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Binding of Bisbenzylisoquinoline Alkaloids to Phosphatidylcholine Vesicles and Alveolar Macrophages: Relationship Between Binding Affinity and Antifibrogenic Potential of These Drugs

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ABSTRACT: *A group of bisbenzylisoquinoline alkaloids has been shown to exhibit various degrees of effectiveness in preventing silica-induced fibrosis in animal models [1, 2]. The objective of the present study was to characterize the binding of several of these alkaloids to phosphatidylcholine vesicles and rat alveolar macrophages using fluorometric and equilibrium dialysis methods, respectively. The lipid binding affinity of these alkaloids was found to depend upon several structural factors including hydrophobic substitutions, chiral configurations, and double oxygen bridge-restricted conformation of the benzylisoquinoline moieties. Tetrandrine, which is a highly effective agent in preventing fibrosis, showed strong binding to both lipid vesicles and alveolar macrophages. In contrast, certain analogues of tetrandrine such as curine and tubocurine, which have little or no effect on silicosis, exhibited only weak binding to lipid vesicles and almost no binding to cells. The moderate binding affinity of fangchinoline to vesicles and cells corresponded to a moderate effectiveness of the compound as an antifibrogenic agent. Methoxyadiantifoline, an alkaloid of unknown antifibrogenic potential, also exhibited high binding affinities for lipid and cells. In conclusion, the results of these studies indicate that alveolar macrophages exhibit large binding capacities for certain members of this class of bisben-*

From the School of Pharmacy, West Virginia University, and the Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia.

Address all correspondence to Dr. Vincent Castranova, Biochemistry Section, NIOSH, 944 Chestnut Ridge Road, MS 211, Morgantown, WV 26505.

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zylisoquinoline alkaloids. A positive correlation was observed between binding affinity to alveolar macrophages and the reported antifibrotic potency of these compounds. These data also suggest that the ability of these drugs to interact with alveolar macrophages may be a key step in inhibition of the progression of silica-induced pulmonary disease.

INTRODUCTION

Inhalation of crystalline silica is associated with the development of silicosis. This fibrotic disease is characterized by the appearance of concentric hyalinized nodular lesions in the lungs with the progressive development of pulmonary dysfunction [3, 4].

Many theories concerning the etiology of silicosis involve silica-induced activation of alveolar macrophages. Evidence indicates that in vitro silica exposures result in the generation of chemiluminescence, and the release of superoxide and hydrogen peroxide from alveolar macrophages [5]. Activation of alveolar macrophages has also been demonstrated after intratracheal instillation or inhalation of silica [5, 6]. Such excess release of reactive products from pulmonary phagocytes has been associated with damage to the lung parenchyma [7, 8]. In addition, silica exposure results in the release of mediators from alveolar macrophages, which enhance proliferation of fibroblasts and collagen synthesis by these pneumocytes [9–12].

Tetrandrine (TE), a bisbenzylisoquinoline (BBIQ) alkaloid isolated from the root of a Chinese medicinal herb, inhibits silica-induced release of reactive oxygen species from alveolar macrophages [5]. Similarly, it inhibits activation of granulocytes [13, 14]. Tetrandrine is also effective in inhibiting the synthesis of collagen by fibroblasts and the formation of silicotic nodules in silica-exposed rats [1, 15, 16]. Therefore, it has been proposed as a possible therapeutic agent for pulmonary fibrosis. Indeed, it is used clinically in China as an antifibrotic drug and has been shown to improve both the appearance of chest radiographs and diffusion capacity in silicosis patients [17].

Several BBIQ alkaloids have been tested for antifibrotic potential in rats intratracheally exposed to 50 mg of silica [2]. Alkaloids were administered orally three times a week for 4 weeks beginning 2 days after silica instillation. Four weeks after silica exposure, lungs were removed, collagen formation was monitored, and lung tissue was scored for macule and nodule formation. As expected, tetrandrine (50 mg/kg) decreased collagen formation by 52%; microscopically, only small macules were observed in tetrandrine-treated, silica-exposed lungs. Cycleanine (CY) was also found to be potent, fangchinoline (FA) and cepharanthine (CE) exhibited slightly lower potency, berbamine (BE) was less effective, while curine (CU) and tubocurine (TU) were relatively ineffective in decreasing collagen formation or preventing the appearance of silicotic nodules. Recently, Kang et al. [18] monitored the ability of selected alkaloids to prevent particle-induced activa-

tion of alveolar macrophages and reported a potency sequence of tetrandrine ≈ methoxyadiantifoline (ME) > tubocurine.

The objective of the present investigation was to characterize the binding of BBIQ alkaloids to biological membranes and determine whether a correlation exists between binding affinity and relative antifibrogenic potential. Alkaloid–membrane interaction was monitored using a membrane model (i.e., phosphatidylcholine vesicles) as well as using alveolar macrophages.

MATERIALS AND METHODS

Materials

Dipalmitoyl phosphatidylcholine (DPPC) and the ammonium salt of 1-anilino-8-naphthalenesulfonate (ANS) were obtained from Calbiochem-Behring Corp (San Diego, CA) and Aldrich Chemical Co (Milwaukee, WI), respectively. The inorganic salts and buffer agents were purchased from Fischer Scientific Company (Pittsburgh, PA). Preparation of DPPC solutions in 0.01 M Tris buffer (pH 7.0) were made by sonication of the lipid solutions under nitrogen for 30 min at 50°C using a heat system W375 sonifier. Small vesicles of these solutions were fractionated from large liposomes by centrifugation at 105,000*g* for 60 min. The concentration of DPPC was then determined via the measurement of inorganic phosphorus. The BBIQ alkaloids listed in Table 1 were kindly supplied by the Institute of Occupational Medicine (Chinese Academy of Preventive Medicine, Beijing, China). For the present study, 10^{-3} M stock solutions of each drug were prepared by first dissolving the drug in diluted HCl and then adjusting the pH to 6.5

Table 1 Structural and Solvent Partition Characteristics of the BBIQ Alkaloids

	Structural Variation							
Compound[a]	C1	C1′	C7	C12	C12′	C7′	Oxygen Bridge	K'[b]
TE	S	S	OMe	OMe			C8–C7′, C11–C12′	108.3
FA	S	S	OH	OMe			C8–C7′, C11–C12′	24.5
BE	R	S	OMe	OH			C8–C7′, C11–C12′	25.8
CE	S	R	$^{-}OCH_2O^{-}C_6$		OMe		C8–C7′, C12–C11′	35.1
TU	R	S	OH	OH			C8–C12′, C13–C7′	2.3
CU	R	R	OH	OH			C8–C12′, C13–C7′	5.9
CY	R	R	OMe			OMe	C8–C12′, C8′–C12	84.6
ME	S	S	(see structure II)				C10–C13′	240.0

[a]*Abbreviations:* TE, tetrandrine; FA, fangchinoline; BE, berbamine; CE, cepharanthine; TU, tubocurine; CU, curine; CY, cycleanine; ME, methoxyadiantifoline.

[b]K' is the apparent partition coefficient measured in *n*-butanol/Tris buffer (pH 7.0).

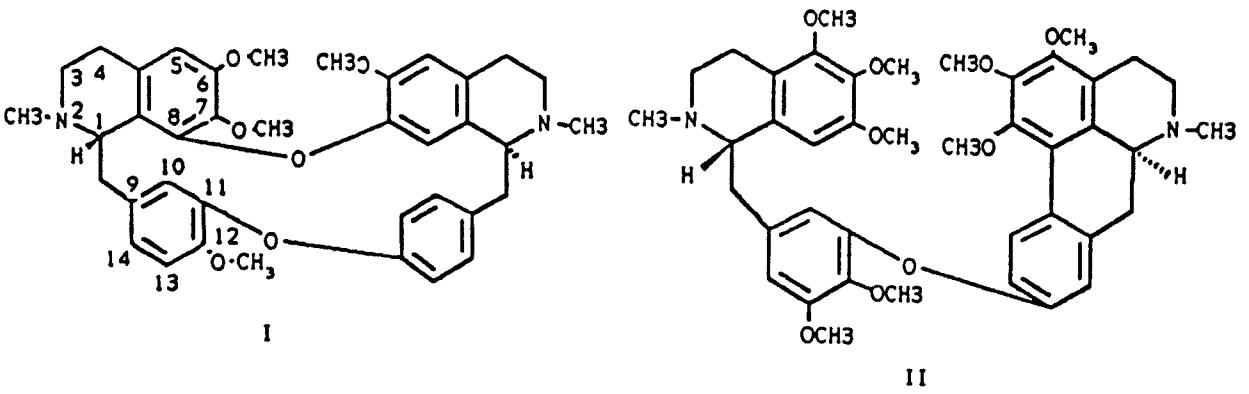

Figure 1 Structural variations are in reference to the structure of tetrandrine (**I**); structure of methoxyadiantifoline is given in (**II**).

using 2N NaOH. Prior to binding studies, drug distribution in *n*-butanol and pH 7.0 Tris buffer was studied and the apparent partition coefficient (K') was determined to provide an indication of relative lipophilicity of these compounds.

Fluorometric Studies of Drug Binding to DPPC

Fluorescence measurements were carried out using an Aminco-Bowman spectrophotofluorometer (American Instrument Co, Silver Spring, MD) equipped with a 150-W xenon lamp, a 1P21 photomultiplier tube, and an *X-Y* recorder. The temperature of the fluorometer cell compartment was controlled at 37 ± 0.1 °C using a circulatory water bath (Forma Scientific Inc, Marietta, OH). Standardization of the fluorescence intensity of the instrument was routinely carried out using a solid sample of tetraphenylbutadiene (excitation, 348 nm; emissions, 422 nm; Atara Scientific, Ltd, Swarthmore, PA).

Binding of the BBIQ alkaloids to DPPC vesicles was studied fluorometrically using 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 DPPC. The fluorescence of phospholipid-bound ANS may be further enhanced by the addition of a cationic amphiphilic amine capable of forming of a more hydrophobic drug–lipid–ANS ternary complex [19]. The binding of the cationic amine involves interaction at the negatively charged oxygen of the phosphocholine moiety.

To obtain binding information, fluorometric titrations of DPPC solutions at various lipid concentrations with a stock solution of ANS were performed in the absence and presence of a BBIQ alkaloid. At 10^{-3} M DPPC, the relatively high concentration of the lipid resulted in total binding of added ANS in the concentration range of 10^{-6} M. The fluorescence intensity showed a linear relationship to ANS concentrations and the slope was calculated to be the emission coefficient (A_b) (i.e., a proportionality constant between intensity and concentration for the binary lipid–ANS complex). In the presence of BBIQ, the titration of 10^{-3} M DPPC with ANS again became linear when drug concentration was 10^{-3} M or higher, suggesting that the measured fluorescence was due to bound ANS in the ternary complex. Similarly, the emission coefficient for the ternary complex (A_t) was also determined. The lipid binding affinities of the BBIQ alkaloids were studied at a DPPC concentration of 5×10^{-5} M DPPC, 10^{-3} M ANS, and varying concentrations of the drug. Under these conditions, the lipid vesicles were saturated with ANS. Addition of drug resulted in an increase in fluorescence intensity. Because the fluorescence increase was due to the formation of the drug–lipid–ANS complex, the concentration of bound drug can be calculated from the $(F_t - F_b)/(A_t - A_b)$ ratio, where F_b and F_t are fluorescence intensities in the absence and presence of the drug, respec-

tively. A_t and A_b are the same as previously defined. The free drug concentration was subsequently determined from the bound concentration and the total drug added.

The binding affinity of the BBIQ alkaloids to DPPC was analyzed using the Scatchard equation:

$$\frac{V}{[D]} = nK - VK \tag{1}$$

where V is the number of moles of bound drug per mole of DPPC, $[D]$ is the free drug concentration, K is the binding constant, and n is the maximum molar binding ratio between the drug and DPPC.

Drug Binding to Alveolar Macrophages

Male Sprague-Dawley rats (175–225 g) obtained from Charles River Laboratories (Wilmington, MA) were anesthetized with sodium pentobarbital (0.2 g/kg) and exsanguinated by cutting the renal artery. Alveolar macrophages were obtained by pulmonary lavage with a Ca^{2+}, Mg^{2+}-free phosphate buffer (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4). Cells were centrifuged at 500g for 5 min, washed, and resuspended in a 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 cell-sizing attachment [20].

The binding of selected BBIQ alkaloids to alveolar macrophages (4.0×10^6 cells/mL) was studied using a 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 h 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 centrifugational removal of alveolar macrophages. Binding of drugs to alveolar macrophages was analyzed using an equation analogous to Eq. (1).

The determination of BBIQ 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 detec-

tor (Waters Associates, Milford, MA). The separation of the BBIQ alkaloid from other substances was achieved using a uBondapak 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 BBIQ alkaloid were obtained over the concentration range of 5×10^{-6} to 8×10^{-5} M.

RESULTS

The structural and solvent partition properties of the BBIQ alkaloids used in this study are shown in Table 1. Characteristic to the structure of tetrandrine and its analogues, fangchinoline and barbamine, is a double oxygen-bridged (C8–C7′ and C11–C12′) ring of 18-bond length. Tetrandrine was found to be highly lipophilic (K' = 108.3). Hydroxyl substitution of either C7 (as in fangchinoline) or C12 (as in berbamine) position resulted in over fourfold reduction of the apparent partition coefficient. Cepharanthine, with a condensed methylenedioxy ring at C6 and C7 and a double oxygen-bridge ring of also 18-bond length, gave a K' of 35.1. Cycleanine, which has a measured K' of 84.6, exhibits an 18-membered ring via oxygen bridges at C8–C12′ and C12–C8′. Studies have shown that the benzylisoquinoline moieties at cycleanine are in a "tub" conformation where the methoxy groups at C7 and C7′ are twisted out of the aromatic ring conjugation [21]. The structures of curine or tubocurine are characterized by hydroxyl substitutions at both C7 and C12 and an oxygen-bridged ring of 20-bond length. These compounds showed the least lipophilicity. Methoxyadiantifoline is a benzylisoquinoline–aporphine dimer with a total of nine methoxy groups [22]. Although it does not possess an oxygen-bridged ring, methoxyadiantifoline was found to exhibit the highest K' (240) among the BBIQ alkaloids. The sequence for lipophilicity is ME > TE > CY > CE > FA ≈ BE > CU ≈ TU.

Figure 2 shows the effect of the BBIQ alkaloids on the fluorescence of lipid-bound ANS in solutions containing 10^{-4} M DPPC and varying concentrations of the fluorescence probe. The titration curve for cycleanine (not shown) was in similar order to that of fangchinoline. Previously, we have shown that amphiphilic amines, capable of binding with phospholipid vesicles via both hydrophobic and ionic interactions, enhance the fluorescence of lipid-bound ANS, and that binding affinity increases with increasing hydrophobic interaction between the drug and the lipid's fatty acid chains [19]. Figure 2 shows that with the exception of curine the titration curves obtained in the presence of 10^{-4} M drug were consistently higher in intensity than that in the absence of drug, thus suggesting a similar binding between the BBIQ alkaloid and DPPC. Figure 3 shows the fluorometric titration curves from solutions containing 5×10^{-5} M DPPC, 10^{-3} M ANS, and varying concentrations of drug. The curve determined for fangchino-

line, which was slightly lower than that of cycleanine, is not shown. Because the concentration of ANS is in large excess, the fluoresence intensity, F_t–F_b, is directly proportional to the concentration of bound drug in the drug-lipid-ANS ternary complex. The results indicate a binding affinity for all drugs in the following decreasing order: ME > TE > CE > CY > FA > BE > TU ≈ CU. It is also interesting to point out that at relatively high

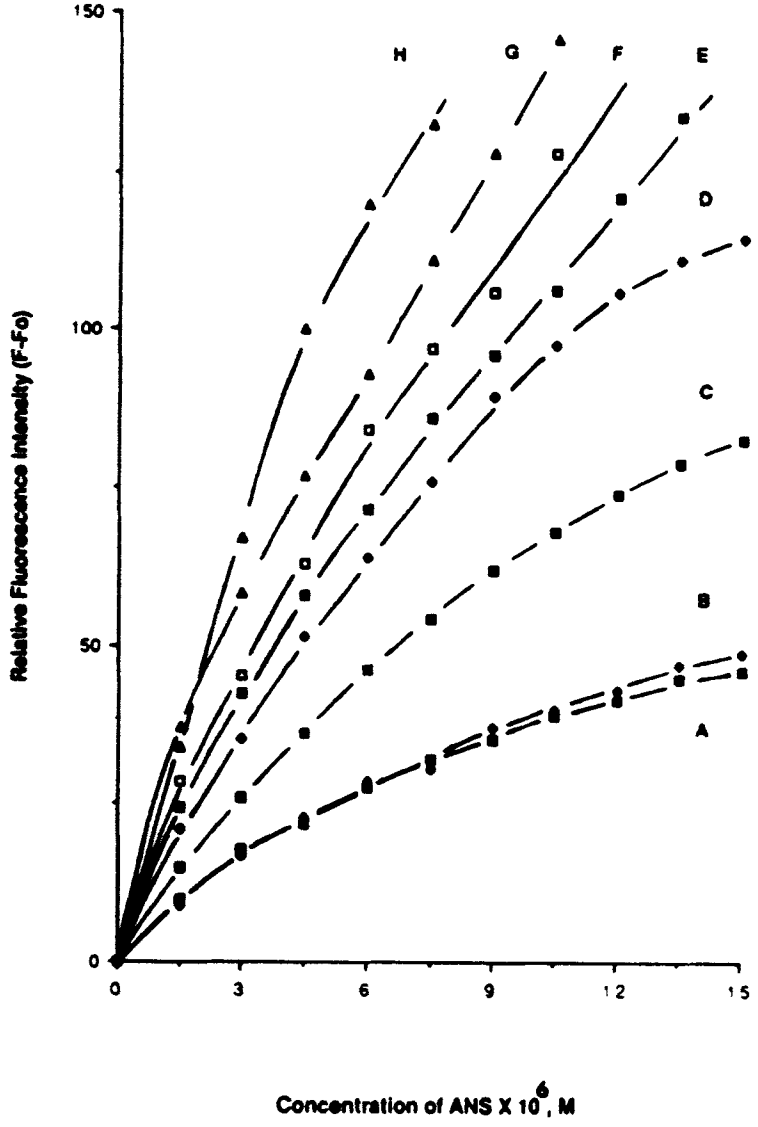

Figure 2 Effect of BBIQ alkaloids (10^{-4} M) on the fluorescence of lipid-bound ANS in solutions containing 10^{-4} M DPPC and varying concentrations of ANS. (A) no drug, (B) CU, (C) TU, (D) BE, (E) FA, (F) CE, (G) TE, and (H) ME. F_0 and F are fluorescence intensities measured in the absence and presence of DPPC, respectively.

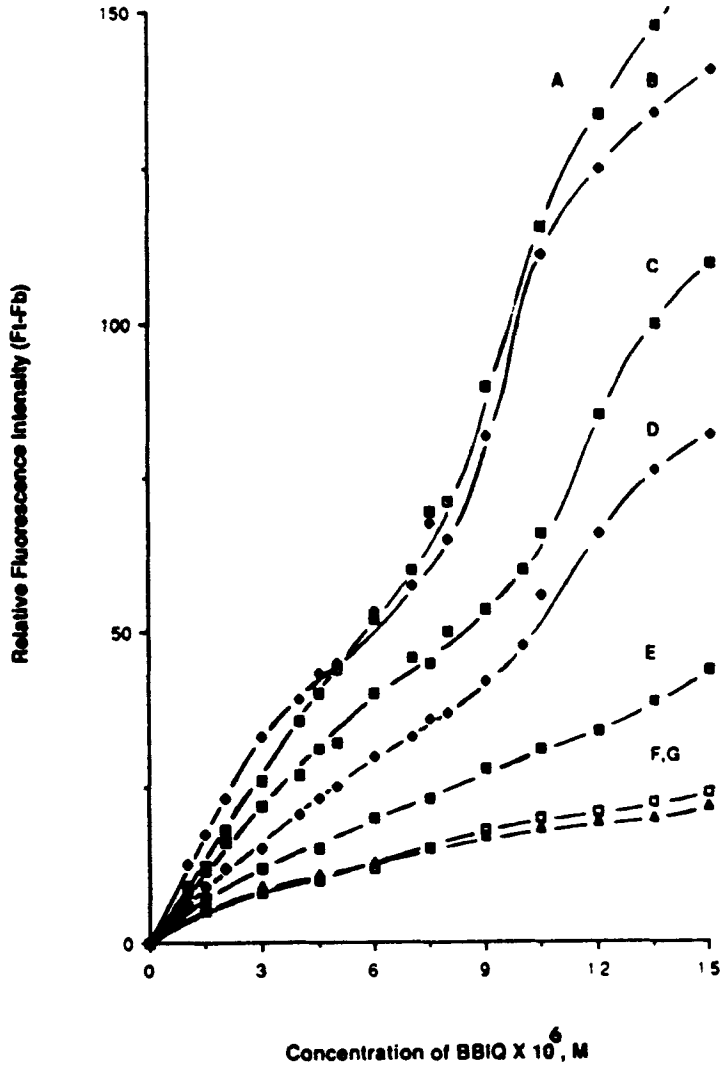

Figure 3 Fluorometric titration curves for solutions containing 5×10^{-5} M DPPC, 10^{-3} M ANS, and varying concentrations of (A) TE, (B) ME, (C) CE, (D) CY, (E) BE, (F) TU, and (G) CU.

drug concentrations, there is a cooperative increase in the concentration of bound drug, suggesting drug binding at secondary sites.

The primary binding between these drugs and DPPC was analyzed using the Scatchard equation. Figure 4 shows the plot of the binding data using Eq. (1). From these data, the binding constant K and binding capacity n for each drug were determined and are shown in Table 2. The overall binding affinity may be indicated by the value of nK. The sequence for nK is: ME > TE > CE > CY > FA > BE > TU $\approx$ CU.

Studies of drug binding to rat alveolar macrophages were carried out with selected alkaloids exhibiting strong (methoxyadiantifoline and tetrandrine),

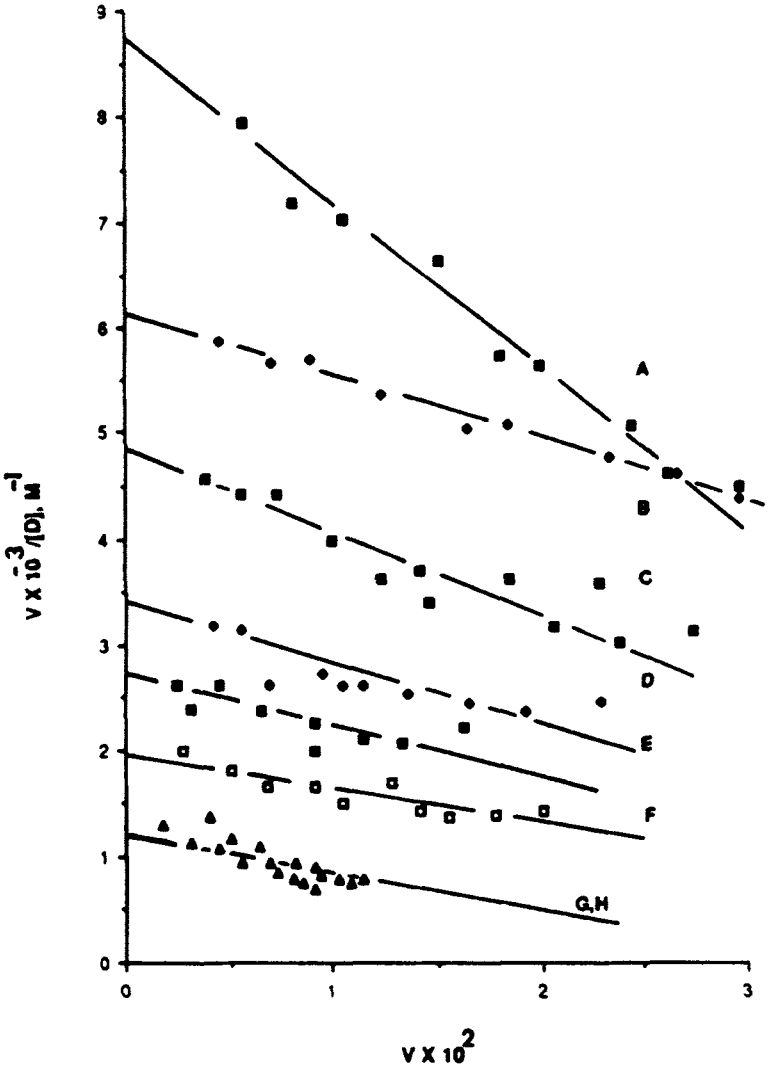

Figure 4 Scatchard plots for the binding of BBIQ alkaloids to DPPC. (A) ME, (B) TE, (C) CE, (D) CY, (E) FA, (F) BE, (G) TU, and (H) CU.

intermediate (fangchinoline), and weak (curine, tubocurine) binding to DPPC vesicles. Figure 5 shows the concentration of bound drug measured in solutions containing 4×10^6 cells/mL as a function of total drug concentration. The data indicate that tetrandrine, fangchinoline, and methoxyadiantifoline are all capable of binding with alveolar macrophages, whereas curine and tubocurine show no binding activity. The binding data obtained for tetrandrine, fangchinoline, and methoxyadiantifoline were analyzed using the Scatchard equation. Figure 6 shows the linear plot of $V/[D]$ vs V for each drug, where V is calculated to indicate millimoles of bound drug per

cell. Based on the linear plots, the cell binding capacity n and binding constants K were determined and are shown in Table 3. The sequence for overall binding affinity nK is TE > ME > FA > TU ≈ CU.

DISCUSSION

The data indicate that BBIQ alkaloids exhibit structure-dependent binding with phosphatidylcholine vesicles and macrophage membranes. Structural characteristics of these alkaloids, which are conducive to higher binding affinity, include hydrophobic substitutions (methoxy groups) at various positions, the chiral configuration, and the double oxygen bridges, which produce a more restricted structure of the benzylisoquinoline moieties.

A strong correlation exists between lipophilicity and binding to DPPC vesicles or alveolar macrophages. For example, high partition coefficients for methoxyadiantifoline and tetrandrine are associated with strong binding to phosphatidylcholine vesicles and alveolar macrophages, while the low partition coefficients for curine and tubocurine correlate with weak membrane binding. Alkaloids such as fangchinoline exhibit an intermediate partition coefficient and moderate membrane binding. The correlation coefficient between the lipophilicity sequence and the sequence of binding affinities is 0.91 for DPPC vesicles and 0.82 for alveolar macrophages, respectively. There is also a strong correlation (r^2 = .85) between binding affinity toward DPPC vesicles and that for macrophages.

The importance of chiral configuration on binding is best demonstrated by fangchinoline and berbamine. These alkaloids exhibit similar partition coefficients, yet fangchinoline, having an S,S configuration of the C1 and C1′ chiral centers, exhibits stronger binding to DPPC vesicles than does berbamine, which possesses an R,S configuration. Interestingly, isotetrandrine (R,S configuration) is significantly lower than tetrandrine (S,S) in

Table 2 Parameters Calculated for the Binding of Various Alkaloids to DPPC

Drug	n	K (M^{-1})	nK (M^{-1})
Methoxyadiantifoline	0.054	1.62×10^5	8.75×10^3
Tetrandrine	0.099	6.22×10^4	6.15×10^3
Cepharanthine	0.062	7.77×10^4	4.82×10^3
Fangchinoline	0.056	4.75×10^4	2.67×10^3
Berbamine	0.055	3.67×10^4	2.01×10^3
Cycleanine	0.055	6.15×10^4	3.38×10^3
Tubocurine	0.031	4.03×10^4	1.25×10^3
Curine	0.031	4.03×10^4	1.25×10^3

n, binding capacity; K, binding constant; nK, overall binding affinity.

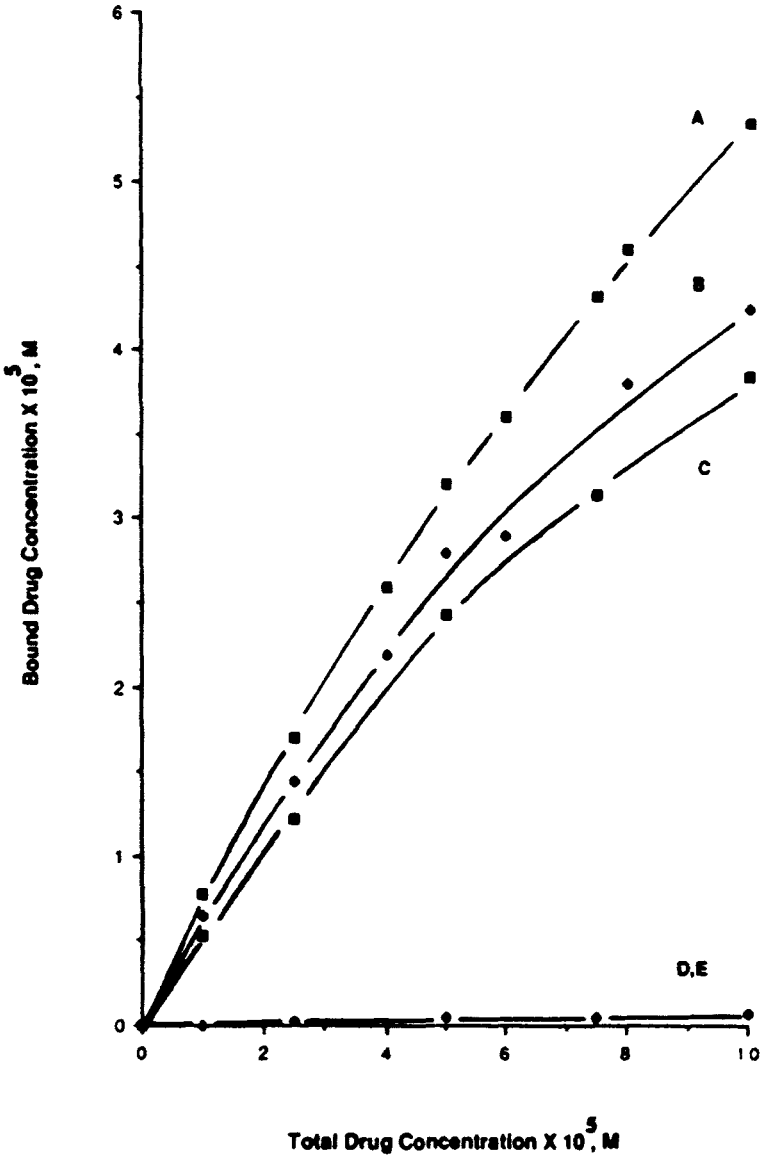

Figure 5 Plots of bound concentration measured in solutions containing 4 × 10^6 cells/mL rat alveolar macrophages as a function of total drug concentration of (A) TE, (B) ME, (C) FA, (D), CU, and (E) TU.

binding affinity to plasma proteins, in antitumor potency, and in antifibrogenic potential [2, 23].

Methoxyadiantifoline is an aporphine–benzylisoquinoline dimer with a single oxygen bridge (C10–C13′). Due to the large number of methoxy groups, it exhibits a significantly higher partition coefficient than tetrandrine. However, tetrandrine, with a double oxygen bridge, exhibits greater binding capacity and affinity toward alveolar macrophages than methoxy-

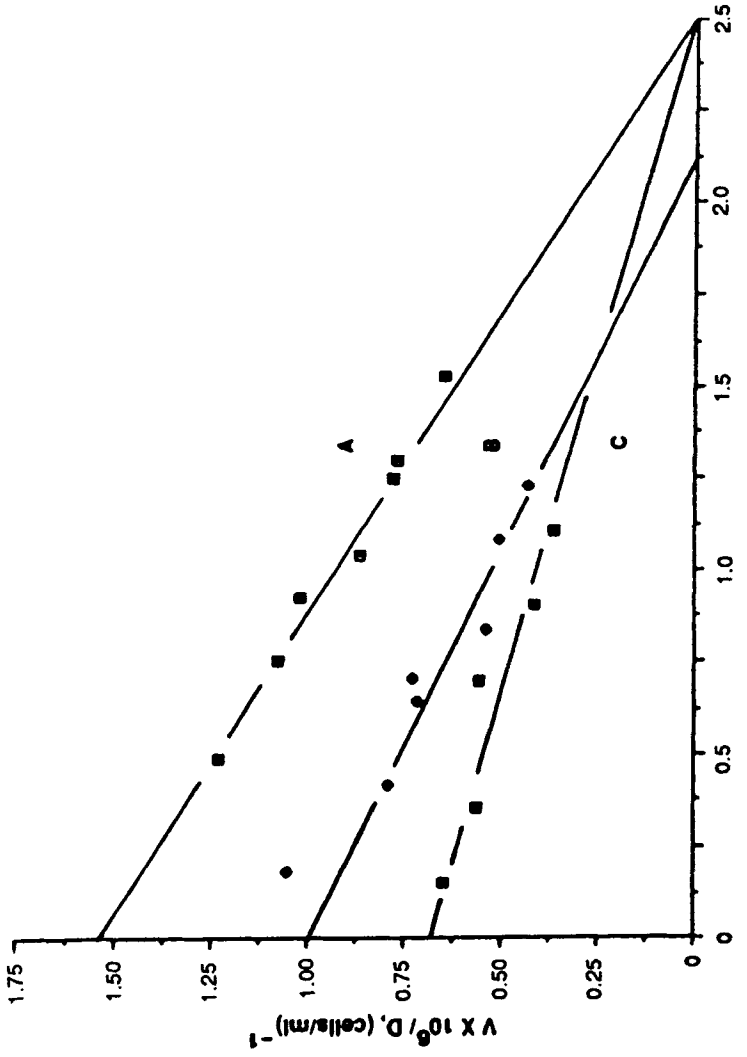

Figure 6 Scatchard plots for the binding of (A) TE, (B) ME, and (C) FA to rat alveolar macrophages.

Table 3 Parameters Calculated for the Binding of Selected Alkaloids to Alveolar Macrophages

Drug	n (mmol/cell)	K (M^{-1})	nK (mL/cell)
Tetrandrine	2.5×10^{-11}	6.16×10^{4}	1.54×10^{-6}
Methoxyadiantifoline	2.12×10^{-11}	4.71×10^{4}	1.00×10^{-6}
Fangchinoline	2.5×10^{-11}	2.75×10^{4}	0.69×10^{-6}
Tubocurine	ND	ND	ND
Curine	ND	ND	ND

n, binding capacity; K, binding constant; nK, overall binding affinity; ND, not detectable.

adiantifoline. The importance of this double oxygen bridge to macrophage binding is supported by the fact that when the double oxygen bridges of alkaloids are altered, as with curine and tubocurine, the ability to interact with cells is substantially reduced.

The relative binding affinities of alkaloids for alveolar macrophages correlates very strongly ($r^2 = .97$) with their reported antifibrotic activity. Tetrandrine strongly inhibits collagen synthesis and nodule formation in silica-treated rats [2] and exhibits strong membrane binding, while curine and tubocurine are not potent antifibrotic agents [2] and exhibit weak binding. Fangchinoline is intermediate both in antifibrogenic activity [2] and in binding affinity with alveolar macrophages. A correlation also exists between binding to alveolar macrophages and the ability of these alkaloids to inhibit particle-induced activation of these phagocytes in vitro; Kang et al. [18] reported that methoxyadiantifoline and tetrandrine are potent inhibitors of particle-stimulated oxygen consumption, superoxide secretion and hydrogen peroxide release, while tubocurine is only minimally effective. Interestingly, tetrandrine and methoxyadiantifoline have been shown to have similar effects on the cardiovascular system and are effective agents against angina and hypertension [24].

The correlation among binding affinity for alveolar macrophages, inhibition of macrophage activation, and antifibrotic potential supports theories that silica-induced activation of alveolar macrophages and the resulting excess oxidant generation leads to damage of the lung parenchyma and silicosis [7, 8, 25]. Therefore, these alkaloids may serve as useful agents to probe mechanisms governing the initiation and progression of the fibrotic process.

The correlation between lipophilicity, phospholipid binding, and interaction with alveolar macrophages suggests that alkaloids bind nonspecifically to membrane lipid rather than to specific membrane receptors. Indeed, the high binding capacity of macrophage membranes for tetrandrine ($n = 2.5 \times 10^{-11}$ mmol/cell) is consistent with the presence of as many as 1.5×10^{10} binding sites per cell.

Alkaloid concentrations used in the present investigation of binding to

macrophages were similar (i.e., 1–30 μm) to those used to inhibit macrophage activation [18]. However, it is unlikely that simple partitioning into the macrophage plasma could explain the inhibitory action of these alkaloids. It is possible that these drugs act by blocking some step or steps in the excitation–secretion process. The specific targets for the inhibitory action of alkaloids on macrophages are currently under study. King et al. [26] have reported that tetrandrine blocks calcium channels in cardiac muscle. However, we have found that tetrandrine does not inhibit stimulant-induced depolarization of alveolar macrophages [27]. These data suggest that tetrandrine does not block stimulant–receptor interaction or the resulting increase in calcium permeability. Seow et al. [28] have reported that tetrandrine inhibits phosphoinositide metabolism to inositol triphosphate in lymphocytes. It is known that production of inositol triphosphate results in an increase in free intracellular calcium. Preliminary data from our laboratory indicate that tetrandrine inhibits stimulant-induced mobilization of calcium from bound stores (data not shown). Lastly, Liu et al. [29] and Chen et al. [30] have shown that tetrandrine avidly binds to isolated microtubules. We have shown that treatment of macrophages with microtubular inhibitors decreases binding of alkaloids to macrophages [31]. Because stimulant-induced actin polymerization is associated with phagocyte activation [32], alkaloids may act to block particle-induced activation via interaction with the cytoskeletal system. Clearly, further investigation concerning the sites of tetrandrine action is warranted.

In conclusion, a relationship exists between reported antifibrotic potential, binding to alveolar macrophages, and deactivation of these phagocytes. Therefore, BBIQ alkaloids may serve as useful probes to elucidate the etiology of fibrotic pulmonary disease.

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