10	51.5 ± 3.4*	33.2 ± 1.6*	5.5 ± 0.3*	4.6 ± 0.1*	6.1 ± 0.2*

Note. Cell proliferation was stimulated with 10% fetal calf serum. Drug effect on cell proliferation is expressed as percentage of control (at zero drug concentration). Data obtained for tetradrine are included to provide comparison of activity for these two classes of compounds. Asterisk indicates significant difference from control ($n = 4$).

DISCUSSION

A large number of bis-basic-substituted polycyclic aromatic compounds have been shown to exhibit in vivo antiviral activity against encephalomyocarditis [28]. The structure–activity relationships of these compounds involves variations of both the side chains and the central nucleus. Potent antiviral activity is generally associated with bis-basic ketones and bis-basic esters of all ring systems, and also with bis-basic ethers of fluorenone, to which tilorone is a member. Mono-basic compounds show very little or no activity. The antiviral activity of bis-basic ethers is, in fact, dramatically influenced by the central nucleus. Of the same side chains, fluorenols and fluorenes are known to be much less effective than

Table 5 Effect of bis-basic ethers of fluorene derivative on the membrane integrity of human fibroblasts

Drug (10 μM)	Cell viability (%)
Control	85.0 ± 1.0
F-9-ol (fluorenol)	87.6 ± 1.2
F-9-H,H (fluorene)	86.7 ± 0.6
F-9-one (fluorenone)	85.0 ± 1.2
F-9-oxime (fluorenoxime)	86.9 ± 1.0
Tetrandrine	89.9 ± 1.5

Note. Fibroblasts were cultured in medium containing 10 μM drug for 30 h at 37°C. Cell viability was determined by monitoring trypan blue exclusion. Values are means ± standard errors of 4 experiments.

fluorenones [10], suggesting that in this bis-basic ether series, the structure of the central nucleus plays an important role in their interaction with various cell types. The mechanisms of cellular action for these drugs have not been reported. However, the potency of drugs appears to depend on the presence of a carbonyl group on the ring system, i.e., fluorenone, xanthenone, etc. [11]. In the present study, we have demonstrated that binding of the bis-basic ethers to AM varies with varying structure of the central nucleus. The fluorenol analog is considerably weaker in binding affinity to AM than other compounds tested, due to the presence of the hydrophilic –OH group, which may account for its reported low activity as either an antiviral or antifibrotic agent. F-9-oxime, on the other hand, shows greater binding affinity than F-9-one to AM, and perhaps to other cell types (such as fibroblasts) as well, suggesting that the presence of an oxime(C=N−OH) moiety on the fluorene nucleus promotes cellular interaction with the bis-basic ethers.

F-9-oxime, 2,7-bis(diethylaminoethoxy)fluoren-9-oxime, is a synthetic derivative of tilorone, which has been tested for potential antifibrotic activity by Cheng et al. [7], along with tilorone (F-9-one) and the corresponding fluorene (F-9-H,H) and fluorenol (F-9-ol) analogs. Both F-9-H,H and F-9-one were reported to reduce the silica-mediated increase in dry lung weight and histologic lung lesions. F-9-ol, on the other hand, was ineffective in the treatment of silicosis. These observations are consistent with those of the antiviral studies. F-9-oxime was reported to be slightly more effective than tilorone and F-9-H,H in the treatment of silicosis, although tilorone was shown to exhibit greater effect on interferon induction than F-9-oxime [7]. F-9-oxime, however, is considerably less toxic than either tilorone or F-9-H,H. The LD_{50} values in rats for F-9-oxime, F-9-one, and F-9-H,H are 1.17, 0.95, and 0.45 g/kg, respectively [7]. Pharmacokinetic studies [14] showed that F-9-oxime has a plasma half-life of 43 to 55 min in rats when given intravenously. This relatively short half-life may be conducive for long-term use with low potential of chronic toxicity. The pharmacological and toxicological effect of F-9-oxime may be attributed to the C=N−OH group, which appears to retain strong binding affinity to cells comparable to the effect of the carbonyl group in tilorone, but facilitates elimination of drug from the tissue compartments.

Only a few classes of compounds have been reported to exhibit inhibitory effects on macrophage respiratory burst and cellular production of cytokines. These compounds include corticosterioids such as dexamethasone, which suppress IL-1 release from a monocyte-like U937 tumor cell line [29], and tetrandrine, which inhibits macrophage activities in response to various stimuli [8, 9, 17, 18]. The present study indicates

that bis-basic ethers of fluorene derivatives also interact with alveolar macrophages with binding affinities in the order of F-9-oxime > F-9-one > F-9-H,H > F-9-ol. These compounds are shown to exhibit structurally dependent capability to inhibit zymosan- and silica-induced respiratory burst activity and the production of IL-1-like cytokines by alveolar macrophages. There is a definite correlation between the binding affinity and the inhibitory effect of the drugs, as these measurements are in the same order of drug potency. Furthermore, these results are also consistent with those reported for the antifibrotic activity and the antiviral effect of these agents. Direct interaction of the bis-basic ethers with pulmonary fibroblasts resulting in inhibition of stimulated proliferation was also observed. This indicates that these compounds can interact with many cell types and their biological effects likely include modification of cellular activities of various cell types in the pulmonary region. F-9-oxime, for example, has been reported to inhibit the biosynthesis of phospholipids and collagen [13].

Although the bisbasic ethers of the fluorene derivatives are structurally very different from tetrandrine, a bisbenzylisoquinoline analog, the actions of these two classes of compounds on alveolar macrophage and fibroblasts are very similar. Both involve strong cellular binding, resulting in an inhibition of stimulant-induced cellular activity. F-9-oxime and other bis-basic ethers tested in the present study are less potent inhibitors of in vitro cell activity than tetrandrine. However, the reported in vivo antifibrotic activity of F-9-oxime seems comparable to that of tetrandrine [7, 24]. Since F-9-oxime is relatively nontoxic for chronic use, investigation of its effectiveness as an antifibrotic agent for the treatment of pulmonary fibrosis is warranted. In summary, the present study demonstrates that bis-basic ethers of fluorene, fluorenone, and fluorenoxime analogs inhibit the release of reactive oxygen species and the production of IL-1 by AM by binding to the cellular membrane. Both the in vitro and in vivo sequences of drug potency observed from this and other studies [7] are consistent with the order of binding affinity. F-9-oxime also inhibits pulmonary fibroblast proliferation through direct interactions with this cell type, further suggesting the potential of this drug as an antifibrotic agent.

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