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Inhibition of Proliferative Activity of Pulmonary Fibroblasts by Tetrandrine

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Tetrandrine, an herbal drug, has been employed in China to treat pulmonary fibrosis. To date, the mechanisms governing the antifibrotic action of tetrandrine are unknown. The present study employs a fibroblast mitogenic assay to determine whether tetrandrine directly inhibits the ability of fibroblasts to respond to stimulation by growth factors. The data indicate that tetrandrine blocks proliferation and the incorporation of tritiated thymidine into DNA by fibroblasts stimulated with human serum, PDGF plus plasma, FGF plus plasma, or TNF plus plasma. Since tetrandrine inhibits the response to a variety of growth factors, its action does not appear to involve the blockade of a specific stimulatory receptor. Tetrandrine is effective in inhibiting thymidine incorporation when added up to 6 hr after stimulation of quiescent cells, suggesting either that tetrandrine does not block the attainment of competence by fibroblasts or that its activity is not limited to blocking the attainment of competence by these cells. Growth factor-induced mitogenesis is also inhibited by nitrendipine, a calcium channel blocker, and by cytochalasin B, a microfilament blocker. However, tetrandrine treatment of fibroblasts neither results in the changes of morphology seen with cytochalasin B nor is limited to the early events of stimulus–response coupling. Therefore, the mechanism of action for tetrandrine is not identical to that for either cytochalasin B or nitrendipine. In summary, these results suggest that the antifibrotic action of tetrandrine may be mediated in part by direct inhibition of fibroblast proliferation normally associated with the development and progression of silicosis.

Silicosis is a fibrotic pulmonary disease produced by inhalation of silica-containing dusts (Peters, 1986). Chronic silicosis may develop 20–40 years after initial exposure to crystalline silica, and the disease may progress from a simple to a complicated form. Few symptoms are evident in simple silicosis with pulmonary function being relatively normal. However, chest radiographs reveal small rounded opacities in the upper lobes of the lungs. These silicotic nodules consist of collagen arranged in a distinctive circular pattern. As fibrotic changes become more severe, the disease progresses to complicated silicosis (progressive massive fibrosis). Complicated silicosis is associated with shortness of breath and decreases in pulmonary function characteristic of restrictive lung disease. Chest x rays reveal numerous large silicotic nodules which may be associated with scar emphysema. Several mechanisms have been proposed to explain the cellular events which result in silica-induced lung damage and fibrosis (Lapp and Castranova, 1993). They include: (1) direct cytotoxicity of silica, (2) activation of oxidant and enzyme release from alveolar macrophages, (3) recruitment and activation of polymorphonuclear leukocytes, and (4) stimulation of fibroblast proliferation and collagen synthesis.

Tetrandrine is a bisbenzylisoquinoline alkaloid derived from the Chinese herb *Radix Stephania tetrandra.* It has a molecular weight of 622.73 and an empirical formula of $C_{38}H_{42}O_6N_2$. The chemical structure of tetrandrine has been published previously by our laboratory (Castranova *et al.*, 1991a). Its structure is characterized by methoxy groups at C_7 and C_{12}, uncharged nitrogens at N_2 and N'_2, and two 17-carbon ring members connected by a double oxygen bridge between C_8–C'_7 and C_{11}–C'_{12}. Tetrandrine has been shown to reduce both silica-induced fibrosis and the elevation of lung collagen content in rats (Huang *et al.*, 1981; Yu *et al.*, 1983; Idel, 1987). Furthermore, tetrandrine has been used as an antifibrotic agent in Chinese clinical trials (Li *et al.*, 1981). Preliminary results suggest that administration of tetrandrine to patients with silicosis results in substantial improvement of symptoms, increases in diffusion capacity, and decreases in the size of shadows on lung x rays.

Recently, the mechanisms by which tetrandrine exerts its antifibrotic activity have begun to be investigated. Tetrandrine does not directly inhibit the *in vitro* cytotoxicity of silica on lung cells (Kang, 1990). However, this drug is a potent inhibitor of pulmonary phagocytes. It inhibits silica-induced release of reactive oxygen species and interleukin-1 from alveolar macrophages (Castranova *et al.*, 1990; Kang *et al.*, 1992), blocks stimulant-induced oxidant release as

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well as prostaglandin and leukotriene production by polymorphonuclear leukocytes (Castranova *et al.*, 1991b; Teh *et al.*, 1990), and exhibits immunosuppressive effects on lymphocytes (Seow *et al.*, 1988). Therefore, it has been proposed that the antisilicotic action of tetrandrine may be mediated in part by its ability to decrease inflammatory-based parenchymal damage following the inhalation of silica.

Several investigations have reported that silica exposure results in the release of fibrogenic factors from alveolar macrophages (Heppleston and Stiles, 1967; Brown *et al.*, 1988; Gritter *et al.*, 1986). Interleukin-1 has been proposed to be one of these silica-induced fibroblast proliferative factors (Schmidt *et al.*, 1984). Tetrandrine inhibits silica-induced interleukin-1 release from alveolar macrophages (Kang *et al.*, 1992). Therefore, it is possible that tetrandrine exhibits antisilicotic activity by preventing the release of fibroblast growth factors from phagocytes.

It is also possible that tetrandrine directly inhibits the ability of fibroblasts to proliferate and/or synthesize collagen. To date, no data are available concerning this question. Therefore, the objective of our investigation was to determine if the antifibrotic action of tetrandrine is mediated in part by direct inhibition of fibroblasts. In particular, we have investigated the effect of tetrandrine on the responsiveness of fibroblasts to known proliferative agents.

MATERIALS AND METHODS

Materials

Fibroblasts

Human fetal lung fibroblasts (HFL1, No. CCL 153) were obtained from the American Type Culture Collection (Rockville, MD). All fibroblast cultures were maintained in closed flasks in minimum essential medium (MEM) containing Earle's salts, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. Media were changed three times weekly. Cultures were incubated at 37°C until confluent. Trypsinization of fibroblasts for subculture was carried out at 22°C using 0.25% trypsin in phosphate-buffered saline. Studies were carried out using 10th through 18th passage fibroblast cultures. Fibroblasts were counted using an electronic cell counter equipped with a cell-sizing attachment.

Growth Factors

Human serum (10–20%) was used as a complete growth factor, i.e., containing both progression and competence activity. Human serum was obtained by collecting blood into nonheparinized tubes, allowing it to clot, removing the clot with a cotton applicator, centrifuging the fluid at 560*g* for 10 min, and collecting the supernate.

Human plasma (1%) was used as a progression factor. It was obtained by collecting blood into heparinized tubes, centrifuging at 560*g* for 10 min, and filtering through 0.45-μm Nalgene and 0.22-μm Milex-gv Millipore filters.

Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and tumor necrosis factor-alpha (TNF-α) were used as competence factors. Human recombinant PDGF (isoform bb) was obtained from either Intergen Co. (Purchase, NY) or Upstate Biotechnology Inc. (Lake Placid, NY). Stock solutions of PDGF made in phosphate-buffered saline (PBS) containing 2 mg/ml bovine serum albumin (BSA). TNF-α and bFGF were obtained from Upstate Biotechnology Inc. Stock solutions of TNF-α and bFGF were prepared in PBS containing 2 or 5 mg/ml BSA, respectively. The final concentration of PDGF, TNF-α, or bFGF used was 50 ng/ml.

Inhibitors

Tetrandrine. Tetrandrine was supplied by Dr. Chang-geng Mo, Institute of Occupational Medicine, Chinese Academy of Preventative Medicine, Beijing, People's Republic of China. Stock solutions were prepared by mixing the powder with a few drops of distilled water, dissolving in HCl, adjusting the pH to 6.0 with NaOH, and then bringing up to volume with distilled water. Final concentrations are noted in the figure legends.

Cytoskeletal blockers. Taxol was supplied by Molecular Probes, Inc. (Eugene, OR). The stock solution was 10 mM in 60% methanol/40% serum-free media. Final concentrations are noted in the tables.

Cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, WI). The stock solution was 10 mM in 30% methanol/70% serum-free media. Final concentrations are noted in the tables.

Ca^{2+} channel blockers. Nitrendipine was purchased from Miles Pharmaceutical (West Haven, CT). Stock solutions were 10 mM in 75% methanol/25% serum-free media. Verapamil was obtained from Knoll Pharmaceutical (Whippany, NJ). Stock solutions were 10 mM in 50% methanol/50% serum-free media. Final concentrations are noted in the tables.

Methods

Development of the Fibroblast Mitogenic Assay

Measurement of the incorporation of tritiated thymidine into DNA. The basic procedural outline of Kumar *et al.* (1988) was used with modifications (Reist *et al.*, 1991) to evaluate the incorporation of tritiated thymidine into the DNA of fibroblast following exposure to various growth factors and/or inhibitors. Human lung fibroblasts were plated in MEM plus 10% fetal calf serum at 50,000 cells/ml at a density of 250,000 cells/25-cm^2 culture plate and cultured for 2 days. Then cells were quiesced for 2 days in serum-free media prior to the addition of growth factors and/or inhibitors at time = 0 hr (note medium alone was used as a negative control). After 6 hr, growth factors and inhibitors were removed, [^{3}H]-thymidine (1 μCi/ml) was added, and the cells were incubated for an additional 32 hr. Thirty-two hours after the addition of tritiated thymidine, i.e., time = 38 hr, cultures were washed with 5 ml of fresh serum-free media for 15 min and then trypsinized at 2°C. Briefly, 3 ml of PBS containing 0.05% trypsin and 0.02% EDTA was added for 1 min and removed by aspiration, and the culture incubated in the remaining trypsin solution until cells began to detach. Cells were recovered by washing three times with 5 ml of MEM plus 10% fetal calf serum. Cells were centrifuged for 10 min at 560*g*, resuspended in 1 ml of PBS, and an aliquot of cell suspension counted as described above using an electronic cell counter. Another aliquot of fibroblasts was used to monitor cell viability using the trypan blue exclusion method (Phillips, 1973).

The remainder of the fibroblast suspension (0.75 ml) was added to an equal volume of 10% TCA. The samples were refrigerated for 15 min and then centrifuged at 1360*g* for 10 min at 2°C. The supernatant was discarded and the precipitate containing the DNA resuspended in 0.5 ml of 0.1 N NaOH. The suspension was vortexed and 1 ml of 10% TCA was added to the mixture. The samples were centrifuged again at 1360*g* for 10 min at 2°C, resuspended in 0.5 ml of 0.1 N NaOH, added to 10 ml of Scintiverse II, and counted for 10 min each using a liquid scintillation counter. Mitogenicity was expressed as dpm/fibroblast.

Measurement of fibroblast proliferation. Fibroblast proliferation was also measured directly by determining the change in cell count following a continuous 7-day treatment with growth factors and/or inhibitors. Briefly, quiesced fibroblasts were exposed to various agents at time = 0. After 4

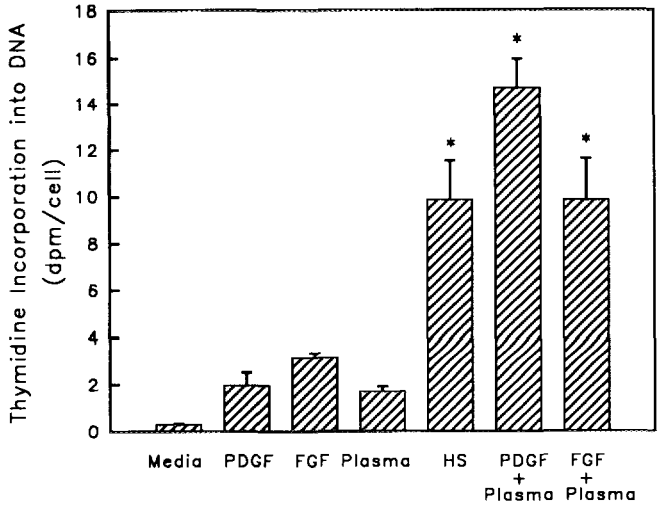

FIG. 1. Incorporation of [^{3}H]thymidine into the DNA of fibroblasts in the absence (media) or presence of growth factors. *Significant increase above the respective control.

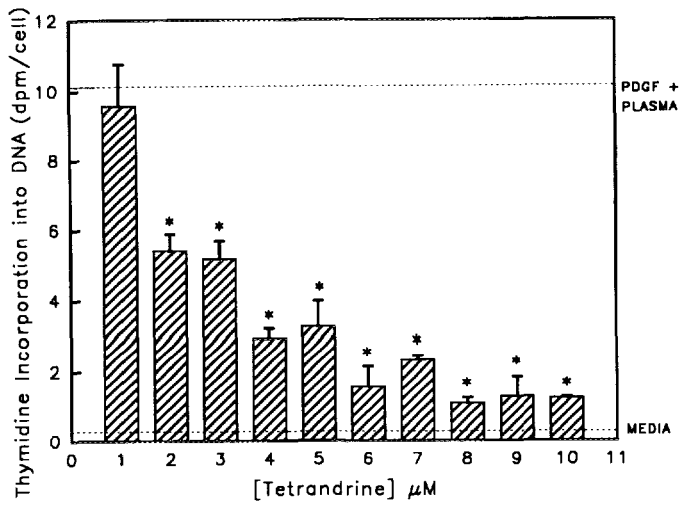

FIG. 2. Inhibitory action of tetrandrine on the mitogenic response of quiesced fibroblasts to PDGF (50 μg/ml) plus human plasma (1%). In the absence of inhibitor, thymidine incorporation was 10.19 ± 1.12 dpm/cell and 0.28 ± 0.03 dpm/cell for the positive (PDGF + plasma) and negative (media) controls, respectively (dashed lines). *Significant decrease from the positive control.

days, media plus plasma was added to all cultures. Fibroblasts were harvested by trypsinization after 7 days in culture and counted using an electronic cell counter as described previously.

Thymidine Influx

Several experiments were designed to determine if tetrandrine or other drugs affected the transport of tritiated thymidine (measured as cytosolic dpm/cell/30 min) across fibroblast plasma membranes. Fibroblasts were preincubated with inhibitors for 6 hr. Thymidine influx then was measured 30 min after the addition of [^{3}H]thymidine to the cell suspension. Briefly, cells were removed from the culture flasks, washed twice with cold serum-free media to remove extracellular label, and centrifuged at 560*g*. Cells were resuspended in 1 ml PBS and an aliquot of the cells was counted using an electronic cell counter. The remaining cells (750 μl) were then lysed with 1 ml 10% TCA. The protein and DNA were precipitated by centrifugation at 560*g* and the radioactive label in the supernatant (cytosolic, unincorporated label) was measured in a liquid scintillation counter. Thymidine influx was expressed as dpm/cell/30 min.

Data Analysis

Data are presented as means ± SE for $n = 2$ to 10 separate determinations. Statistical analysis employed an ANOVA followed by a Tukey's test with significance set at $p < 0.05$ (Dowdy and Wearden, 1983).

RESULTS

The utility of the thymidine incorporation assay to monitor mitogenic potential of pulmonary fibroblasts was assessed using a variety of growth factors. These data are summarized in Fig. 1. The incorporation of tritiated thymidine was low in the negative control (media alone). Competence factors, such as PDGF or FGF, alone resulted in a small increase in thymidine incorporation. Similar results were obtained with plasma alone which contains only progression activity. The addition of both competence and progression activity, as found in human serum, PDGF plus plasma, or FGF plus plasma, resulted in a significant elevation in the incorporation of tritiated thymidine into fibroblast DNA.

Tetrandrine was a potent inhibitor of the mitogenic response of pulmonary fibroblasts to growth factors. Figure 2 shows that tetrandrine exhibited a dose-dependent inhibition of [^{3}H]thymidine incorporation in response to PDGF plus plasma with significant inhibition occurring with as little as 2 μM tetrandrine. Tetrandrine also inhibited thymidine incorporation in response to human serum, TNF plus plasma, and FGF plus plasma. Table 1 lists values for per-

TABLE 1
Inhibitory Action of Tetrandrine on the Mitogenic Response of Pulmonary Fibroblasts to Growth Factors[a]

Growth factor[b]	Maximal Inhibition (%)[c]	ID_{50} (μM)
PDGF + plasma	92 ± 3*	2.5
Human serum	86 ± 3*	3.5
FGF + plasma	99 ± 9*	1.5
TNF + plasma	100 ± 0*	2.5

[a] Values calculated from dose–response curves similar to the one shown in Fig. 2.

[b] Thymidine incorporation of quiesced pulmonary fibroblasts in media (negative control) was 0.28 ± 0.03 dpm/cell. Positive controls were 10.19 ± 1.12 dpm/cell for PDGF (50 ng/ml) + 1% plasma, 10.23 ± 1.03 dpm/cell for 10% human serum, 14.70 ± 0.74 dpm/cell for FGF (50 ng/ml) + 1% plasma, and 2.55 ± 0.33 dpm/cell for TNF (50 ng/ml) + 1% plasma.

[c] Maximal concentration of tetrandrine used was 10 μM.

* Indicates a significant decrease from the positive control.

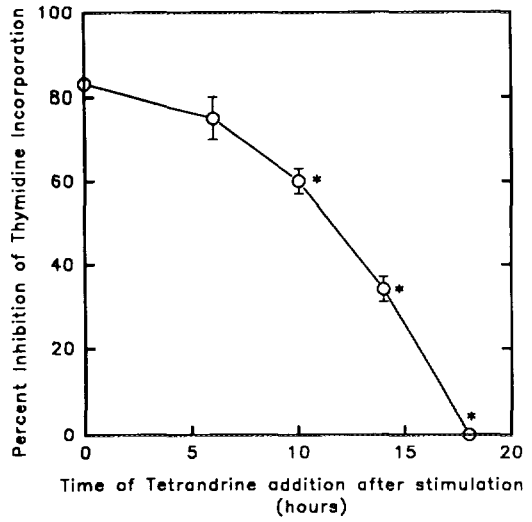

FIG. 3. Effect of delayed treatment of fibroblasts with tetrandrine on human serum-induced [^{3}H]thymidine incorporation. Pulmonary fibroblasts were stimulated with 10% human serum at time = 0 and were treated with 10 μM tetrandrine at various times after stimulation. *Significant decrease in the inhibitory potency of tetrandrine when added after stimulation compared to that measured when tetrandrine and serum were added simultaneously.

centage maximal inhibition and half maximal inhibitory concentrations of tetrandrine obtained with each of these growth factors. This inhibitory effect of tetrandrine on growth factor-induced [^{3}H]thymidine incorporation into DNA was also reflected in a direct decrease in proliferative activity of these pulmonary fibroblasts. Indeed, 2.5 μM tetrandrine decreased proliferation of quiesced pulmonary fibroblasts in response to 10% human serum by 22.5 ± 1.9% (compared to a 40% inhibition of thymidine incorporation at 2.5 μM tetrandrine). This inhibition of growth factor-induced thymidine incorporation and proliferation was not due to an effect of tetrandrine on fibroblast viability, since trypan blue exclusion of pulmonary fibroblasts treated for 6 hr with 10 μM tetrandrine was 94 ± 1% compared to 88 ± 2% for the untreated controls.

The ability of tetrandrine to inhibit growth factor-induced incorporation of [^{3}H]thymidine into DNA of pulmonary fibroblasts was not limited to a particular stage in the cell cycle. Indeed, 5 μM tetrandrine inhibited human serum-induced thymidine incorporation in nonquiesced, competent fibroblasts, which presumably were in various phases of the cell cycle, by 51.5 ± 2.6% compared to 67 ± 1% in quiesced fibroblasts, where most cells were in the G_0 stage. Furthermore, tetrandrine continued to significantly block thymidine incorporation even when added 14 hr after addition of 10% human serum to previously quiescent pulmonary fibroblasts, i.e., well after competence had been achieved (Fig. 3). However, the addition of tetrandrine more than 6 hr after stimulation resulted in a significant decline in the ability of tetrandrine to inhibit [^{3}H]-thymidine incorporation.

The inhibitory effect of tetrandrine on growth factor-induced [^{3}H]thymidine incorporation into the DNA of pulmonary fibroblasts was irreversible as shown in Fig. 4. A 15-min pretreatment and removal of extracellular tetrandrine inhibited subsequent PDGF plus plasma-induced thymidine incorporation by 95.0 ± 0.4% compared to a 99 ± 0% inhibition when tetrandrine was allowed to remain in the culture medium during stimulation.

Pulmonary fibroblasts were also treated with selected calcium channel blockers or cytoskeletal modifiers and their effects on growth factor-induced [^{3}H]thymidine incorporation were compared with the inhibitory effects of tetrandrine (Table 2). The calcium channel blocker nitrendipine significantly inhibited [^{3}H]thymidine incorporation while verapamil did not. Cytochalasin B, a microfilament blocker, significantly decreased the incorporation of [^{3}H]-thymidine into DNA, while taxol, a microtubule modifier, was ineffective.

As with tetrandrine, cytochalasin B not only inhibited growth factor-induced thymidine incorporation but also decreased proliferation of pulmonary fibroblasts, i.e., 5 μg/ml cytochalasin B decreased the number of fibroblasts harvested 7 days after *in vitro* stimulation with 10% human serum by 76 ± 1%. However, the mode of action of cytochalasin B and tetrandrine seem to be different, since unlike tetrandrine cytochalasin B dramatically modified the morphology of the fibroblast monolayer evaluated by light microscopy with the plated fibroblasts appearing shrunken and deformed in the presence of cytochalasin B.

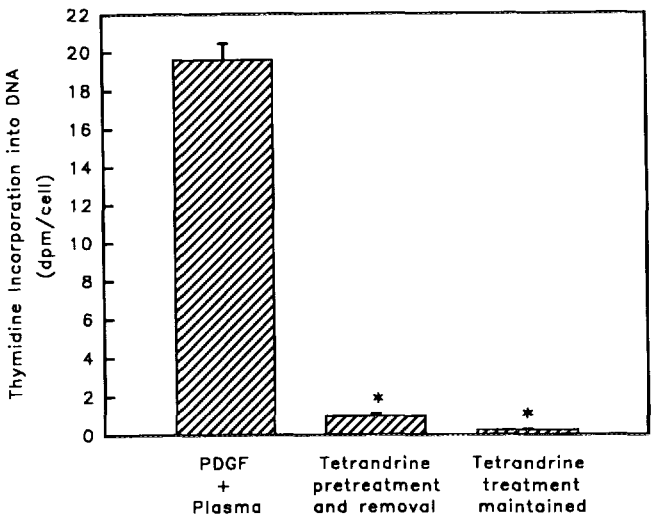

FIG. 4. The inhibitory action of tetrandrine on PDGF plus plasma-induced [^{3}H]thymidine incorporation: the effectiveness of a 15-min pretreatment vs maintained exposure. Fibroblasts were stimulated with PDGF (50 ng/ml) plus 1% plasma for 1 hr. Cells were either pretreated with 10 μM tetrandrine for 15 min and then washed prior to stimulation or treated during stimulation. *Significant decrease from the uninhibited control.

Stimulation of pulmonary fibroblasts with 10% human serum not only induced mitogenesis but also enhanced the influx of thymidine by 3.8 (±0.3)-fold. Tetrandrine, nitrendipine, verapamil, and cytochalasin B all significantly inhibited the influx of thymidine, measured as uptake of [^{3}H]-thymidine in 30 min. These data are given in Table 3.

DISCUSSION

Data from this investigation indicate that tetrandrine inhibits both the mitogenic and the proliferative response of pulmonary fibroblasts to stimulation by growth factors, i.e., tetrandrine inhibits the incorporation of tritiated thymidine into DNA and cell multiplication in response to stimulants. Tetrandrine effectively inhibits the mitogenic response to a variety of growth factors, including human serum, PDGF plus plasma, FGF plus plasma, and TNF plus plasma. This suggests that tetrandrine does not act by blockage of a specific membrane receptor but rather via a more generalized route. A similar conclusion was drawn with alveolar macrophages, since tetrandrine inhibits stimulant-induced respiratory burst activity but not depolarization of these phagocytes (Castranova *et al.*, 1991b). In addition, Seow and co-workers (1988) reported that tetrandrine does not block the binding of concanavalin A or monoclonal reagents surface receptors of lymphocytes.

The inhibitory action of tetrandrine on the mitogenic activity of fibroblasts is not secondary to cell death, since trypan blue exclusion is unaffected at doses and treatment times of tetrandrine which decrease fibroblast mitogenesis by approximately 90%. Similarly, Kang and co-workers (1992) reported that tetrandrine inhibits the ability of thymocytes to respond to mitogenic stimulation by IL-1 or concanavalin A. In both the fibroblast and the thymocyte systems, tetrandrine exhibits ID_{50} values in the range 2–4 μM.

TABLE 2

Inhibitory Action of Calcium Channel Blockers or Cytoskeletal Modifiers on the Mitogenic Response of Pulmonary Fibroblasts to Growth Factors

Modifier[a]	Maximal inhibition (%)[b]	ID_{50}
Calcium channel blockers		
(1) Nitrendipine	82 ± 3*	25 μM
(2) Verapamil	26 ± 8	—
Cytoskeletal modifiers		
(1) Cytochalasin B	93 ± 11*	3.5 μg/ml
(2) Taxol	0	—

[a] Maximal concentrations of modifiers were: 100 μM nitrendipine, 100 μM verapamil, 20 μg/ml cytochalasin B, and 20 μM taxol.

[b] [^{3}H]Thymidine incorporation was not affected by methanol at concentrations used as vehicle for these modifiers.

* Indicates a significant decrease from the positive control (10% human serum in the absence of inhibitor).

TABLE 3

Effect of Drugs on the Influx of [^{3}H]thymidine into Pulmonary Fibroblasts[a]

Drug	Thymidine influx (%)[b]
Tetrandrine (10 μM)	16.5 ± 0.9*
Nitrendipine (100 μM)	20.0 ± 1.4*
Verapamil (100 μM)	21.3 ± 1.3*
Cytochalasin B (20 μg/ml)	38.0 ± 8.0*

[a] Pulmonary fibroblasts were treated with 10% human serum in the absence or presence of drugs for 6 hr prior to the addition of [^{3}H]thymidine and measurement of thymidine influx into the cytoplasm for 30 min.

[b] Percentage of the positive control (10% human serum in the absence of drugs). The methanol vehicle had no effect on thymidine influx.

* Indicates a significant decrease below the positive control.

Two lines of evidence suggest that the inhibitor action of tetrandrine is not limited to blocking the attainment of competence by stimulated fibroblasts. First, tetrandrine decreases the incorporation of tritiated thymidine in growing, nonquiesced fibroblasts treated with human serum. These cells may attain the equivalence of competence in the G_2 phase of the previous cycle. Secondly, tetrandrine remains effective in inhibiting mitogenesis of quiesced fibroblasts treated with growth factors even when added as much as 6 hr after cell stimulation. Hendrickson and Scher (1983) reported that competence is achieved within 2 hr of stimulation. Therefore, tetrandrine is effective long after the fibroblasts have reached competence, and therefore, it is likely that tetrandrine blocks progression.

The data indicate that tetrandrine binds rapidly and irreversibly to pulmonary fibroblasts. Indeed, the inhibitory activity of tetrandrine is maintained even when cells are pretreated with tetrandrine for 15 min at 37°C, washed, and placed in a medium free of tetrandrine. Similarly, Ma and co-workers (1992) reported that the binding of tetrandrine to alveolar macrophages is rapid (with a $T_{1/2}$ of approximately 5 min) and irreversible.

Growth factors have been associated with an increase in cytoplasmic calcium which precedes mitogenesis (Rozengurt, 1986). Thus, it is of interest that the calcium channel blocker nitrendipine inhibits tritiated thymidine incorporation in stimulated fibroblasts. Tetrandrine has been reported to be a calcium channel blocker (King *et al.*, 1988). However, the inhibitory action of tetrandrine on fibroblast mitogenesis cannot be limited to blockade of stimulant-induced calcium uptake, since tetrandrine exhibits antimitogenic potential even when added up to 6 hr after stimulation, i.e., when intracellular calcium has returned to baseline (Rozengurt, 1986).

Liu and co-workers (1988) reported that tetrandrine can bind to isolated microtubules. Further, Ma and co-workers

(1992) found that the binding of tetrandrine to alveolar macrophages is inhibited by pretreatment of the cells with microtubular agents (taxol or vinblastine) or substances which affect microfilaments (cytochalasin B). Our data indicate that cytochalasin B is effective in decreasing stimulation-induced mitogenesis and proliferation of pulmonary fibroblasts. However, the microtubular agent, taxol, does not alter the incorporation of tritiated thymidine by stimulated fibroblasts. Although cytochalasin B and tetrandrine both inhibit fibroblast proliferation, it appears that they act by different mechanisms. Indeed, fibroblast morphology evaluated by light microscopy is not altered 32 hr after a 6-hr treatment with tetrandrine but is dramatically modified after exposure to cytochalasin B.

Our data indicate that stimulation of pulmonary fibroblasts with human serum increases the influx of thymidine. Treatment of fibroblasts with tetrandrine, nitrendipine, verapamil, or cytochalasin B depresses the rate of thymidine uptake in serum-stimulated cells but not in unstimulated fibroblasts. It is possible that inhibition of thymidine uptake is related to the calcium blocking action of these drugs.

Several lines of evidence argue that the inhibition of growth factor-induced mitogenesis is not due simply to a decrease in the influx of [^{3}H]thymidine. First, tetrandrine and cytochalasin B not only inhibit the incorporation of [^{3}H]thymidine into DNA but also inhibit proliferation, measured as cell counts 7 days after stimulation with growth factors. Secondly, although these drugs inhibit the rate of thymidine influx, incorporation experiments were assayed after a 32-hr incubation with [^{3}H]thymidine. Experiments indicate that incorporation of [^{3}H]thymidine into DNA rises as incubation time with label is increased from 0 to approximately 16 hr, maintaining a maximum from 16 to 32 hr (data not shown). Therefore, our experimental design allows 16 additional hr for [^{3}H]thymidine to enter the cells and be incorporated in DNA, thus decreasing the impact of a lower rate of uptake for the label in drug-treated fibroblasts. Lastly, verapamil is very effective in inhibiting the uptake of [^{3}H]thymidine into fibroblasts, yet is rather ineffective in inhibiting growth factor-induced [^{3}H]-thymidine incorporation into DNA. This suggests that labeled thymidine is not the limiting factor in these incorporation experiments.

Hashizume and co-workers (1991) have shown that tetrandrine suppresses the activity of phospholipase C. Data reported by Seow and co-workers (1988) support this conclusion with evidence that tetrandrine inhibits phosphoinositide metabolism in concanavalin A-stimulated lymphocytes. However, the fact that tetrandrine remains effective when added 6 hr after fibroblast stimulation suggests that the antimitogenic action of tetrandrine is not limited to blockage of these early events in the stimulus–response coupling scheme.

In conclusion, the direct inhibitory action of tetrandrine on fibroblast proliferation and mitogenesis in the late G_1 phase suggests a potential mechanism to explain its reported antifibrotic activity. This inhibition by tetrandrine is not completely explained by blockade of calcium channels or modification of the cytoskeletal system. However, tetrandrine may also affect other mechanisms of fibrogenesis. For example, tetrandrine is an effective inhibitor of particle-induced secretion of reactive oxygen species from alveolar macrophages and neutrophils (Castranova *et al.*, 1991a,b). Such action may act to moderate silica-induced lung damage. Clearly, further investigation is required to fully understand the potential activities of tetrandrine and the mechanisms by which it may reduce fibrosis.

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