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Effects of Bisbenzylisoquinoline Alkaloids on Alveolar Macrophages: Correlation between Binding Affinity, Inhibitory Potency, and Antifibrotic Potential

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Effects of Bisbenzylisoquinoline Alkaloids on Alveolar Macrophages: Correlation between Binding Affinity, Inhibitory Potency, and Antifibrotic Potential. CASTRANOVA, V., KANG, J-H., MA, J. K. H., MO, C-H., MALANGA, C. J., MOORE, M. D., SCHWEGLER-BERRY, D., AND MA, J. Y. C. (1991). *Toxicol. Appl. Pharmacol.* **108,** 242–252. The Chinese have conducted extensive studies concerning the medicinal properties of plant products. In this investigation the ability of three bisbenzylisoquinoline alkaloids to inhibit particle-induced activation of alveolar macrophages was evaluated and this inhibitory potential was correlated with the ability of those drugs to bind to membrane components. Tetrandrine, i.e., an herbal medicine used as an antifibrotic agent in China, was a potent inhibitor of particle-stimulated oxygen consumption, superoxide release, and hydrogen peroxide secretion by alveolar macrophages. Tetrandrine also exhibited substantial binding affinity for membrane lipids and alveolar macrophages. In contrast, tubocurine, an analogue with little antifibrotic potential, exhibited low binding affinity and had little effect on macrophage activation. Methoxyadiantifoline, an alkaloid of unknown antifibrotic potential, exhibited inhibitory and binding properties similar to those of tetrandrine. The data indicate that a strong relationship exists between the antifibrotic potential of these alkaloids and their ability to bind to alveolar macrophages and inhibit particle-induced activation of these phagocytes. These drugs should serve as useful probes to evaluate the role of alveolar macrophages in pulmonary fibrosis.

Chronic silicosis is a fibrotic disease resulting from occupational exposure to silica dust. It is manifested initially by an increase in the quantity of pulmonary surfactant (Dethloff *et al.*, 1986). However, in later stages it is characterized by an increase in collagen formation and the development of concentric hyalinized nodular lesions in the lung (Ziskind *et al.*, 1976). Although mechanisms for the progression of silicosis have not yet been completely defined, activation of alveolar macrophages by respirable silica particles is believed to play an important role in the etiology of this disease. Studies from our laboratory have shown that both *in vitro* and *in vivo* silica exposures result in a stimulation of macrophage release of reactive products capable of damaging the lung parenchyma (Castranova *et al.*, 1990; Goodman *et al.*, 1990). In addition, exposure of alveolar macrophages to silica results in the release of fibrogenic factors which enhance the proliferation of fibroblasts and the synthesis of collagen by these pneumocytes (Leibovich and Ross, 1976; Bitterman *et al.*, 1982, 1983).

The bisbenzylisoquinoline alkaloids, which represent the largest group among the isoquinoline compounds isolated from the plant kingdom, are known to exhibit a broad range

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of pharmacological activities. Tetrandrine, for example, an alkaloid isolated from the root of *Stephania tetrandra,* has been tested for its antitumor, antihypertensive, and antisilicotic effects (Kupchan and Atland, 1973; Zeng *et al.,* 1985; Yu *et al.,* 1983). It is currently used in China as a treatment for angina, hypertension, and silica-induced pulmonary fibrosis (Li *et al.,* 1981). Several related bisbenzylisoquinoline alkaloids such as methoxyadiantifoline and tubocurine have also been evaluated for medicinal effects. Methoxyadiantifoline has been shown to exhibit effects on the cardiovascular system similar to those reported for tetrandrine (Jia *et al.,* 1988). However, to date, the antifibrotic activity of methoxyadiantifoline has not been evaluated. In contrast to tetrandrine, tubocurine, a structural analogue of this drug, has been reported to be relatively ineffective against fibrosis (Mo *et al.,* 1982).

One objective of this investigation was to determine the *in vitro* effects of selected bisbenzylisoquinoline alkaloids on the activation of alveolar macrophages by particles. The three alkaloids chosen for study exhibit a range of antifibrotic potency, i.e., tetrandrine (effective), tubocurine (ineffective), and methoxyadiantifoline (unknown potency). Structures of these alkaloids are given in Fig. 1. A second objective was to characterize the binding of these alkaloids to membrane lipid and alveolar macrophages. Finally, correlations between binding, inhibition of macrophage function, and/or antifibrotic potential were determined.

MATERIALS AND METHODS

Animals. Pathogen-free male Sprague–Dawley rats (200–250 g) were obtained from Charles River Laboratories (Wilmington, MA) and quarantined for 1 week after arrival before use in the experiments. Animals were housed in polycarbonate cages (three rats/cage) and placed in laminar flow hoods within the NIOSH animal facility (AAALAC approved). The lighting cycle was maintained at 16 hr light–8 hr dark. Relative humidity averaged 60 ± 4% and the environmental temperature was controlled at 70–72°F.

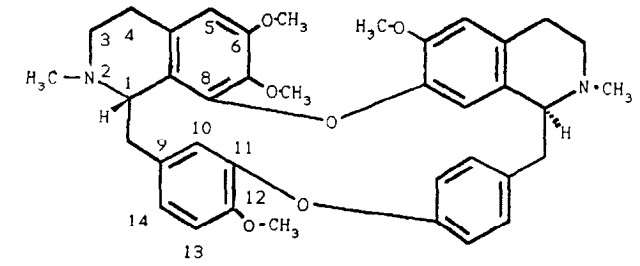
Cellular isolation. Alveolar macrophages were harvested by bronchoalveolar lavage (Sweeney *et al.,* 1981). Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (200 mg/kg body wt) and exsanguinated by cutting the left renal artery. The trachea was cannulated and the lungs lavaged 10 times with 8-ml aliquots of Ca^{2+}–Mg^{2+}-free Hank's balanced salt solution. Cell suspensions were centrifuged at 500*g* for 5 min at 4°C. The cell pellets then were washed twice by alternate resuspension and centrifugation in Hepes-buffered medium (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose; pH 7.4). Cell number, purity, and mean cell volume of macrophage preparations were determined using an electronic cell counter with a cell sizing attachment (Castranova *et al.,* 1979). The average values for these parameters were: yield, 5.8 ± 0.2 × 10^6 alveolar macrophages/rat; purity, 91.4 ± 0.4%; and mean cell volume, 1410 ± 11 μm^3.

In vitro treatment of phagocytes with bisbenzylisoquinoline alkaloids. Tetrandrine, tubocurine, and methoxyadiantifoline were kindly supplied by Dr. Chang-geng Mo, Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Beijing, People's Republic of China. Stock solutions were prepared by mixing the drug with a few drops of H_2O, adding HCl until the drug was dissolved, adjusting pH to 6.5 with NaOH, and then bringing up to volume with H_2O. For *in vitro* studies, alkaloid was added to the cell suspension just prior to assay of cellular activity.

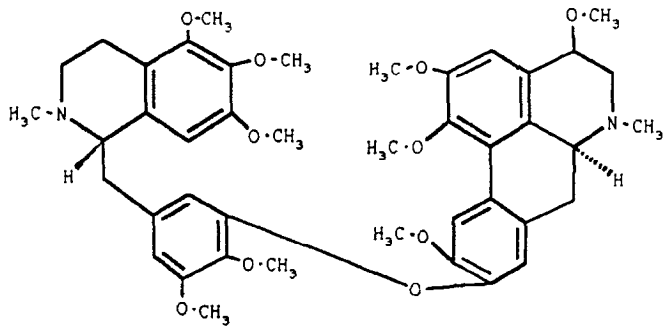
Measurement of cellular activity after in vitro treatment with drugs. Membrane integrity was determined microscopically by monitoring the exclusion of trypan blue dye (Phillips, 1973). Briefly, rat alveolar macrophages (8 × 10^5 cells/0.2 ml of Hepes-buffered medium) were preincubated for 20 min at 37°C in the absence or presence of various concentrations of the drug (5–60 μg/ml). After preincubation, 0.04% trypan blue dye was added and the cells were incubated at 22°C for 4 min. Then cells were fixed with 1% buffered formalin, observed under a light microscope, and cell viability was determined as the percentage of cells excluding dye.

Oxygen consumption was measured with an oxygraph equipped with a Clark electrode (Castranova *et al.,* 1980). Rat alveolar macrophages (4 × 10^6) were preincubated at 37°C in 1.75 ml of Hepes-buffered medium for 10 min. After preincubation, the cell suspension was transferred to a temperature controlled (37°C) chamber and resting oxygen consumption monitored. Then alkaloid (5–60 μg/ml) was added to determine the cytotoxic effects of the drug. Particle-stimulated oxygen consumption was determined by the addition of zymosan (2 mg/ml) to cells preincubated for 5 min at 37°C in the absence or presence of the drug. The oxygraph was calibrated using media equilibrated with gases of known oxygen content.

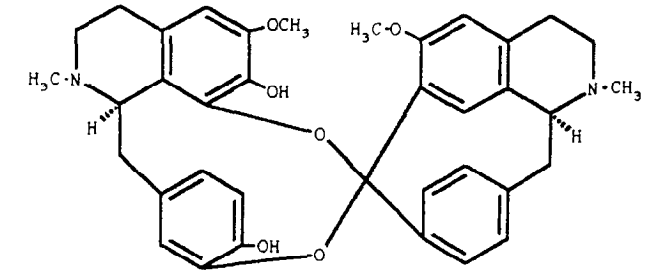
Superoxide anion release was determined spectrophotometrically at 550 nm as the reduction of cytochrome *C* (Sweeney *et al.,* 1981). Briefly, alveolar macrophages (4 × 10^6) were preincubated at 37°C for 10 min in 6 ml of Hepes-buffered medium containing 0.12 mM cytochrome *C*. After preincubation, cells were treated with alkaloid (5–50 μg/ml) and/or zymosan (2 mg/ml) and superoxide



TETRANDRINE



METHOXYADIANTIFOLINE



TUBOCURINE

FIG. 1. Chemical structures of tetrandrine, methoxyadiantifoline, and tubocurine.

release over the next 30 min was measured. Absorbance changes were converted to nanomoles of reduced cytochrome *C* using an extinction coefficient of 18.7 mM^{-1} cm^{-1}.

Hydrogen peroxide secretion was determined fluorometrically at an excitation wavelength of 350 nm and an emission wavelength of 460 nm as the oxidation of scopoletin in the presence of horseradish peroxidase (Van Scott *et al.*, 1984). Alveolar macrophages (4×10^6) were preincubated at 37°C in 3 ml of Hepes-buffered medium for 10 min. After preincubation, scopoletin (1.2 μM) and type IX horseradish peroxidase (6.6 units/ml) were added and fluorescence of the cell suspension was monitored in the absence or presence of alkaloid (5–55 μg/ml) and/or zymosan (0.4 mg/ml) for 10 min. Fluorescence changes were converted to nanomoles of H_2O_2 using a standard curve.

In vivo treatment with bisbenzylisoquinoline alkaloids. Pathogen-free male Sprague–Dawley rats (175–225 g) were treated orally with the drug in conjunction with inhalation of silica. The silica used was 98.5% pure with a specific surface area of 3.97 m^2/g and with 80% of the particles having a diameter of less than 5 μm (Min-U-Sil, Pennsylvania Glass and Sand Corp., Berkeley Springs, WV).

Briefly, rats were treated orally by gavage with tetrandrine (33 μg/g body wt) or H_2O daily for 4 days. On the fifth day, rats were exposed by inhalation to either filtered air or silica for 6 hr. An aerosol of silica dust was generated

using a fluidized bed dust generator and animals were exposed in a nose-only chamber. The exposure level, determined gravimetrically from filter samples, was 117.5 ± 12.5 mg/m^3 and the mass median diameter of the silica dust generated was 1.54 ± 0.01 μm, determined with an aerodynamic particle sizer (Model APS 33, TSI, Inc., St. Paul, MN). After exposure, rats were returned to daily oral treatments with H_2O or the drug for 4 more days prior to euthanization. After euthanization alveolar macrophages were collected by bronchoalveolar lavage and prepared for microscopic study.

For transmission electron microscopy, alveolar macrophages were pelleted and fixed with 3% Karnovsky's fixative for 2 hr followed by 2% tannic acid and 2% osmium tetroxide for 60 min each. The pellets were then processed for embedding with LX112. Thin sections were cut on a LKB NOVA ultramicrotome and placed on 200-mesh copper grids. These were stained with 5% alcoholic uranyl acetate for 25 min and Reynold's lead citrate for 15 min.

Quantitation of drug binding to phospholipid. Binding of the drugs to phospholipid vesicles was studied fluorometrically at 37°C using 1-anilino-8-napthalene sulfonate (ANS) as a fluorescence probe. ANS shows weak fluorescence at 510 nm (excitation, 380 nm), but exhibits enhanced emission intensity at 480 nm when bound to phospholipid. The fluorescence of phospholipid-bound ANS may be further enhanced by the addition of a cationic amphiphilic amine capable of forming a more hydrophobic drug–lipid–ANS ternary complex (Ma *et al.*, 1985). The binding of the cationic amine involves interaction at the negatively charged oxygen of the phosphocholine moiety.

Phospholipid vesicles were prepared by sonicating a suspension of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in 0.01 M Tris buffer (pH 7.0) under nitrogen for 30 min at 50°C. Small vesicles were fractionated from large liposomes by centrifugation at 105,000*g* for 60 min. The concentration of DPPC was determined by measuring inorganic phosphorus.

To obtain binding information, solutions containing 10^{-4} M DPPC in the absence or presence of 10^{-4} M drug were titrated with aliquots of ANS (10^{-3} M stock solution) and fluorescence intensities (F) recorded. ANS titrations of solutions in the absence of DPPC were also performed to yield baseline fluorescence (F_0). The difference in fluorescence intensity ($F - F_0$) was calculated to obtain the relative binding affinity of these alkaloids to DPPC.

Quantitation of drug binding to alveolar macrophages. The binding of selected alkaloids to alveolar macrophages (4.0×10^6 cells/ml) was studied using an equilibrium dialysis method. Matched pairs of dialysis cells (1 ml in size, Thomas Scientific Company, Swedesboro, NJ) were separated by a dialysis membrane (Spectra/Por, Spectrum Medical Industries, Inc., Los Angeles, CA) with a molecular weight cutoff of 12,000–14,000. Equilibrium dialysis of drug from Hepes-buffered medium to cell solution was carried out at room temperature for 8 hr under constant shaking. The equilibrium condition was established in control experiments without the presence of alveolar macrophages. After dialysis, solutions from both sides of the dialysis cell compartments were centrifuged and the supernatants were analyzed for drug content. The results showed that upon binding to alveolar macrophages, the bound drug was effectively removed from the solution after the centrifugation of alveolar macrophages. Relative binding affinities of drugs to alveolar macrophages were determined from the relationships of bound vs total drug concentration.

The determination of drug concentrations in the dialysis samples was carried out via reverse-phase high-performance liquid chromatography (HPLC) using a Waters HPLC system equipped with a Model 440 uv detector (Waters Associates, Milford, MA). The separation of the alkaloid from other substances was achieved using a μBondapak C18 column and a mobile phase of CH_3CN/Hepes buffer (pH 5)/butanol (60:40:10) delivered at 1 ml/min. The samples were detected by uv at 254 nm. Linear standard curves for each alkaloid were obtained over the concentration range 5×10^{-6} to 8×10^{-5} M.

Statistics. Data were expressed as means ± standard errors of separate experiments. Data were compared to controls using one-way analysis of variance. Significance was set at $p < 0.05$.

RESULTS

An objective of this investigation was to compare the ability of various bisbenzylisoquinoline alkaloids to inhibit particle-stimulated respiratory burst activity of alveolar macrophages. Therefore, it was essential to demonstrate that these agents were not cytotoxic under conditions employed in this study. Cellular viability was monitored as trypan blue exclusion and oxygen consumption at rest. Membrane integrity of alveolar macrophages was not compromised by a 20 min *in vitro* exposure to 60 μg/ml tetrandrine or tubocurine; i.e., no significant decline in trypan blue exclusion was noted at 96 μM tetrandrine or 88 μM tubocurine from the control value of 92.0 ± 1.5%. The same was true for methoxyadiantifoline at a dose of 40 μg/ml or lower, i.e., concentrations lower than 53 μM. Significant leakage of trypan blue dye was recorded at doses of 50 and 60 μg/ml methoxyadiantifoline, i.e., concentrations of 66 and 79 μM, where trypan blue exclusion was decreased by 16 and 35%, respectively. However, mem-

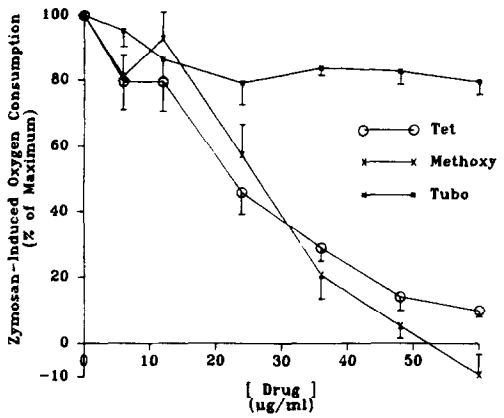


FIG. 2. Effect of various bisbenzylisoquinoline alkaloids on oxygen consumption by zymosan-stimulated alveolar macrophages. Cells were treated with various concentrations of alkaloids for 5 min at 37°C prior to the addition of zymosan (2 mg/ml). Data are presented as percentage of the zymosan-stimulated increase in oxygen consumption measured in the absence of alkaloids. In the absence of alkaloids, oxygen consumption was 28.07 ± 1.37 nmol/10^6 cells/30 min and 76.91 ± 3.65 nmol/10^6 cells/30 min for resting and zymosan-stimulated alveolar macrophages, respectively. Values represent means ± standard errors of four separate experiments.

brane integrity was unaffected even at these concentrations when exposure was limited to 10 min. These alkaloids were also judged to be nontoxic as evaluated by their inability to decrease the resting rate of oxygen consumption; i.e., resting oxygen consumption by alveolar macrophages was not significantly affected by exposure to 60 μg/ml tetrandrine (96 μM), tubocurine (88 μM), or methoxyadiantifoline (79 μM) where the control level was 28.07 ± 1.37 nmol/10^6 cells/30 min.

Since these alkaloids were not toxic to alveolar macrophages after short term *in vitro* treatment, it was possible to determine if these agents specifically inhibited the ability of stimulants to activate alveolar macrophages, i.e., whether the magnitude of zymosan-induced respiratory burst and secretion of reactive oxygen species was depressed. Tetrandrine and methoxyadiantifoline were potent inhibitors of zymosan-stimulated oxygen consumption (Fig. 2). Maximal inhibition was approximately 90% at 60 μg/ml tetrandrine (96 μM) and 100% at 60 μg/ml methoxyadiantifoline (79 μM). The ID_{50} values for tetrandrine and methoxyadiantifoline were 22 μg/ml and 26 μg/ml, respectively. On a concentration basis the ID_{50} values were identical, i.e., 34 μM. In contrast, tubocurine was much less effective, i.e., 60 μg/ml tubocurine (88 μM) reduced zymosan-induced oxygen consumption by only 21%. Similar results were obtained with zymosan-stimulated superoxide and hydrogen peroxide release. Superoxide was maximally inhibited by 73% at 60 μg/ml tetrandrine (96 μM) and by 85% in the presence of 60 μg/ml methoxyadiantifoline (79 μM) (Fig. 3). ID_{50} values were 16 μg/ml for tetrandrine and 40 μg/ml methoxyadiantifoline, i.e., 26 μM and 53 μM, respectively. Tubocurine had no effect on zymosan-stimulated superoxide

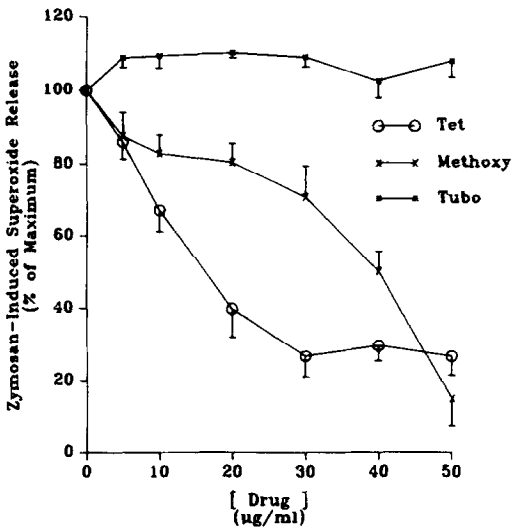


FIG. 3. Effect of various bisbenzylisoquinoline alkaloids on superoxide anion release from zymosan-stimulated alveolar macrophages. Zymosan (2 mg/ml) and various concentrations of alkaloids were added at zero time and cytochrome *C* reduction was monitored for 30 min at 37°C. Data are presented as percentage of the zymosan-stimulated increase in superoxide release measured in the absence of alkaloids. In the absence of alkaloids, superoxide release was 15.0 ± 2.9 nmol/10^6 cells/30 min and 88.5 ± 14.6 nmol/10^6 cells/30 min for resting and zymosan-stimulated alveolar macrophages, respectively. Values represent means ± standard errors of four separate experiments.

release. Similarly, hydrogen peroxide secretion by alveolar macrophages was significantly inhibited by tetrandrine and methoxyadiantifoline (Fig. 4). Maximal inhibition was 75% at 60 μg/ml tetrandrine (96 μM) and 100% at 25 μg/ml methoxyadiantifoline (33 μM) with ID_{50} values of 24 μg/ml (38 μM) and 6 μg/ml (8 μM), respectively. Unfortunately, tubocurine directly interacted with the fluorescent probe (scopoletin) used in the hydrogen peroxide assay. Therefore, its effect on hydrogen peroxide release by alveolar macrophages could not be determined.

Figure 5 shows transmission electron micrographs of alveolar macrophages obtained from silica-exposed rats receiving oral treatments of either water (5A) or tetrandrine (5B). Cell inclusions of silica were readily seen in both the control and tetrandrine groups. However, lipid material associated with these silica inclusions was apparent in the tetrandrine group. Alveolar macrophages in the tetrandrine-treated group were also characterized by a distinctly dark plasma membrane.

Since differences in staining properties reflect changes in membrane characteristics, these results suggest that tetrandrine was interacting with the macrophage membrane. To test this possibility, we monitored the binding of various alkaloids to the membrane lipid, disaturated phosphatidylcholine (DPPC). Figure 6 shows that there was a dose-dependent interaction of the bisbenzylisoquinoline alkaloids with DPPC vesicles. The binding affinity of these alkaloids was: methoxyadiantifoline > tetrandrine > tubocurine. We also quantitated the binding of these alkaloids to alveolar macrophages *in vitro* (Fig. 7). Both tetrandrine and methoxyadiantifoline exhibited strong binding to alveolar macrophages with the binding affinity of tetrandrine being slightly greater than that of methoxyadiantifoline. In contrast, tubocurine exhibited little binding to alveolar macrophages.

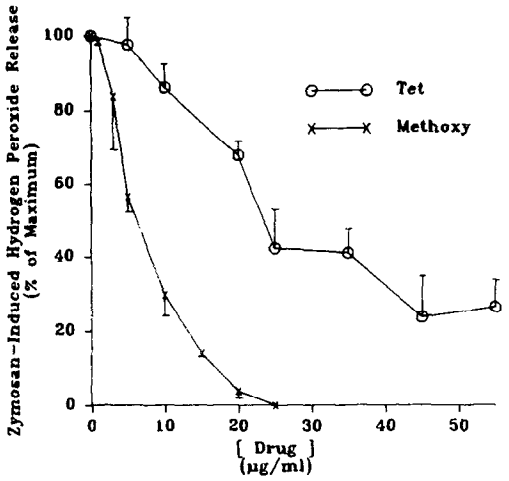


FIG. 4. Effect of various bisbenzylisoquinoline alkaloids on hydrogen peroxide secretion from zymosan-stimulated alveolar macrophages. Cells were treated with various concentrations of alkaloids for 1 min at 37°C prior to the addition of zymosan (0.4 mg/ml). Data are presented as percentage of the zymosan-stimulated increase in hydrogen peroxide secretion measured in the absence of alkaloids. In the absence of alkaloids, hydrogen peroxide release was less than 0.1 nmol/min/3 × 10^6 cells at rest and 10.0 ± 1.9 nmol/min/3 × 10^6 cells in zymosan-stimulated alveolar macrophages. Values represent means ± standard errors of three separate experiments.

DISCUSSION

This investigation measured the ability of three bisbenzylisoquinoline alkaloids to bind to alveolar macrophages and to inhibit particle-stimulated activation of these phagocytes. Tetrandrine was chosen for study because of its reported antifibrotic activity. This drug has been shown to decrease both the synthesis of collagen and the formation of fibrotic nodules in rat lungs after intratracheal instillation of silica (Liu *et al.*, 1983; Huang *et al.*, 1981; and Yu *et al.*, 1983). Tetrandrine has also been used clinically in China as a treatment for silicosis (Li *et al.*, 1981). Reports suggest that patients treated with tetrandrine show marked clinical improvement, i.e., clearing of shadows on chest x rays and an improvement of diffusion capacity, with few side effects. In contrast, tubocurine, a structural analogue of tetrandrine, has been shown to be ineffective in reducing collagen formation or preventing nodule development in silicotic rats (Mo *et al.*, 1982). Therefore, it was used as a negative control. Methoxyadiantifoline is an alkaloid

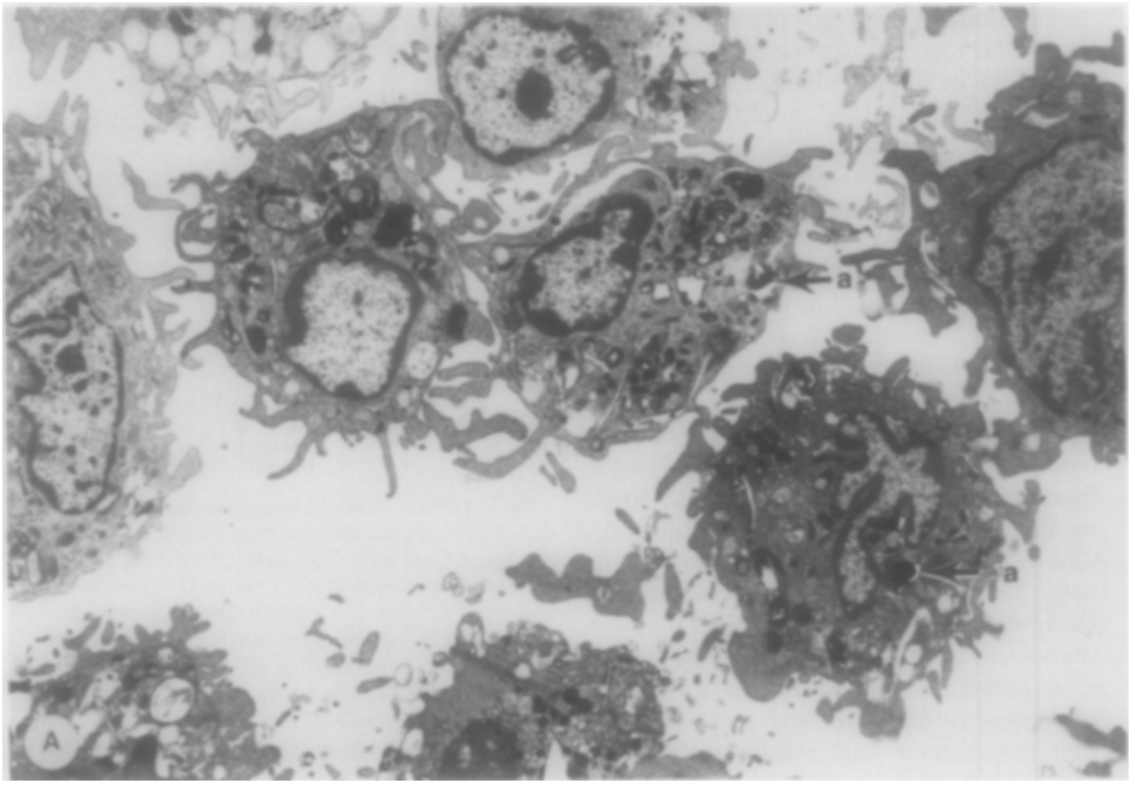


FIG. 5. Transmission electron micrographs of alveolar macrophages obtained from rats after (A) inhalation of silica and oral treatment with water, or (B) inhalation of silica and oral treatment with tetrandrine. Rats were given water or tetrandrine (33 μg/g body wt/day) orally for 4 days prior and 4 days after inhalation exposure (6 hr to 117 mg/m^3). Key: (a) silica inclusions, (b) lipid inclusions, and (c) darkly stained plasma membrane.

of untested antifibrotic potential. However, like tetrandrine, it has been shown to be hypotensive and reduce the contractility of coronary smooth muscle (Jia *et al.*, 1988).

The results of the present investigation indicate that tetrandrine, an alkaloid with antifibrotic potential, binds strongly to lipid vesicles and alveolar macrophages. Further, tetrandrine is a potent *in vitro* inhibitor of particle-induced activation of alveolar macrophages. In contrast, tubocurine, an alkaloid which is ineffective in preventing fibrosis, exhibits weak binding to lipids, no binding to macrophages, and no inhibitory activity toward alveolar macrophages. Methoxyadiantifoline, a drug of untested antifibrotic potential, exhibits binding and inhibitory activities *in vitro* which are similar to those of tetrandrine.

Although the present study demonstrates that tetrandrine is a potent inhibitor of particle-induced activation of the respiratory burst in alveolar macrophages, evidence suggests that particle uptake is not inhibited by tetrandrine treatment. Indeed, *in vivo* treatment of silica-exposed rats with tetrandrine did not greatly affect the number of silica particles observed by transmission electron microscopy in lavaged alveolar macrophages (Fig. 5). Furthermore, *in vitro* treatment of alveolar macrophages with tetrandrine has been found to have no effect on the phagocytosis of diisodecyl phthalate particles containing oil red O (Castranova *et al.*, 1991).

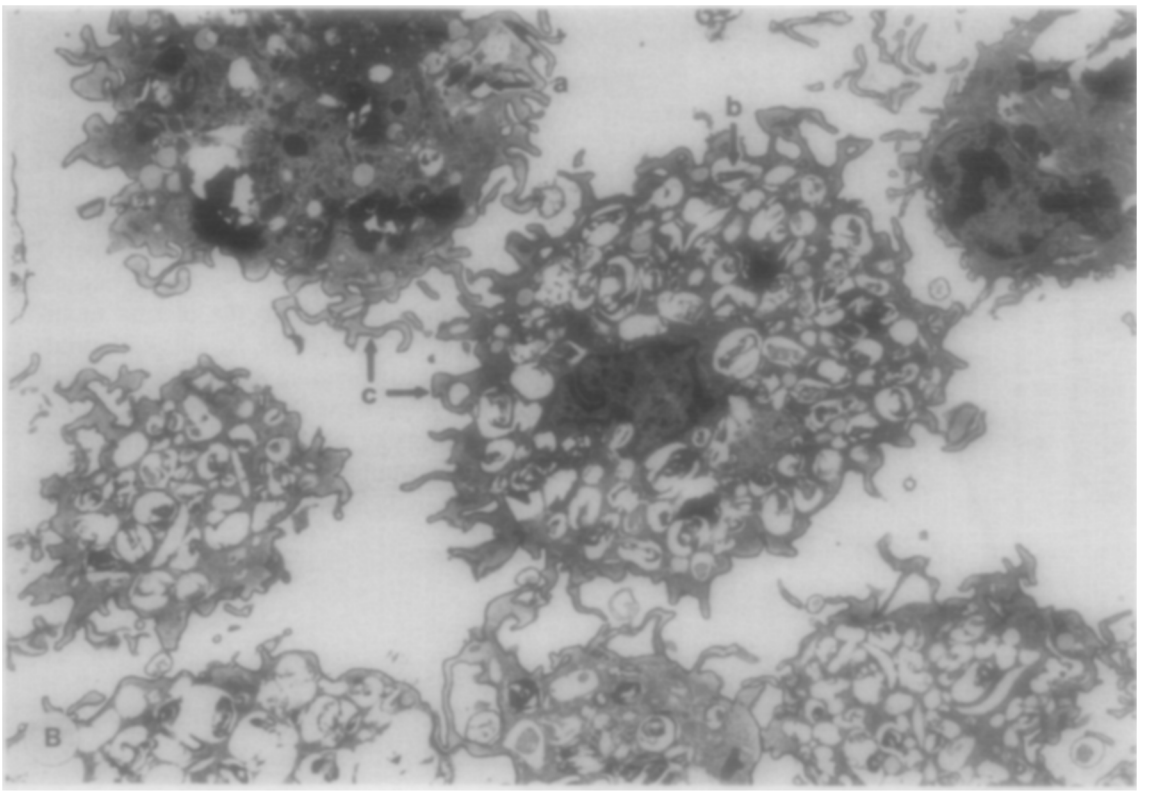


FIG. 5—*Continued*

Data in Fig. 5 indicate that alveolar macrophages harvested from rats treated by gavage with tetrandrine and exposed by inhalation to silica contained lipid inclusions. The reason for this increase in intracellular lipid is unclear. However, data from our laboratory suggest that it is not due to either an increase in surfactant production or a decrease in the ability of macrophages to metabolize surfactant, since DPPC obtained by bronchoalveolar lavage was not significantly elevated after oral administration of tetrandrine and *in vitro* treatment with tetrandrine did not inhibit degradation of DPPC by macrophages.

The present study concerns the *in vitro* effects of three bisbenzylisoquinoline alkaloids on the ability of alveolar macrophages to become activated by particles. Data indicate that tetrandrine and methoxyadiantifoline inhibit particle-induced release of reactive oxygen species while tubocurine has little effect. Our lab has also found that tetrandrine was effective *in vivo*. Briefly, alveolar macrophages harvested from rats exposed to silica either by inhalation or by intratracheal instillation exhibited enhanced zymosan-stimulated hydrogen peroxide release. Oral administration of tetrandrine (33 μg/g body wt/day) significantly inhibited this silica-induced activation (Castranova *et al.* 1990; Castranova *et al.*, 1991). In contrast, oral treatment with tetrandrine failed to prevent the inflammatory response to particulate inhalation. That is, inhalation of silica resulted in a 3.4-fold increase in the number of leukocytes harvested from rats 1 day after silica exposure. Oral treatment with tetrandrine did not significantly affect this inflammatory response (Moore, 1988). Similarly, *in vivo* treatment of guinea pigs with tetrandrine did not significantly decrease the in-

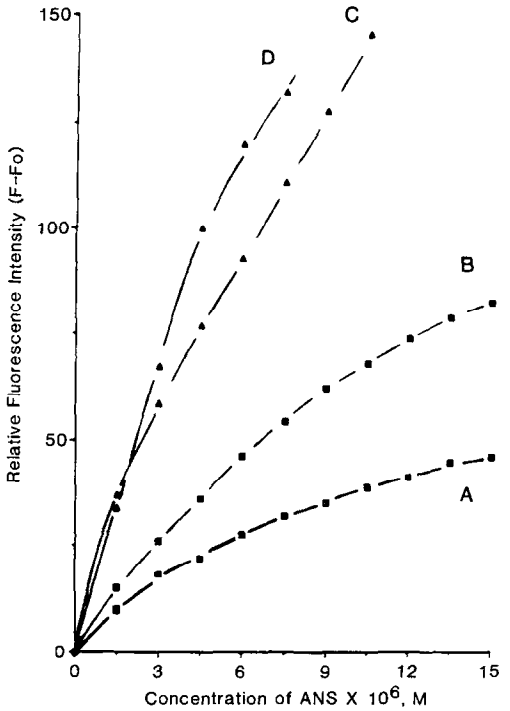


FIG. 6. Binding of methoxyadiantifoline (D), tetrandrine (C), and tubocurine (B) to DPPC vesicles. Fluorometric titration curves were obtained for solutions containing 10^{-4} M DPPC in the absence (A) or presence of alkaloids (B–D) and varying concentrations of ANS. Fluorescence was monitored at an excitation wavelength of 380 nm and an emission wavelength of 480 nm. F_0 and F are fluorescence intensities measured in the absence and presence of DPPC, respectively.

flux of leukocytes into air spaces which occurred in response to inhalation of cotton dust (Castranova *et al.*, 1989).

Recently, the potential antifibrotic activity of tetrandrine and several other bisbenzylisoquinoline alkaloids has been tested using a silicotic rat model (Mo *et al.*, 1982). The potency reported was tetrandrine ≈ cycleanine > cepharanthine > fangchinoline > berbamine > curine ≈ tubocurine. Ma *et al.* (1990) found that this potency sequence correlated very well with the binding affinity of these alkaloids with membrane lipid and with alveolar macrophages. However, to date, an investigation of the ability of cycleanine, cepharanthine, fangchinoline, berbamine, or curine to inhibit particle-induced activation of alveolar macrophages has not been conducted.

The data suggest a strong relationship between binding, inhibition of macrophages, and antifibrotic potential. Transmission electron micrographs of alveolar macrophages harvested from tetrandrine-treated rats show a darkly stained plasma membrane compared to controls (Fig. 5). These results may suggest that tetrandrine binds to the plasma membrane of these phagocytes. Results with binding to vesicles of membrane lipid are consistent with this hypothesis (Fig. 6). Recent studies have shown that tetrandrine avidly binds to isolated microtubules and to these cytoskeletal structures in 3T3 cells (Liu *et al.*, 1988; Chen

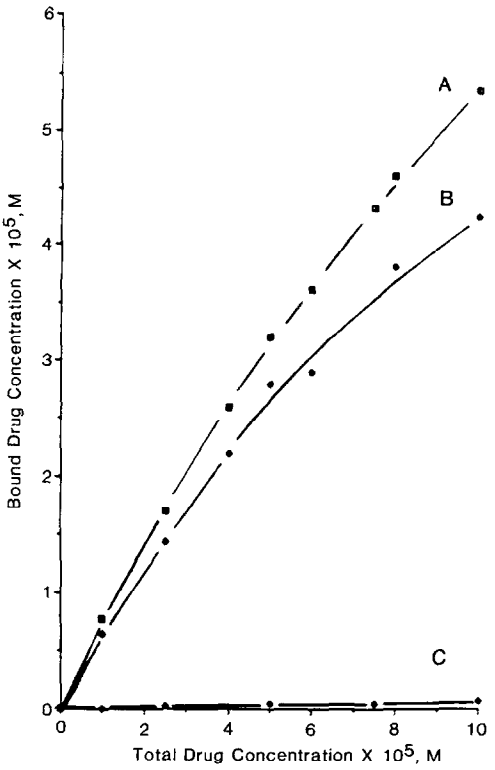


FIG. 7. *In vitro* binding of tetrandrine (A), methoxyadiantifoline (B), and tubocurine (C) to alveolar macrophages. Solutions contained 4×10^6 cells/ml and various concentrations of alkaloids. Binding was monitored using an equilibrium dialysis method and alkaloid concentration determined using an HPLC system equipped with a uv detector.

et al., 1988). Several studies indicate that activation of the respiratory burst of phagocytic cells is associated with the rapid conversion of monomeric actin to polymeric actin (Rao and Varani, 1982; Fechheimer and Zigmond, 1983; Howard and Meyer, 1984; Wallace *et al.*, 1984). Furthermore, inhibition of stimulant-induced actin polymerization correlates with inhibition of superoxide anion secretion, hydrogen peroxide release, and chemiluminescence generation by neutrophils (Rao and Castranova, 1988). Therefore, it is possible that tetrandrine acts to block particle-induced polymerization of microtubules and in this manner prevents the activation of NADPH oxidase in the macrophage membrane. Clearly, further investigation concerning the site of tetrandrine action in the stimulus-secretion coupling scheme is required.

Results from our laboratory indicate that both *in vitro* and *in vivo* silica exposures result in the release of reactive forms of oxygen from alveolar macrophages (Castranova *et al.*, 1990; Goodman *et al.*, 1990). Such excess oxidant generation has been associated with damage to the lung parenchyma and silicosis (Brain, 1980; Weiss and LoBuglio, 1982; Ghio *et al.*, 1990). These results support the theory that activation of alveolar macrophages plays an important role in the development of silicosis. In this study, we have shown that an antifibrotic agent, tetrandrine, is a potent *in vitro* inhibitor of the zymosan-induced respiratory burst and secretion of reactive oxygen species (Figs. 2–4). Data from our laboratory indicate that tetrandrine is also effective in preventing activation of alveolar macrophages by silica dust (Castranova *et al.*, 1990). Furthermore, reports indicate that tetrandrine is effective in inhibiting stimulant-induced chemotaxis, degranulation, and superoxide secretion by neutrophils (Seow *et al.*, 1988; Matsuno *et al.*, 1987). Therefore, tetrandrine may decrease silica-induced damage of the lung parenchyma by preventing hypersecretion of reactive products from macrophages and neutrophils.

Our results support the potential usefulness of tetrandrine and methoxyadiantifoline as antifibrotic drugs. In addition, tetrandrine (effective) and its analogue tubocurine (ineffective) should prove to be useful positive and negative probes to investigate the mechanisms involved in phagocyte activation and to elucidate the etiology of pulmonary fibrosis.

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