Applied Occupational and Environmental Hygiene

ISSN: 1047-322X (Print) 1521-0898 (Online) Journal homepage: http://www.tandfonline.com/loi/uaoh20

Multiple Emulsion-Mediated Enhancement of the Therapeutic Effect of Tetrandrine against Silicosis

De-Hwa Chao , Jane Y.C. Ma , Carl J. Malanga , Daniel E. Banks , Ann F. Hubbs , Yongyut Rojanasakul , Vincent Castranova & Joseph K.H. Ma

To cite this article: De-Hwa Chao , Jane Y.C. Ma , Carl J. Malanga , Daniel E. Banks , Ann F. Hubbs , Yongyut Rojanasakul , Vincent Castranova & Joseph K.H. Ma (1996) Multiple Emulsion-Mediated Enhancement of the Therapeutic Effect of Tetrandrine against Silicosis, Applied Occupational and Environmental Hygiene, 11:7, 1008-1018, DOI: 10.1080/1047322X.1996.10390003

To link to this article: http://dx.doi.org/10.1080/1047322X.1996.10390003

Published online: 24 Feb 2011.

Multiple Emulsion-Mediated Enhancement of the Therapeutic Effect of Tetrandrine Against Silicosis

De-Hwa Chao,[A] *Jane Y.C. Ma,*[B] *Carl J. Malanga,*[A] *Daniel E. Banks,*[C] *Ann F. Hubbs,*[B] *Yongyut Rojanasakul,*[A] *Vincent Castranova,*[B] *and Joseph K.H. Ma*[A,D]

[A]School of Pharmacy, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506; [B]Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; [C]Section of Pulmonary and Critical Care Medicine, School of Medicine, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506; [D]To whom correspondence should be addressed

Using a water-in-oil-in-water multiple emulsion system developed for pulmonary drug targeting, the effectiveness of tetrandrine as an antifibrotic agent and the therapeutic advantage of a tetrandrine emulsion over drug in solution for the treatment of silicosis were investigated in rats. Previously we have shown that the action of tetrandrine is attributed to its ability to inhibit the release of reactive oxygen metabolites and inflammatory cytokines by alveolar macrophages, and that targeted delivery of tetrandrine to alveolar macrophages using a multiple emulsion system minimizes drug toxicity, maintaines the drug's pharmacological activity, and enhances tetrandrine distribution in the lungs while reducing systemic drug distribution. The purpose of the present study is to provide *in vivo* evidence of emulsion-mediated enhancement of drug action in the lungs against silica-induced lung injury using a rat model. The antifibrotic action of tetrandrine was evaluated by examinations of lung histology, alveolar cell differentials, *in vivo* drug effect on macrophage respiratory burst, and the measurements of lung weight and collagen content. Tetrandrine was shown to inhibit the macrophage-orchestrated inflammatory process in response to silica exposure, preventing infiltration of neutrophils into the alveolar space. In addition, the drug was also shown to protect the cells from silica-induced toxicity and stimulation, and to restore healthy alveolar macrophage populations in the alveolar region. Intervention of the silica effect with tetrandrine markedly decreased light microscopic lung lesions. These results were also supported by quantitative inhibition of a silica-induced increase in lung weight and collagen content by tetrandrine. In all experiments the tetrandrine emulsion system was shown to be consistently more efficacious than the solution dosage form in the treatment of silica-induced granulomatous pneumonia, alveolar lipoproteinosis consistent with acute silicosis, and fibrosis. These results confirm our hypothesis regarding the functional and morphologic interactions of emulsions with alveolar macrophages *in vivo*. CHAO, D.-H.; MA, J.Y.C.; MALANGA, C.J.; BANKS, D.E.; HUBBS, A.F.; ROJANASAKUL, Y.; CASTRANOVA, V.; MA, J.K.H.: MULTIPLE EMULSION-MEDIATED ENHANCEMENT OF THE THERAPEUTIC EFFECT OF TETRANDRINE AGAINST SILICOSIS. APPL. OCCUP. ENVIRON. HYG. 11(7):1008–1018; 1996.

The sequential cellular and biochemical events in silica-induced lung injury involve (1) primary dust reactions at the epithelium; (2) reactions of dusts with lung interstitial cells; and (3) steps from cytokine-mediated fibrogenesis to fibrosis. The alveolar wall consists of a specialized epithelium and a closely apposed network of capillaries supported by a delicate interstitial matrix. Nearly 95 percent of the alveolar surface area is covered by a single layer of epithelial type I pneumocytes joined by tight junctions. These terminally differentiated cells are vulnerable to a variety of injuries and have little, if any, regenerative or replicative potential. Type II cells, on the other hand, are more resistant to oxidative injury[1] and have the capacity to proliferate and repair the epithelial lesions.[2,3] Alteration of the epithelial structure by silica can lead to the migration of dust into the interstitium and the development of fibrosis. As seen in acute silicosis, silica causes severe damage to the lining cells of the airways where lipoprotein exudate accumulates, obliterating the air space. These events are followed by type II pneumocyte hypertrophy and hyperplasia, phospholipidosis, and impaired phagocytosis.[4]

Alveolar macrophages (AM) are a key cell type involved in the fibrotic process. Silica stimulates AM to release proteolytic enzymes and reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl free radical.[5,6] Hypersecretion of these products in response to intense or prolonged silica exposure may overwhelm the lung's natural protective mechanisms, such as those by antioxidant enzymes and antiproteases, and result in cell damage and destruction of the lung parenchyma.[7,8] Hydrogen peroxide, for example, has been shown to damage tight junctions between epithelial cells in culture.[9]

Macrophage-derived cytokines, such as interleukin-1 (IL-1),[10,11] tumor necrosis factor (TNF),[12] platelet-derived growth factor (PDGF),[13] macrophage-derived growth factor,[14] and fibronectin have been shown to stimulate fibroblast growth *in vitro*. Goldring and Krane[10] showed that IL-1 also enhances fibroblast production of collagen in culture. Alveolar macrophages also release chemotactic factors such as interleukin-8 and leukotriene B4, which, along with IL-1, TNF, and PDGF, recruit polymorphonuclear leukocytes and activate their phagocytic and secretory processes, resulting in alveolar granuloma and the release of more oxidative species including hypochlorous acid by these phagocytes.[8,15,16] Indeed, neu-

trophil-derived oxidative products have been linked to damage of lung explant tissue and have been implicated in fibrotic lung diseases.[17] The net result of these cell–cell interactions and hypersecretion is an escalating inflammatory cycle which may be directly linked to pulmonary fibrosis. Thus, ample evidence suggests that AM play an important role in the pathogenesis of silicosis. The silica-mediated release of oxidative products and secretion of inflammatory and fibrogenic factors by AM are important events in understanding and treating silicosis.

Despite the difficulties in treating fibrotic lung diseases, continuing efforts have been made to develop therapeutic strategies to treat silicosis by preventing the development of associated diseases, by pharmacologically controlling, reversing, or halting the progression of fibrosis, or by removing the fibrogenic dust stimuli, activated AM, and other stimulated effector cells and cytokines which perpetuate the fibrotic process.[18] Corticosteroid therapy, for example, has shown an apparent short-term potential to suppress inflammation in the lungs of those with acute silicosis,[19] suggesting that drugs which inhibit the macrophage-orchestrated inflammatory cycle can be reasonably expected to have therapeutic benefits.[19]

Tetrandrine is a plant alkaloid which has been shown to inhibit silica-induced pulmonary fibrosis in experimental animals.[20] The chemical activity of this drug is characterized by its two benzylisoquinoline moieties connected in a head-to-head, tail-to-tail fashion via two ether linkages, resulting in a ring of 18 carbons with α, β stereochemistry at the isoquinoline chiral centers. Dozens of bisbenzylisoquinoline alkaloids have been tested in experimental models for the treatment of silicosis. Tetrandrine was the most potent antifibrotic agent among these alkaloids.[21] Reports indicated that tetrandrine resulted in a decrease in the synthesis of collagen by pulmonary fibroblasts and in the formation of silicotic nodules.[20–22] Studies in our laboratory showed that tetrandrine bound strongly and selectively to viable AM at the cellular plasma membrane.[23] Binding of tetrandrine to AM was blocked by cytochalasin B, vinblastine, and taxol,[24] suggesting that this binding involves drug interaction with the cellular cytoskeletal system. In addition, binding of tetrandrine to AM was enhanced when AM were stimulated by zymosan or phorbol ester,[24] indicating a dynamic interaction between tetrandrine and AM which depends on the cellular cytoskeletal control of membrane activity. Tetrandrine has no effect on the release of oxidative products or cytokines by nonstimulated AM, but shows strong inhibition on particle-stimulated macrophage respiratory burst and the secretion of IL-1-like cytokines by AM challenged with silica and other stimuli.[24–26] Our data on the binding and inhibitory effects of tetrandrine and other bisbenzylisoquinoline alkaloids show a strong correlation between the drug's binding affinity to AM and its *in vitro* inhibitory effect, and between the drug's *in vitro* inhibitory effect and its reported *in vivo* antifibrotic potential.

In a clinical study tetrandrine was reported to improve diffusion capacity and decrease the size of lesions visualized on chest radiographs of patients suffering from silicosis,[27] but the therapeutic value of tetrandrine as an antifibrotic agent remained inconclusive. Some pharmaceutical and toxicological factors may have contributed to the difficulty in clinically assessing this drug. Tetrandrine was given orally in a drug regimen which called for 3-month treating periods followed by 2- to 3-month resting periods of no drug treatment. These resting periods were to avoid drug toxicities reported to be reversible when the drug was discontinued. Most noticeably, patients receiving tetrandrine developed dose-related brownish-black skin pigmentation following 2 to 3 months of drug treatment. Transient enlargement of the liver and elevation of serum transaminase were also seen in some cases associated with tetrandrine treatment. Oral delivery of lipophilic drugs is subjected to significant first-pass hepatic extraction. This may account for the observed liver toxicity. The skin pigmentation indicates a wide tissue distribution of the drug with a long biological half-life. Indeed, studies in animals have shown that the accumulation of tetrandrine in viscera was dose dependent and decreased gradually to one tenth of the original level over 2 months after discontinuation of the drug.[28] The low therapeutic index associated with the oral regimen has limited the use of tetrandrine for the treatment of silicosis. A lack of sound pharmaceutical approaches in drug delivery strategies may have also obscured the observation of the drug's pharmacological effect.

Selective delivery of therapeutic agents to specific sites in the body has the potential to provide increased drug exposure at the site of action and reduced systemic drug toxicity. The concept of using a colloidal carrier for site-specific drug delivery has been demonstrated using a variety of particulate systems, including liposomes,[29] polymeric microspheres, and multiple emulsions.[30] Multiple emulsions are complex systems which contain small droplets within large dispersed drops where the external liquid phase is miscible or identical with the continuous phase. They are, therefore, emulsions of emulsions. With recent advances in emulsion technology, stable multiple emulsions have been developed for diverse applications.[31,32] As drug carriers, multiple emulsions have been shown to sustain drug release and to enhance uptake of anticancer agents by tumor cells.[33–35]

Emulsion-entrapped drugs will follow the fate of the carrier, resulting in drug release at the site of emulsion destruction. The multiple emulsion system is recognized as foreign by the mononuclear phagocyte system (i.e., they may be rapidly removed from the circulation after intravenous administration and accumulated in tissues with high phagocytic activities). With desirable particle size, multiple emulsions will be sequestered by the lung through filtration mechanisms. The intravenous route offers further advantage over an oral dose for pulmonary drug delivery by eliminating the first-pass hepatic extraction. Previously we have developed a stable multiple emulsion system containing peanut oil as the liquid membrane, which reduced tetrandrine toxicity to AM, enhanced AM uptake of drug and drug distribution in the lungs, and reduced systemic drug levels.[36,37] The purpose of the present research is to investigate the hypothesis that the multiple emulsion system, through its effect on pulmonary drug targeting, enhances the therapeutic activity of tetrandrine against silicosis. To achieve this objective, *in vitro* and *in vivo* experiments have been carried out to evaluate (1) effects of emulsion on *in vivo* drug–cell interactions, (2) effects of emulsion on the antifibrotic action of tetrandrine as indicated by measurement of lung weight, collagen content, alveolar cell differentials, and lung histology, and (3) correlations between drug targeting, cellular activities, and the therapeutic effect.

Methods

Preparation of Multiple Emulsions

Span 80 (sorbitan monooleate), Tween 80 (polysorbate 80), peanut oil, and hydrogenated soybean oil (HSO) were obtained from Ruger Chemical Co., Inc. (Irvington, New Jersey). Tetrandrine was obtained as a gift from the Institute of Occupational Medicine, Chinese Academy of Prevention Medicine (Beijing, China). All other chemicals were obtained from commercial sources as specified.

Multiple water-in-oil-in-water (w/o/w) emulsions were prepared using a two-step emulsification method modified from that of Matsumoto *et al.*[38] Tween 80 and Span 80, initially dispersed separately in the internal aqueous phase and the oil phase, respectively, were used as emulsifiers in the primary w/o emulsion, which was proportioned to give a hydrophile–lipophile balance of 5.5 at the liquid interface. The oil phase contained 27 percent (wt/vol) of the lipophilic emulsifier and 73 percent of a blend of peanut oil with HSO. One milliliter of a tetrandrine solution (70 mg/ml) containing 10 percent (wt/vol) of Tween 80 was introduced into 3 g of the oil phase and emulsified by sonication at 4°C using a sonicator–cell disruptor (model W-225, Heat Systems-Ultrasonics, Plainview, New York). For the second-step emulsification, 8 ml of an aqueous solution containing Tween 80 (1%) and Triton X-100 (0.05%) as the hydrophilic emulsifier was added to the primary emulsion, and the mixture was agitated with a vibrator–mixer (Gerald K. Heller Co., Las Vegas, Nevada) for 3 minutes in an ice bath. Particle size analysis was performed on all freshly prepared and aged formulations to determine the number and mean diameter of the droplets in the emulsions, using an electronic Coulter counter (model Z_B, Coulter Instruments) equipped with a sizing attachment.[39] To determine drug content in w/o/w multiple emulsion, samples of emulsions (6 ml) introduced into cellulose dialysis tubes were dialyzed against 400 ml of an isotonic phosphate buffer solution (pH 7.4) for 6 hours at 4°C. At this temperature emulsions were stable and without drug release. The dialysis sack was agitated and maintained in a vertical floating position by attaching a regular liquid-tight closer on the top and a magnetic weighted closure on the bottom to submerse the tube completely into the buffer solution. Drug concentration in emulsion following dialysis was determined by high performance liquid chromatography[24] using a reverse-phase μBondapak C_{18} column (Waters Associates, Milford, Massachusetts) and a mobile phase of acetonitrile:HEPES buffer (pH 4.5):butanol in a 32:65:3 ratio, delivered at a flow rate of 1.0 ml/min. Tetrandrine was detected by ultraviolet light at 254 nm using albendazole as an internal standard. The multiple emulsion system thus prepared contained peanut oil and HSO (7:3) as the lipid membrane, Tween 80 and Span 80 in the internal aqueous phase, and Tween 80 plus Triton X-100 in the external aqueous phase. The mean emulsion droplet diameter was 5.5 μm, with 8×10^{10} droplets/ml and a final drug concentration of 5.25 mg/ml.

Silica Exposure and Drug Treatment

Male Sprague–Dawley rats (180 to 200 g) were anesthetized with sodium brevital, and silica (40 mg of silica/0.5 ml of saline) or saline (nonsilicotic control) was instilled into the lungs via the trachea. The silica used was 98.5 percent pure with a specific surface area of 3.97 m^2/g and with 80 percent of the particles having a diameter of less than 5 μm (Min-u-sil; Pennsylvania Glass and Sand Corp., Berkeley Springs, West Virginia). Following intratracheal instillation, a daily single dose of tetrandrine either in solution or in emulsion (13 mg/kg) was administered through the tail vein for 4 days. The animals were under normal care for 2 or 4 weeks. Animals were sacrificed and alveolar cells were harvested by bronchoalveolar lavage (BAL) and analyzed for cell counts and differentiation. The lungs were isolated, and the left lungs were used for lung weight measurement and the right lungs were minced and further prepared for quantitative measurement of collagen content by hydroxyproline analysis. Lavaged lung specimens were also prepared for histological examination of the silicotic development. Evidence of *in vivo* drug–cell interactions was determined via measurements of resting and zymosan-stimulated oxygen consumption by AM from silicotic and nonsilicotic control rats and from drug-treated animals.

Bronchoalveolar Lavage

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (0.2 g/kg body weight) and exsanguinated by cutting the left renal artery. Alveolar cells were obtained by pulmonary lavage using an ice-cold, Ca^{++}-free medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.5 mM glucose; pH 7.4). Cells were centrifuged at 500 *g* for 5 minutes at 4°C, washed, and resuspended in a HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, and 1 mM $CaCl_2$; pH 7.4). Cell differentials, number, and mean cell volume were measured using an electronic cell counter equipped with a sizing attachment. The average values of parameters determined for AM in control animals were: yield, $8.6 \pm 0.3 \times 10^6$ AM/rat; purity, 90.3 ± 3.2 percent; mean cell volume, 1282 ± 8 μm^3. Cell viability was determined microscopically by monitoring the membrane integrity using the Trypan blue exclusion method.

Oxygen Consumption Studies

Oxygen consumption was monitored at 37°C using an oxygraph equipped with a Clark electrode.[40] The oxygraph was calibrated using media equilibrated with gases of known oxygen content. AM (4×10^6 cells) were incubated at 37°C in 1.75 ml Dulbecco's Modified Eagle Medium (GIBCO Laboratories, Life Technologies, Inc., Grand Island, New York) for 10 minutes, transferred to a temperature-controlled (37°C) chamber, and measured for oxygen consumption by AM at rest or for a zymosan (0.2 mg/ml)-stimulated level of oxygen consumption.

Measurement of Lung Collagen Content

The presence of hydroxyproline in animal tissues is found mainly in collagen. Hence, hydroxyproline has been widely used as an indicator for the synthesis and metabolism of collagen. Analysis of hydroxyproline in rat lungs was carried out using a method modified from that of Kivirikko *et al.*[41] A number of reagents and buffers were prepared. Potassium borate buffer was prepared by dissolving 61.84 g of boric acid and 225 g of KCl in 800 ml of distilled water. The solution was

TABLE 1. Effects of *In Vivo* Silica and Tetrandrine on BAL Cell Differentiation Measured 2 and 4 Weeks After Silica Treatment

	Cell Counts in Millions			
	AM		PMN	
Treatment Group	**2 Weeks**	**4 Weeks**	**2 Weeks**	**4 Weeks**
Nonsilicotic				
Saline	16.3 ± 0.5 (87.2 ± 3.2)[A]	—	1.5 ± 0.5 (7.7 ± 2.2)[A]	—
Tetrandrine solution	17.7 ± 2.2 (86.2 ± 2.1)	—	2.9 ± 1.8 (11.5 ± 2.8)	—
Tetrandrine emulsion	17.9 ± 2.7 (89.9 ± 0.8)	—	2.7 ± 1.0 (9.5 ± 2.2)	—
Silicotic				
Silica	18.4 ± 3.3 (63.0 ± 7.5)	3.7 ± 0.5 (44.3 ± 3.9)[B]	9.2 ± 2.0 (22.1 ± 4.4)[C]	2.1 ± 0.9 (15.6 ± 2.4)
Tetrandrine solution	16.6 ± 2.0 (62.0 ± 6.8)	—	5.7 ± 3.0 (13.5 ± 3.8)	—
Tetrandrine emulsion	18.1 ± 3.1 (71.3 ± 6.3)	14.1 ± 1.0 (59.2 ± 15.1)[D]	3.3 ± 1.1 (12.0 ± 2.9)[D]	6.2 ± 2.7 (15.1 ± 3.3)

[A]Values in parenthesis indicate percentage of cells counted in BAL. In silica-exposed groups, percentage cell counts not shown include the presence of silica particulate, red blood cells, and lymphocytes whose purities were not differentiated in the Coulter counter.
[B]Value is significantly lower than the saline control.
[C]Value is significantly higher than the saline control.
[D]Data are significantly different from those of the silicotic (no tetrandrine) control.

adjusted for pH to 8.7 with KOH (10 N or 1 N) and diluted to 1 L. Alanine solution was prepared by dissolving 10 g of DL-alanine (Aldrich Chemical Co., Milwaukee, Wisconsin) in 90 ml of distilled water, followed by adjusting pH to 8.7 with KOH and diluting the solution to 100 ml. A borate–alanine buffer was prepared by mixing the potassium borate buffer and alanine solution in a 2:1 ratio. Chloramine T solution (0.2 M) was prepared daily by dissolving chloramine T in methyl cellusolve (Sigma Chemical Co., St Louis, Missouri), whereas sodium thiosulfate solution (3.6 M) was prepared by dissolving reagent in distilled water and stored under toluene at room temperature. The Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) was prepared as follows: concentrated H_2SO_4 (27.4 ml) was slowly added to 200 ml of absolute alcohol, the mixture was allowed to cool, and a solution of 120 g of *p*-dimethylaminobenzaldehyde (Sigma Chemical Co.) in 200 ml of absolute alcohol was then added slowly to the sulfuric acid–alcohol mixture. Stock solution of hydroxyproline was prepared by dissolving 10 mg of trans-4-hydroxyl-L-proline (Sigma Chemical Co.) in 100 ml distilled water. Standards of 2 to 20 μg/2.5 ml were then prepared by diluting the stock with borate–alanine buffer.

Rat lungs were weighed and chopped with scissors into fine pieces. Two milliliters of 6 N HCl was added to each sample to hydrolyze the tissue for 48 to 72 hours at 110°C. Samples were neutralized with 10 N KOH and adjusted to 10 ml with distilled water and centrifuged. An aliquot of 0.1 ml of the centrifuged hydrolysates was pipetted into capped tubes and the volume was then adjusted to 2.5 ml with borate–alanine buffer. Standards and blank solutions were appropriately prepared in 2.5 ml of the same buffer as above. Saturated amounts of solid potassium chloride were added into each sample, and following vortex this imino acid mixture was oxidized at room temperature with 0.6 ml of freshly made 0.2 M chloramine T solution. After 30 minutes the oxidation was stopped by adding 2 ml of the sodium thiosulfate solution. Samples in capped tubes were further mixed with 3 ml toluene and heated in a boiling water bath for 30 minutes to form pyrrole. After cooling, the tube was shaken vigorously and centrifuged briefly at low speed, and a 1-ml portion of the toluene phase was placed in a separate tube. Addition of 0.4 ml of the Ehrlich's reagent to the toluene layer was quickly made by stirring, and the resulting color was measured after 30 minutes at 560 nm on a Hitachi U-2000 spectrophometer (Hitachi Instruments, Inc.).

Micrographic and Histological Examination

Samples of AM collected from bronchoalveolar lavage were prepared for microscopic study. For transmission electron microscopy (JOEL, 100CX), AM were pelleted and fixed with 3 percent Karnovsky's fixative in 0.1 M sodium cacodylate buffer for 2 hours, followed by treatment with 2 percent tannic acid and 2 percent osmium tetroxide for 60 minutes 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 percent alcoholic uranyl acetate for 25 minutes and Reynold's lead citrate for 15 minutes. The same procedure was carried out for the preparation of lung tissues. For light microscopy, hematoxylin/eosin stain and trichrome stain were used for observation of silicotic granuloma and collagen formation in lung tissues, respectively. Tissues were routinely processed, embedded in paraffin, and stained with hematoxylin, eosin, and Gomori's trichrome.[42] Pathological scoring of lung injury was made from coded specimens by an independent pathologist unaware of the exposure and treatment conditions.

Data and Statistical Analysis

All studies were carried out in repeated experiments to attain reproducibility, and the results were expressed as means ± standard errors of data from at least four animals. Statistical comparison of results was analyzed using analysis of variance and two sample Student's *t*-tests with the significance level set at $p \leq 0.05$.

Results and Discussion

The effects of intratracheal silica exposure and tetrandrine treatment on the bronchoalveolar cell differentials 2 and 4 weeks after silica instillation are shown in Table 1. In nonsilicotic rats, treatment of the animals with tetrandrine in solution

or in emulsion did not result in any change in the BAL cell counts when measured 2 weeks following the initial treatment. In comparison, lavaged cells obtained from silicotic rats showed similar numbers of alveolar macrophages representing a decreased percentage of the total cell population, suggesting infiltration of other cell types [red blood cells, polymorphonuclear leukocytes (PMN), lymphocytes, etc.] into the alveolar space. The number of PMN increased sixfold as compared with the nonsilicotic control group. Table 1 also shows that the tetrandrine treatment has a significant effect on silica-induced cell differentials 2 weeks after silica exposure. The tetrandrine solution resulted in a reduced influx of PMN, which were 62 percent of that in the untreated, silica-exposed rats (silicotic control). In rats receiving tetrandrine emulsion, the PMN were only 36 percent of those in the silicotic control. These results suggest that the silica-induced pulmonary inflammation was partially inhibited by tetrandrine, and more so by tetrandrine in the emulsion dosage form.

At 4 weeks after silica exposure, both the number and percentage of AM were substantially decreased in the silicotic control animals (Table 1). This decrease in AM yield may be due to the extensive granulomatous response in these lungs, which may trap AM in the lungs and prevent their efficient removal by lavage. Treatment with the tetrandrine emulsion, however, protected and restored AM populations in the injured lung. The PMN levels in silicotic control and drug-treated rats are similar to those seen in nonsilicotic animals, but represent higher percentages in total cell population.

The role of PMN in tissue injury secondary to silica exposure has been reported in several studies. Accumulation of PMN in the alveolar space was reported even at 6 months after a single intratracheal instillation of silica (50 mg) in guinea pigs.[43] As per early discussion, these phagocytes release enzymes and free radicals and contribute significantly to the silica-induced lung lesions. In normal lungs AM are the resident cells, whereas PMN are almost absent. Under silica exposure, AM may release chemotactic factors (IL-1, TNF, etc.) which recruit PMN from the capillary lumen to the alveolar space.[15] Indeed, migration of PMN from the intravascular space to extravascular sites largely depends on the adherence of PMN to endothelial cells. Both IL-1 and TNF have been shown to enhance adherence by a mechanism involving the regulation of expression of cell surface molecules on neutrophils and vascular endothelial cells.[12] Previously we have shown that tetrandrine inhibits silica-induced production of IL-1-like mediators by AM,[26] suggesting that the drug's effect on the PMN population in silica-exposed lung may be mediated through AM.

Evidence of *in vivo* interaction of tetrandrine with AM has not been clearly demonstrated. With the aid of the emulsion system, Figure 1 shows the effects of tetrandrine on the oxygen consumption by AM harvested from silica-exposed and nonsilicotic rats 2 weeks after silica exposure. In the nonsilicotic groups, neither the resting nor the zymosan-induced oxygen consumptions were affected by the tetrandrine treatment. This is expected since tetrandrine has been shown to have no effect on nonstimulated AM *in vitro*.[25] It is also possible that tetrandrine may have been substantially eliminated from the lungs 2 weeks after treatment. The estimated half-lives of tetrandrine solution and tetrandrine emulsion in nonsilicotic rat lungs are

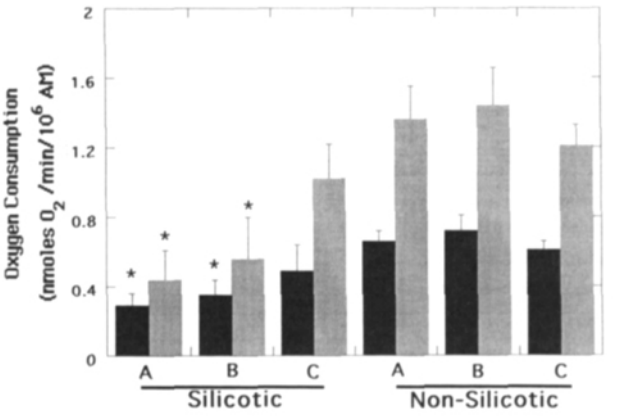

FIGURE 1. Effect of *in vivo* silica and tetrandrine treatments on the resting (dark bar) and zymosan-stimulated (light bar) oxygen consumption of AM from the BAL fluid. Treatment group: (A) control; (B) tetrandrine solution; (C) tetrandrine emulsion. Animals received 40 mg silica or saline intratracheally, followed by four daily doses of tetrandrine (13 mg/kg). Cells were harvested 2 weeks after silica treatment. *indicates a significant decrease from the corresponding nonsilicotic value.

23 and 36 hours, respectively.[37] In silicotic rats, AM exhibit reduced levels of resting and zymosan-stimulated oxygen consumption when compared with the nonsilicotic control (Figure 1). This suggests that silica alters normal cell activity or cell population due to silica-mediated cell death or epithelial lung damage. The effect of tetrandrine on silica-mediated cell activity is of interest. Tetrandrine appears to restore the normal function of AM, as indicated by the increased levels of both resting and zymosan-induced oxygen consumption in comparison to the silicotic control. This effect is especially clear in the case of treatment with tetrandrine emulsion, where the oxygen consumption levels approach normal values. Similar results indicating the protective effect of tetrandrine emulsion on macrophage respiratory burst were also obtained at 4 weeks after exposure (data not shown). These results are consistent with the data of Table 1, which show that rats receiving silica and tetrandrine emulsion maintained a high level of AM in the bronchoalveolar region when measured at 2 and 4 weeks after silica exposure.

The observed drug effect at 2 and 4 weeks after silica exposure indicates that tetrandrine inhibits the initiation of the silicotic process, since tetrandrine was given in the first 4 days of the treatment schedule. The reason for the drug effect may be twofold. First, the presence of tetrandrine in lung tissue may prevent the acute silica toxicity against alveolar macrophages and epithelial cells. This can be seen from the data on cell differentials where the PMN population in drug-treated animals is significantly lower than that in the silicotic control. The absence of accumulative inflammatory effect in the tetrandrine emulsion-treated animals may account for the relatively long-term drug activity. Second, tetrandrine exhibits unique binding characteristics with alveolar macrophages. Our studies indicated that binding of tetrandrine to AM and the drug effect on macrophage respiratory burst were significantly increased when AM were first stimulated with either phorbol-12-myristate-13-acetate or zymosan (data not shown). This suggests

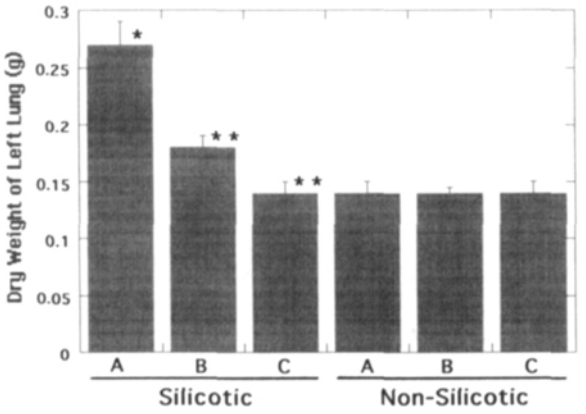

FIGURE 2. Effect of tetrandrine on the silica-induced increase in the dry weight of rat lung: (A) Control; (B) treated with tetrandrine solution; (C) treated with tetrandrine emulsion. *indicates a significant difference from nonsilicotic control; **indicates a significant difference from silicotic control.

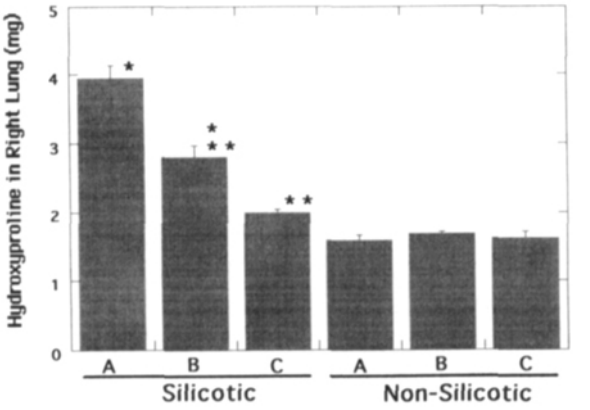

FIGURE 3. Effect of tetrandrine treatment on the silica-induced increase in hydroxyproline in lung tissue. (A) Control; (B) treated with tetrandrine solution; (C) treated with tetrandrine emulsion. *indicates a significant difference from nonsilicotic control; **indicates a significant difference from silicotic control.

that tetrandrine has a preferential affinity to activated cells or sites, which may account for its protective action on AM and the anti-inflammatory effect. It is also possible that the distribution of tetrandrine in the lungs is enhanced and the half-life prolonged under silica exposure due to the dust–cell and drug–cell interactions.

At necropsy the lungs of silica-exposed rats receiving no drug treatment were extremely heavy, firm, and gray. In contrast, treatment of silicotic rats with tetrandrine solution or emulsion diminished gross lesions. Figure 2 shows the measured dry weight of lungs from silicotic and nonsilicotic control rats and from rats treated with tetrandrine 2 weeks after exposure. Tetrandrine was found to have no effect on the lung weight of nonsilicotic animals, but to prevent weight increase associated with silica exposure, with a greater effect observed in the emulsion system than with the solution dosage form.

The silica-induced increase in dry lung weight indicates abnormal production of insoluble materials such as collagen. Figure 3 shows that lungs of the silicotic control exhibit a twofold increase in hydroxyproline content in comparison to those of the nonsilicotic control 2 weeks after silica exposure. The increase in lung hydroxyproline (i.e., collagen) content indicates an ongoing fibrotic process. The silica effect was inhibited by tetrandrine, and the inhibitory effect of the tetrandrine emulsion was significantly greater than that of the tetrandrine solution. These results are very consistent with those of the dry weight measurement. Reduction of lung hydroxyproline content by tetrandrine emulsion was also observed 4 weeks after exposure (Figure 4), further indicating that targeted delivery of tetrandrine using the emulsion system prolongs drug activity.

Ultrastructurally, AM harvested from nonsilicotic rats exhibited normal cell characteristics. Several organelles and numerous phagocytic vacuoles were shown in the cytoplasm. Alveolar macrophages obtained from the silicotic control rats 4 weeks after exposure contained phagocytized silica, surfactant, and crystal-like material morphologically consistent with cholesterol (Figure 5). Heppleston[44] reported that the material which accumulates within alveoli in lipoproteinosis has biochemical features similar to surfactant, but lacks surface tension reduction properties. The mechanism of accumulation of this material is unknown, but could be related to both the synthesis and metabolic degradation of pulmonary surfactant. A hallmark of the acute action of silica–lung interaction is the development of lipidosis, involving abnormal production of phospholipids in type II cells. Studies by Miller *et al.*[45] showed that lungs of rats exposed to 10 mg of intratracheal silica exhibit a bimodal population of type II cells: one population that resembled type II cells from control animals, and one that had an increased cell volume with an increased number of lamellar bodies per cell. In addition, the average volume of lamellar bodies was also increased in these hypertrophic type II pneumocytes. Figure 6 shows one isolated type II cell obtained from a silicotic control rat, which demonstrates the surfactant-like content.

Figure 7 shows the light microscopic examinations of rat

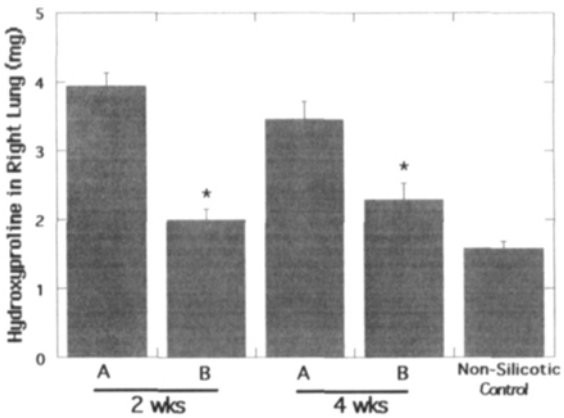

FIGURE 4. Comparison of the effect of tetrandrine treatment (given as emulsion) on the silica-induced increase in lung hydroxyproline at 2 and 4 weeks after silica exposure. (A) Silicotic control; (B) treated with tetrandrine emulsion. *indicates a significant decrease from the silicotic control.

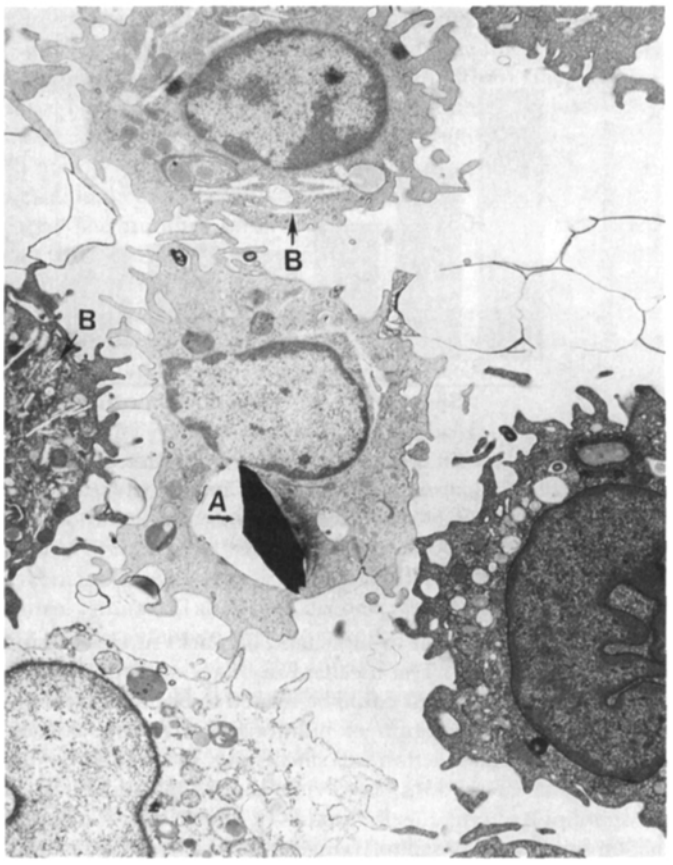

FIGURE 5. Electron micrograph of AM from a rat exposed to 40 mg silica 4 weeks after intratracheal instillation. Increased numbers of macrophages are within the alveolar space, which is shown to contain phagocytized silica (A) and cytoplasmic crystalline structure (B) consistent with cholesterol. (×10,000)

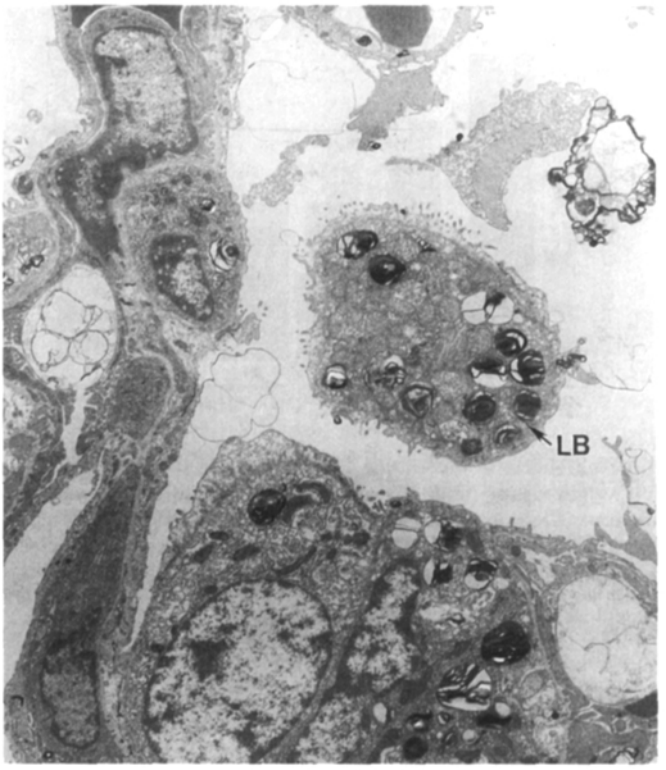

FIGURE 6. Transmission electron micrograph of an alveolar type II cell from a rat exposed to 40 mg silica 4 weeks after intratracheal instillation. This type II cell contains numerous lamellar bodies (LB). (×10,000)

lungs following silica and drug treatments using hematoxylin and eosin-stained sections of lung tissues. Rats receiving saline displayed normal lungs (Figure 7a), whereas for the silicotic control, lesions were characterized by granulomatous pneumonia (which varied from mild, focal to marked, multifocal, and coalescent) and alveolar lipoproteinosis (from mild to moderate, multifocal) consistent with acute silicosis. Variably sized silicotic granulomas composed of epitheloid macrophages and immature fibrous connective tissue were found in most of the rats with granulomatous pneumonia. In Figure 7b, for example, a lung section from a silicotic control rat 2 weeks after silica exposure shows alveoli in multiple scattered foci containing eosinophilic, granular debris and large, vacuolated macrophages consistent with alveolar lipoproteinosis. This is in line with early observation of increased production of lung surfactant, which is probably responsible for the light microscopic evidence of lipoproteinosis. Although the total number of macrophages recovered by BAL was not increased (Table 1), Figure 7b indicates a clear increase in the number of macrophages in the interstitium of silica-exposed rats.

Intervention of the silica effect with tetrandrine emulsion or tetrandrine solution dramatically decreased light microscopic lesions. Except for a small aggregate of epitheloid macrophages adjacent to a pulmonary artery in one rat and mild perivascular inflammation in another rat, no significant lesions were found in the tetrandrine emulsion treatment group 2 weeks after silica exposure (Figure 8a). Treatment of silicotic rats with tetrandrine solution (Figure 8b) also diminished lesions of acute silicosis, but was found to be less effective than the tetrandrine emulsion. Mild, multifocal granulomatous pneumonia and a few small foci composed of epitheloid macrophages were found in one rat treated with tetrandrine solution. Another rat had locally extensive, moderate subacute bronchitis. There were very few lesions in other rats. Figure 9 further demonstrates the emulsion effect on the antifibrotic activity of tetrandrine. The presence of collagen in the silicotic granulomatas was demonstrated using trichrome-stained sections (Figure 9a). Here, evidence of organized granulomatous development with moderate deposition of collagen, which appears as lightly stained, immature fibrous tissue, is clearly seen in the silicotic control. In contrast, the tetrandrine emulsion-treated lung shows collagen only at expected places, but no granulomas (Figure 9b).

Four weeks after exposure, granulomatous pneumonia varied from moderate, multifocal to severe, multifocal, and coalescent, and alveolar lipoproteinosis varied from mild, multifocal to multifocal and coalescent in silicotic control rats similar to those of Figures 7 and 9. Lesions in some rats were centered around bronchioles. Treatment with the tetrandrine emulsion eliminated microscopic lesions in four of six rats. The presence of silicotic granulomas in two rats may be due to treatment irregularities (such as injection loss or insufficient dose) or

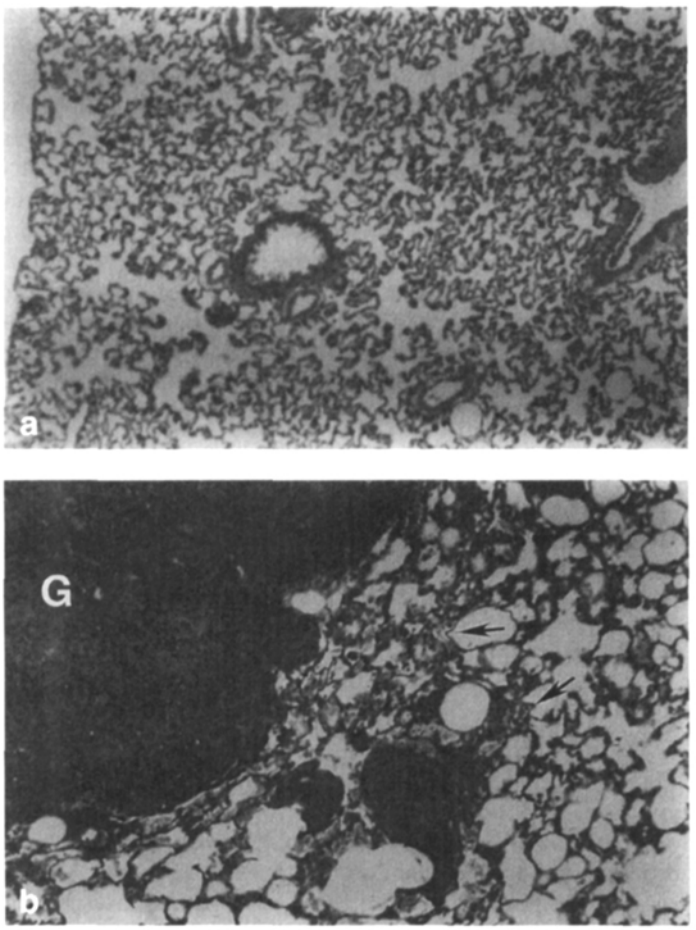

FIGURE 7. Light micrographs of hematoxylin and eosin-stained lung sections from (a) a nonsilicotic rat (EM93-30NL) showing normal pulmonary histology (×1000); and (b) a silicotic rat (EM93-30-2) (2 weeks after exposure) showing silica-induced granuloma (G) and lipoprotein in alveoli (arrow). (×1000)

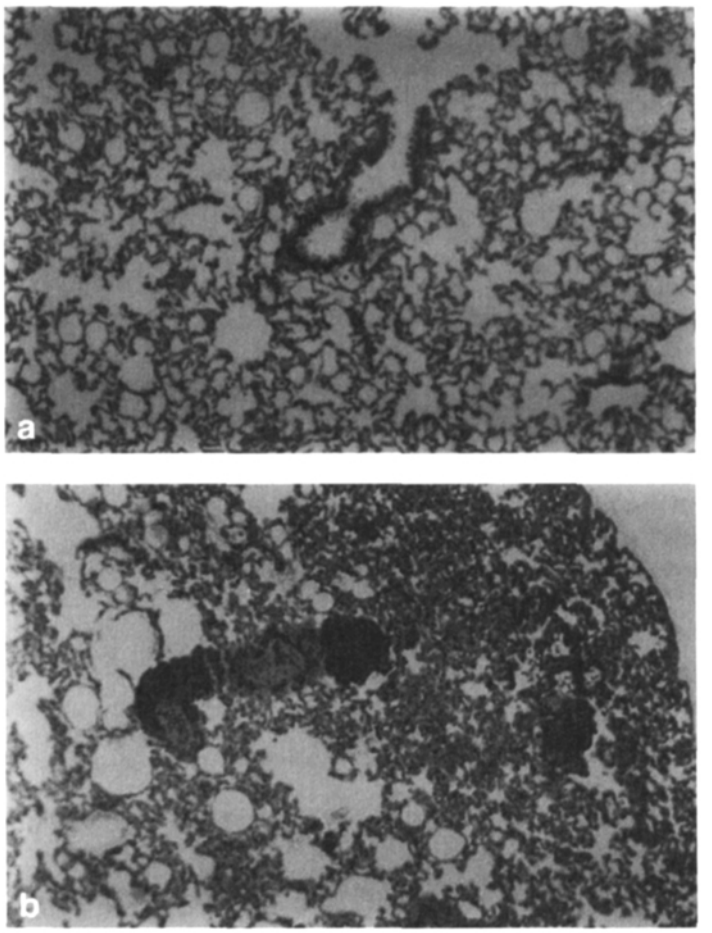

FIGURE 8. Hematoxylin- and eosin-stained light micrographs of tetrandrine-treated, silica-exposed rat lungs 2 weeks after silica exposure: (a) Treated with tetrandrine emulsion (EM93-30-1, no significant lesions); (b) treated with tetrandrine solution (EM93-30-9, mild granulomatous pneumonia). (×1000)

individual rat variation. The longer time interval since silica exposure and tetrandrine treatment may also have contributed to the development of silicotic granulomas in these rats.

The average pathological scores of lung lesions with respect to their distribution and severity for the control and drug-treated silicotic rats are summarized in Table 2. There is strong indication that the emulsion system enhances the inhibitory effect of tetrandrine on silica-induced granulomatous pneumonia and lipoproteinosis. In the present study, lungs were lavaged prior to fixation of tissues for microscopic examinations. Some of the material in the alveolar space may, therefore, have been removed but never artifactually introduced. However, the lavage should not have affected change within the interstitium, including the granulomatous pneumonia. Alveolar lipoproteinosis, when observed microscopically, is, therefore, important in our study, but quantitative comparisons between 2- and 4-week groups are avoided.

Conclusion

The hypothesis that multiple emulsions may be used as a drug delivery system to enhance the antifibrotic activity of tetrandrine against silicosis was investigated. *In vitro* tetrandrine has been shown to inhibit the release of reactive oxygen species and the secretion of inflammatory cytokines by AM, a key cell type central to the fibrotic process. Hence, targeted delivery of tetrandrine to AM is of value.

Previously we have demonstrated that a w/o/w multiple emulsion system containing peanut oil and HSO as the oil liquid membrane exhibited long-term stability and controlled drug release, and was readily recognized and endocytosed by the phagocytes.[36] Tetrandrine, which is toxic to AM on prolonged contact through drug binding to the cellular plasma membrane, may be incorporated into the multiple emulsion system and delivered into the intracellular space. This emulsion-delivered drug was shown by both *in vitro* and *in vivo* experiments to remain pharmacologically active, but without cellular toxicity.[36] In addition, we have demonstrated that this multiple emulsion system also facilitated tetrandrine distribution in the lungs, while it reduced the drug's systemic circulation.[37]

Multiple emulsions are liposome-like particulates which may be regulated *in vivo* by the monocyte–macrophage phagocytic system, suggesting that their distribution in tissue is likely to play an important role in prolonging the bioavailability of

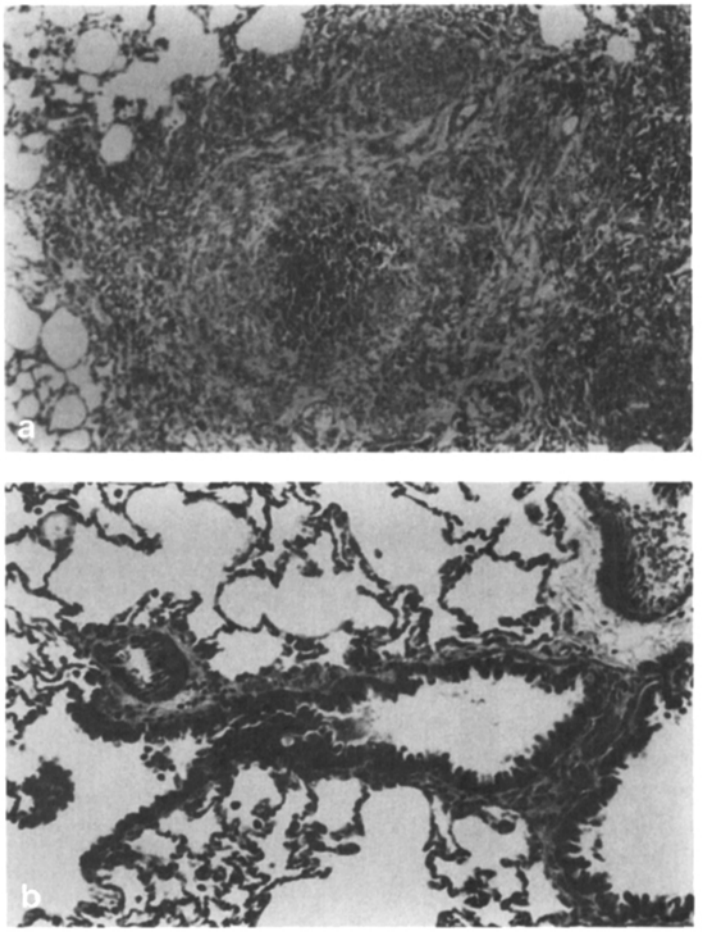

FIGURE 9. Light micrographs (trichrome-stained sections) of lungs from (a) a silicotic control (EM90-30-6) and (b) a silica-exposed rat treated with tetrandrine emulsion (EM90-30-1) 2 weeks after exposure. Panel a shows organization of the granuloma with moderate collagen deposition appearing as lightly stained fibrous structure (×1000); panel b shows no significant lesions (×2000).

drugs. The present investigation shows a positive correlation between the pharmaceutical-based drug targeting and the therapeutic effect of tetrandrine. As demonstrated by alveolar cell differentials, AM respiratory burst, alterations in lung weight and collagen content, and histological examinations, tetrandrine has a marked inhibitory effect on silica-induced granulomatous pneumonia, alveolar lipoproteinosis, and fibrosis. Despite several *in vivo* and clinical studies, direct evidence of *in vivo* action of tetrandrine on lung cells had not been characterized prior to this study. Tetrandrine was shown in this study to inhibit the macrophage-orchestrated inflammatory cycle in response to silica exposure, preventing infiltration of PMN into the alveolar space. Studies on the oxygen consumption by AM further showed that tetrandrine protected the cells from silica stimulation and restored healthy AM populations even at 4 weeks after silica exposure. The inhibition of a silica-induced increase in lung weight and hydroxyproline content by tetrandrine was supported by histological evidence of the tetrandrine effect on granulomatous pneumonia, alveolar lipoproteinosis, and collagen formation. In all experiments, the emulsion dosage form was consistently more efficacious than the solution dosage form in the treatment of silica-induced lung lesions.

Multiple emulsions of the w/o/w type have emerged as an attractive drug delivery system to achieve increased drug efficacy in contemporary biomedical research. The present study showed that such preparations have a marked effect on the *in vivo* drug–cell interactions and the pharmacodynamic consequences of the drug. With the aid of the emulsion system, this study established the *in vivo* antifibrotic activity of tetrandrine. Although fibrosis has been considered to be irreversible, the present study indicates that when the drug is given at the onset of silica exposure, the fibrotic process can be delayed by inhibition of abnormal cellular activities leading to fibrosis and by restoration of normal cell functions using an effective antifibrotic agent. Such a delay would allow time for the lung to clear its dust problem. Given the long time required for the development of silicosis in humans, a delay or inhibition of the fibrotic process might have a very significant effect on the disease development.

Acknowledgments

This research, supported in part by a grant from the U.S. Bureau of Mines administered through the Generic Mineral Technology Center for Respirable Dust (BOM1195142-5430), and by a grant from the National Institute for Occupational Safety and Health (U60/OCU306149), was carried out in partial fulfillment of the requirements for the Ph.D. degree for Dr. De-Hwa Chao at the School of Pharmacy, West Virginia University.

TABLE 2. Summary of Histological Lung Injuries in Silicotic Rats 2 and 4 Weeks After Silica (40 mg) Exposure

	2 Weeks				4 Weeks			
	Granulomatous Pneumonia		Lipoproteinosis		Granulomatous Pneumonia		Lipoproteinosis	
Treatment Group	**D**	**S**	**D**	**S**	**D**	**S**	**D**	**S**
Silicotic control	3	3	3	3	3	3	3	3
Treatment with Tetrandrine								
Solution	1	1	0	0	—	—	—	—
Emulsion	0	0	0	0	1	1	1	1

D denotes distribution; scores for distribution of lung lesions are: 0 = not present; 1 = focal; 2 = locally extensive; 3 = multifocal; 4 = multifocal and coalescent; 5 = diffuse. S denotes severity; scores for severity are: 0 = not present; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; 5 = severe. Average score of 4 (2 weeks group) and 6 (4 weeks group).

References

1. Freeman, B.A.; Mason, R.J.; Williams, M.C.; Crapo, J.D.: Antioxidant Enzyme Activity in Alveolar Type II Cells after Exposure of Rats to Hyperoxia. Exp. Lung Res 10:203–222 (1986).
2. Adamson, I.Y.; Bowden, D.H.: The Type 2 Cell as Progenitor of Alveolar Epithelial Regeneration: A Cytodynamic Study in Mice after Exposure to Oxygen. Lab. Invest. 30:35–42 (1974).
3. Bowden, D.H.: Alveolar Response to Injury. Thorax 36:801–804 (1981).
4. Heppleston, A.G.: Silicotic Fibrogenesis: A Concept of Pulmonary Fibrosis. Ann. Occup. Hyg. 26:449–452 (1982).
5. Davis, G.S.: The Pathogenesis of Silicosis. Chest 89:166S–169S (1986).
6. Castranova, V.; Kang, J.H.; Moore, M.D.; et al.: Inhibition of Stimulant-induced Activation of Phagocytic Cells with Tetrandrine. J. Leukocyte Biol. 50:412–422 (1991).
7. Weiss, S.J.; Buglio, A.F.: Biology of Disease: Phagocyte-Generated Oxygen Metabolites and Cellular Injury. Lab. Invest. 47: 5–18 (1982).
8. Borm, P.J.A.; Henderson, P.T.: Symposium on Health Effects of Occupational Exposure to Inorganic Dusts. Exp. Lung Res. 16:1–3 (1990).
9. Welish, M.J.; Shasby, D.M.; Husted, R.M.: Oxidants Increase Paracellular Permeability in a Cultured Epithelial Cell Line. J. Clin. Invest. 76:1155–1168 (1985).
10. Goldring, M.B.; Krane, S.M.: Modulation of Collagen Synthesis in Human Chondrocyte Cultures by Interleukin-1. J. Bone Min. Res. 1(suppl.):56 (1986).
11. Schmidt, J.A.; Oliver, C.N.; Lape-Zuniga, J.L.; et al.: Silica-Stimulated Monocytes Release Fibroblast Proliferation Factors Identical to Interleukin-1: A Potential Role for Interleukin-1 in the Pathogenesis of Silicosis. J. Clin. Invest. 73:1462–1472 (1984).
12. Pohlman, T.H.; Stanness, K.A.; Beatty, P.G.; et al.: An Endothelial Cell Surface Factor(s) Induced In Vitro by Lipopolysaccharide, Interleukin 1, and Tumor Necrosis Factor-Alpha Increases Neutrophil Adherence by a CDw18-Dependent Mechanism. J. Immunol. 136:4548 (1986).
13. Reist, R.H.; Bryner, K.; Wearden, P.; et al.: Development of a Bioassay for Pulmonary Cell Production of Fibrogenic Factors. Toxicol. Meth. 1:53–65 (1991).
14. Bitterman, P.B.; Adelberg, S.; Crystal, R.G.: Mechanisms of Pulmonary Fibrosis: Spontaneous Release of Alveolar Macrophage Derived Growth Factor in the Interstitial Lung Disorders. J. Clin. Invest. 72:1801–1813 (1983).
15. Sibille, Y.; Reynolds, H.Y.: Macrophages and Polymorphonuclear Neutrophils in Lung Defense and Injury. Am. Rev. Respir. Dis. 141:471–50 (1990).
16. Martin, T.R.; Altman, L.C.; Albert, R.K.; Henderson, W.R.: Leukotriene B4 Production by the Human Alveolar Macrophages: A Potential Mechanism for Amplifying Inflammation in the Lung. Am. Rev. Respir. Dis. 129:106–111 (1984).
17. Futone, J.C.; Ward, P.A.: Role of Oxygen-Derived Free Radicals and Metabolites in Leukocyte-Dependent Inflammatory Reactions. Am. J. Pathol. 107:397–418 (1982).
18. Banks, D.E.; Cheng, Y.H.; Weber, S.L.; Ma, J.K.H.: Strategies for the Treatment of Pneumoconiosis. In: Occupational Medicine, the Mining Industry, pp. 205–232. Hanley & Belfus, Philadelphia (1993).
19. Goodman, G.B.; Kaplan, P.D.; Stachura, I.; et al.: Acute Silicosis Responding to Corticosteroid Therapy. Chest 101:366–370 (1992).
20. Yu, X.F.; Zou, C.Q.; Lim, M.B.: Observation of the Effect of Tetrandrine on Experimental Silicosis of Rats. Ecotoxicol. Environ. Safety 7:306–312 (1983).
21. Mo, C.G.; Yu, X.F.; Zou, C.Q.: Study on Relationship Between Chemical Structure and Biochemical Action of Bisbenzylisoquinoline Alkaloids. J. Hyg. Res 11:118–130 (1982).
22. Huang, T.; Liu, Y.; Zhao, S.; Li, Y.: Changes of Acid-Soluble Collagen from Lungs of Silicotic Rats and Tetrandrine-Treated Silicotic Rats. Acta Biochem. Biophys. Sintca 13:61–68 (1981).
23. Ma, J.K.H.; Mo, C.G.; Malanga, C.J.; et al.: Binding of Bisbenzylisoquinoline Alkaloids to Phosphatidylcholine Vesicles and Alveolar Macrophages: Relationship Between Binding Affinity and Antifibrogenic Potential of These Drugs. Exp. Lung Res. 17:1061–1077 (1991).
24. Ma, J.Y.C.; Barger, M.W.; Ma, J.K.H.; Castranova, V.: Inhibition of Respiratory Burst Activity in Alveolar Macrophages by Bisbenzylisoquinoline Alkaloids: Characterization of Drug-Cell Interaction. Exp. Lung Res. 18:829–843 (1992).
25. Castranova, V.; Kang, J.H.; Ma, J.K.H.; et al.: Effects of Bisbenzylisoquinoline Alkaloids on Alveolar Macrophages: Correlation Between Binding Affinity, Inhibitory Potentcy, and Antifibrotic Potential. Toxicol. Appl. Pharmacol. 108:242–252 (1991).
26. Kang, J.H.; Lewis, D.M.; Castranova, V.; et al.: Inhibitory Action of Tetrandrine on Macrophage Production of Interleukin-1 (IL-1)-like Activity and Thymocyte Proliferation. Exp. Lung Res. 18:715–729 (1992).
27. Lu, X.R.; Li, Q.R.: Clinical Studies of the Therapeutic Effect of Tetrandrine on Silicosis. Chin. J. Ind. Hyg. Occup. Med. 2:106–110 (1983).
28. Li, T.N.; Hu, T.Y.; Zou, C.Q.: Studies of the Chronic Toxicity of Tetrandrine in Dogs. Ecotoxicol. Environ. Safety 6:528–534 (1983).
29. Schroit, A.J.; Hart, I.R.; Madsen, J.; Fidler, I.J.: Selective Delivery of Drugs Encapsulated in Liposomes: Natural Targeting to Macrophages Involved in Various Disease States. J. Biol. Response Mod. 2:97–100 (1983).
30. Florence, A.T.; Omotosho, J.A.; Whateley, T.L.: Multiple w/o/w Emulsions as Drug Vehicles. In: Controlled Release of Drugs: Polymers and Aggregate Systems, pp. 163–184. M. Rosoff, Ed. VCH, New York (1989).
31. Law, T.K.; Whateley, T.L.; Florence, A.T.: Stabilization of W/O/W Multiple Emulsions by Interfacial Complexation of Macromolecules and Nonionic Surfactants. J. Controlled Rel. 3:279–290 (1986).
32. Iso, M.; Shirahase, T.; Haramura, S.; et al.: Application of Encapsulated Enzyme as a Continuous Packed-Bed Reactor. J. Microencapsul. 6:285–299 (1989).
33. Miyakawa, T.; Zhang, W.; Uchida, T.; et al.: In Vivo Release of Water-Soluble Drug from Stabilized Water-in-Oil-in-Water (w/o/w) Type Multiple Emulsions Following Intravenous Administrations Using Rats. Biol. Pharm. Bull. 16:268–272 (1993).
34. Omotosho, J.A.; Whateley, T.L.; Florence, A.T.: Methotrexate Transport from the Internal Phase of Multiple w/o/w Emulsions. J. Microencapsul. 6:183–192 (1989).
35. Omotosho, J.A.; Florence, A.T.; Whateley, T.L.: Absorption and Lymphatic Uptake of 5-Fluorouracil in the Rat Following Oral Administration of w/o/w Multiple Emulsions. International J. Pharmaceutics 61:51–56 (1990).
36. Chao, D.H.; Peng, X.S.; Rojanasakul, Y.; et al.: Evaluation of a Multiple Emulsion System for Delivery of Tetrandrine to the Lung I. Formulation Stability, Drug Release, and Cellular Activity. Pharm. Res. 7:S–150 (1990).
37. Chao, D.H.; Pan, W.F.; Rojanasakul, Y.; et al.: Evaluation of a Multiple Emulsion System for Delivery of Tetrandrine to the Lung II. Emulsion-Mediated Drug Distribution and Activity in Rat Lungs. Pharm. Res. 9:S–201 (1992).
38. Matsumoto, S.; Khoda, M.; Murata, S.: Preparation of Liquid Vesicles on the Basis of a Technique for Providing W/O/W Emulsions. J. Colloid Interface Sci. 62:149–157 (1977).
39. Davis, S.S.; Purewal, T.S.; Burbage, A.S.: The Particle Size

Analysis of Multiple Emulsions. J. Pharm. Pharmacol. 28(Suppl.): 60P (1976).
40. Castranova, V.; Bowman, L.; Miles, P.R.; Reasor, M.J.: Toxicity of Metal Ions to Alveolar Macrophages. Am. J. Ind. Med. 1:349–357 (1980).
41. Kivirikko, K.I.; Laitinen, O.; Prockop, D.J.: Modifications of a Specific Assay for Hydroxyproline in Urine. Anal. Biochem. 19:249–255 (1967).
42. Prophet, E.B.; Mills, B.; Arrington, J.B.; Sobin, L.H. Eds. Laboratory Methods. In: Histotechnology, p. 1–279. Armed Forces Institute of Pathology, Washington, DC (1992).
43. Dauber, J.H.; Roseman, M.D.; Pietra, G.G.; et al.: Experimental Silicosis: Morphologic and Biochemical Abnormalities Produced by Intratracheal Instillation of Quartz into Guinea Pig Lungs. Am. J. Pathol. 101:595–612 (1980).
44. Heppleston, A.G.: Atypical Reaction to Inhaled Silica. Nature 213:199 (1967).
45. Miller, K.; Dethloff, L.A.; Hook, G.E.R.: Silica-Induced Hypertrophy of Type II Cells in the Lungs of Rats. Lab. Invest. 55:153–163 (1986).