Chapter 21

Studies of the Binding of Bisbenzylisoquinoline Alkaloids to Phosphatidylcholine Vesicles and Alveolar Macrophages in Relation to their Antifibrogenic Potential

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ABSTRACT

A group of bisbenzylisoquinoline (BBIQ) alkaloids has been shown to exhibit various degrees of effectiveness in preventing silica-induced fibrosis in animal models (1, 2). The capability of these drugs to interact with phosphatidycholine vesicles and rat alveolar macrophages was studied using fluorometric and equilibrium dialysis methods. The lipid binding affinity of these alkaloids was found to depend upon several structural factors including hydrophobic substitutions, the chiral configurations, and the double oxygen bridge-restricted conformation of the benzylisoquinoline moieties. Tetrandrine, which is highly effective in preventing fibrosis, showed strong binding to both lipid vesicles and alveolar macrophages. In contrast, 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 summary, this study indicates that alveolar macrophages exhibit large binding capacities for certain members of the BBIQ alkaloids. A positive correlation was observed between binding affinity to alveolar macrophages and the reported antifibrotic potency of these compounds, suggesting 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

Many theories concerning the etiology of silicosis invole silica-induced activation of alveolar macrophages. Evidence indicates that both *in vitro* and *in vivo* silica exposures result in the generation of chemiluminescence, and the release of superoxide and hydrogen peroxide from alveolar macrophages (3.4). Such excess release of reactive products from pulmonary phagocytes has been associated with damage to the lung parenchyma (5,6). In addition, silica exposure results in the release of mediators from alveolar macrophages which enhance proliferation of fibroblasts and collagen synthesis (7-9).

Tetrandrine, a BBIQ alkaloid with reported antifibrotic activity, inhibits silica-induced release of reactive oxygen species from alveolar macrophages (3). It also inhibits activation of granulocytes (10,11), the synthesis of collagen by fibroblasts, and the formation of silicotic nodules in silica-exposed rats (12,13). When used clinically in China, tetrandrine has been shown to improve both the appearance of chest radiographs and diffusion capacity in silicosis patients (14).

Several bisbenzylisoquinoline alkaloids have been tested for antifibrotic potential in rats intratracheally exposed to 50mg of silica (2). In comparison with control, tetrandrine (50mg/kg) decreased collagen formation by 52%; microscopically only small macules wre observed in tetrandrine-treated silica-exposed lungs. Cycleanine was also found to be potent; fangchinoline and cepharanthine exhibited slightly lower potency; berbamine was less effective; while curine and tubocurine were relatively ineffective in decreasing collagen formation or preventing the appearance of silicotic nodules. The objective of the present investigation was to characterize the binding of bisbenzyli-soquinoline alkaloids to biological membranes using phosphatidylcholine vesicles and alveolar macrophages and determine

whether a correlation exists between binding affinity and relative antifibrogenic potential.

MATERIALS AND METHODS

Materials

Dipalmitoyl-phosphatidylcholine (DPPC) and the ammonium salt of 1-anilino-8-naphthalenesulfonate (ANS) were obtained from Calbiochem-Behring Corp. and Aldrich Chemical Co., respectively. Preparation of DPPC vesicles in 0.01M 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, followed by fractionation of large liposomes by centrifugation at 105,000g 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. 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 alkaloids.

Fluorometric Studies of Drug Binding to DPPC

Binding of the BBIQ alkaloids to DPPC vesicles was studied using ANS as a fluorescence probe. (15). ANS shows weak fluorescence at 510 nm (excitation, 380 nm), it 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 a more hydrophobic drug-lipid-ANS ternary complex through ionic interaction at the negatively charged oxygen of the phosphocholine moiety (15).

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. The binding parameters were determined at concentrations 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, indicating the binding of the BBIQ alkaloids to DPPC vesicles.

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

$$\frac{V}{[D]} = nK - VK \qquad (1)$$

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 gm) obtained from Charles River Laboratories (Wilmington, MA) were anesthetized with sodium pentobarbital (0.2gm/kg) and exsanguinated by cutting the renal artery. Alveolar macrophages were obtained by pulmonary lavage with a Ca^{++}, Mg^{++}-free phosphate buffer (145mM NaCl, 5mM KCl, 1.9mM NaH_2PO_4, 9.35mM Na_2HPO_4, and 5.5mM glucose; pH 7.4), according to a method previously described (16).

The binding of selected BBIQ alkaloids to alveolar macrophages (4.0×10^6 cells/ml) was studied using an equilibrium dialysis method. Equilibrium dialysis of drug from HEPES-buffered medium to cell solution was carried out using matched pairs of 1 ml dialysis cells at room temperature for 8 hrs under constant shaking. After dialysis, solutions from both sides of the dialysis cell compartments were centrifuged and the supernatants were analyzed for drug content. The BBIQ concentrations were determined via high performance liquid chromatography (HPLC) using a Waters HPLC system equipped with a Model 440 uv detector set at 254 nm, a uBondapak C18 column, and a mobile phase of CH_3CN-HEPES buffer (pH 5) butanol (60:40:10) delivered at 1ml/min. Binding of drugs to alveolar macrophages was analyzed using an equation analogous to equation 1.

RESULTS AND DISCUSSION

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 berbamine, 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 at either C7 (as in fangchinoline) or C12 (as in berbamine) position resulted in over 4-fold reduction of the apparent partition coefficient. Cepharanthine, with a condensed methylenedioxy ring at C6 and C7 and a double oxygen-bridged ring of also 18-bond length, gave a K′ of 35.1. Cycleanine, which has a measured K′ of 84.6, exhibits a 18-membered ring via oxygen bridges at C8-C12′ and C12-C8′. 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. Methyoxyadiantifoline is a benzylisoquinoline-aporphine dimer with a

total of nine methoxy groups (17). 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.

Fluorescence studies of binding between these drugs and DPPC was analyzed using equation 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 > CU ~ TU, which is consistent with that of the lipophilicity as indicated by K´.

Studies of drug binding to rat alveolar macrophages were carried out with selected alkaloids exhibiting strong (methoxyadiantifoline and tetrandrine), intermediate (fangchinoline) and weak (curine, tubocurine) binding to DPPC vesicles. 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 parameters determined for tetrandrine, fangchinoline, and methoxyadiantifoline using the Scatchard equation are shown in Table 3. The sequence for overall binding affinity (nK) is: TE > ME > FA > TU ~ CU.

The above results show a strong correlation (r^2 = 0.97) between the relative binding affinities of alkaloids for alveolar macrophages and 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 and exhibit weak binding. Fangchinoline is intermediate both in antifibrogenic activity 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*. Recently, we have reported that methoxyadiantifoline and tetrandrine are potent inhibitors of particle-stimulated oxygen consumption, superoxide secretion and hydrogen peroxide release while tubocurine is only minimally affective (18).

The correlation among binding affinity for alveolar macrophages, inhibition of macrophage activation, and antifibrotic potential support theories that silica-induced activation of alveolar macrophages and the resulting excess oxidant generation leads to damage of the lung parenchyma and silicosis (5,6,19). Therefore, these alkaloids may serve as useful agents to probe mechanisms governing the initiation and progression of the fibrotic process.

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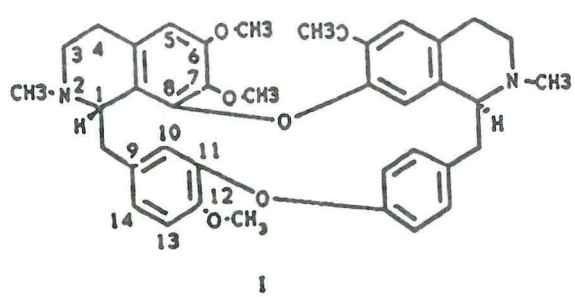
TABLE 1

The Structural and Solvent Partition Characteristics of the BBIQ Alkaloids

	Structural Variation[a]							
Compound[b]	C1	C1′	C7	C12	C12′	C7′	Oxygen Bridge	K′[c]
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] Structural variation are in reference to the structure of tetrandrine (I); structure of methoxyadiantifoline is given in (II):



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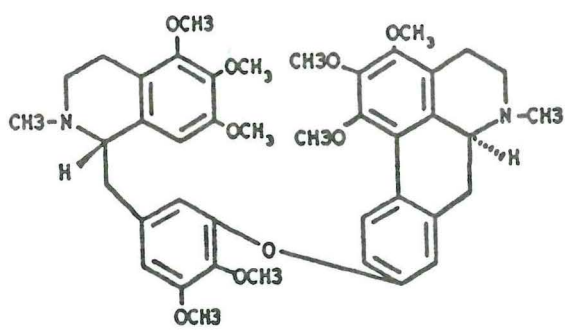

II

[b] Abbreviations: TE-tetrandrine; FA-fangchinoline; BE:berbamine; CE-cepharanthine; TU-tubocurine; CU-curine; CY-cycleanine; ME-methoxyadiantifoline

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

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

TABLE 3

Parameters Calculated for the Binding of Selected Alkaloids to Alveolar Macrophages*

Drug	n (mmole/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[1]	ND[1]	ND[1]
Curine	ND[1]	ND[1]	ND[1]

* n = binding capacity
K = binding constant
nK = overall binding affinity

[1] ND = not detectable

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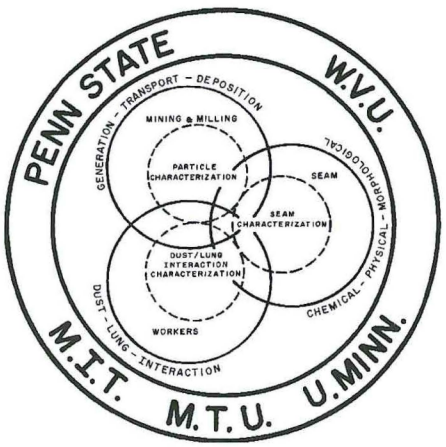

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