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Cytotoxicity to Macrophages of Tetrandrine, an Antisilicosis Alkaloid, Accompanied by an Overproduction of Prostaglandins

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ABSTRACT. Tetrandrine, an anti-inflammatory immunosuppressive bisbenzylisoquinoline alkaloid of Chinese herbal origin, is widely used to treat silicosis and interferes with the regulation of calcium in many cell types. We investigated its effect on the cellular integrity of macrophages and on their ability to generate prostaglandins and nitric oxide, mediators of inflammation with immunomodulatory roles. Tetrandrine at 10^{-7} M to 10^{-4} M caused dose- and time-dependent loss of cell viability of mouse peritoneal macrophages, guinea-pig alveolar macrophages and mouse macrophage-like J774 cells. Loss of viability (50%) occurred within 1–3 hr and required ≈5 × 10^{-6} M tetrandrine. Loss of macrophage viability after tetrandrine treatment was accompanied by the generation of large amounts of prostaglandin E_2 (PGE_2), to levels 285–877% of control. Coincubation with indomethacin abolished PGE_2 generation, but did not prevent cell death. Tetrandrine did not cause generation of nitric oxide. Verapamil also reduced the viability of mouse peritoneal macrophages and J774 cells, but did not cause PGE_2 overproduction, except at 10^{-4} M in mouse peritoneal macrophages. In macrophages cultured with lipopolysaccharide and interferon-γ to induce the generation of large amounts of both PGE_2 and nitric oxide, tetrandrine reduced mediator release and their forming enzymes (cyclo-oxygenase-2 and inducible nitric oxide synthase), secondary to cytotoxicity. The predominant action of tetrandrine is to exert a cytotoxic effect on macrophages, perhaps by interfering with calcium homeostasis; this leads to overproduction of immunomodulatory but proinflammatory prostaglandin. This may be relevant to its protective actions in human fibrosing silicosis, in which there is alveolar macrophage involvement. BIOCHEM PHARMACOL 53;6:773–782, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. tetrandrine; bisbenzylisoquinoline alkaloids; macrophages; cytotoxicity; nitric oxide; inducible nitric oxide synthase; prostaglandin E_2; cyclo-oxygenase-2; Western blot

Tetrandrine is one of a large number of known plant-derived bisbenzylisoquinoline alkaloids [1] and is obtained from the roots of *Stephania tetrandria*. This plant is used as an analgesic, diuretic, and anti-inflammatory agent in Chinese traditional medicine [2], but the main use now for pure tetrandrine in China is to treat silicosis, an important occupational disease [3–5]. Tetrandrine has antihypertensive, antianginal, anti-inflammatory, and immunosuppressive properties, which many pharmacological studies suggest could be due to calcium antagonist-like actions (reviewed in [1, 6, 7]; see also [8–10]). Indeed, in HL-60 leukocytic cells, tetrandrine both mobilises an intracellular calcium storage pool and blocks the voltage-sensitive entry pathway, thus disrupting cytosolic calcium homeostasis [11]. It has also been found that tetrandrine and related alkaloids inhibit nitric oxide production in mouse peritoneal macrophages activated by bacterial lipopolysaccharide [12].

The purpose of the present paper was to investigate the effects of tetrandrine on macrophage function, with emphasis on its actions on cell viability as well as on the generation of the proinflammatory immunomodulatory mediators, nitric oxide and prostaglandins. We compared some of tetrandrine's actions with those of verapamil, an established calcium channel antagonist.

MATERIALS AND METHODS

Cell Culture

The mouse macrophage-like J774 line was obtained from the European Collection of Animal Cell Cultures, Porton Down, Wiltshire, U.K. Macrophages from 20 g male Swiss Webster mice were obtained by peritoneal lavage using 3 mL ice-cold PBS. Alveolar macrophages from urethane-anaesthetised 400 g male Dunkin-Hartley guinea pigs were lavaged into 5 mL sterile saline. In both cases, the washings were centrifuged at 500 *g* for 10 min (4°C) and the pellets resuspended gently in 2–10 mL DMEM‡.

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‡ *Abbreviations:* COX-2, inducible form of cyclo-oxygenase; DMEM, Dulbecco's modified Eagle's medium; IFN-γ, interferon-γ; iNOS, inducible form of nitric oxide synthase; LPS, bacterial lipopolysaccharide; MTT, Thiazolyl Blue; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; TNF, tumour necrosis factor.

J774 cells were added at 5×10^5 cells in 1 mL DMEM into 24-well culture plates or at 10^5 cells in 250 μL DMEM to 96-well culture plates. In the case of peritoneal and alveolar washings, cells were added at these densities to similar wells, and the macrophages allowed to adhere for 2 hr prior to repeat washing to remove nonadherent cells. DMEM containing 5% fetal calf serum was then added and incubations with tetrandrine and other drugs performed as described.

Measurement of Nitric Oxide and Prostaglandin E_2 Released Into the Medium and COX Activity

Following incubation, the culture media were sampled for assay of released nitric oxide and prostaglandin E_2. For PGE_2, 50 μL aliquots of medium were subjected to radioimmunoassay using a polyclonal antibody prepared from rabbits (final dilution 1:2000 and 0.01 μCi [$^3H_7$]-PGE_2) and the bound label-antibody complexes separated using charcoal to precipitate unbound label. Nitric oxide was measured as NO_2^- and quantified by adding 100 μL aliquots of medium to 100 μL of Griess reagent (1% sulphanilamide, 0.1% naphthylethyldiamine, in 5% phosphoric acid), and comparing the resulting OD_{550} values in a microplate reader to those obtained from a standard curve of sodium nitrite also prepared in medium.

Cyclo-oxygenase (COX) activity was assayed functionally by washing the cells 3 times in PBS and then adding 10^{-6} M arachidonic acid for a further 1-hr incubation in culture medium. These samples were subjected to radioimmunoassay for PGE_2.

Measurement of Cell Viability

Cell viability was measured by adding 20 μL 5 mg/mL MTT to cells in 96-well plates and incubating for 1 hr at 37°C. After removing the medium, 100 μL DMSO was added to solubilize the blue-coloured tetrazolium and the plates were shaken for 5 min. The OD_{550} values were read in a microplate reader. Viability was set as 100% in control cells.

Expression of iNOS and COX-2 Determined by Western Blotting

Identification of COX-2 and iNOS by Western blotting was performed by culturing J774 cells in 6-well plates. After washing the cells with PBS, they were incubated for 5 min with an extraction buffer (0.9% NaCl, tris-HCl 20 mM, pH 7.6, triton X-100 0.1%, phenylmethylsulphonyl fluoride 1 mM, leupeptin 0.01%) with gentle shaking. The cell extract was centrifuged (4000 *g*, 4°C, 10 min), and the protein concentration in the supernatant measured using the Folin-Ciocalteu reagent. Sufficient aliquots of sample were mixed 1:1 with sample buffer (tris-HCl 20 mM pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.025% Bromophenol Blue) and boiled for 5 min prior to electrophoresis in 7.5% SDS-polyacrylamide gel (5 hr, 500V, 36 mA, 30 μg protein per track). Separated proteins were transferred to Biorad nitrocellulose membranes (2 hr, 40V) and the blot incubated for 1 hr with primary antibodies, *viz.* rabbit antisera to murine COX-2 (Cayman, 1:1000) and to murine iNOS (gift of Dr. V. Riveros-Moreno, 1:1000). The blot was incubated with secondary antibody (sheep anti-rabbit IgG linked to horseradish peroxidase conjugate, Sigma, 1:2000) for 1 hr and, finally, incubated with ECL reagent (Amersham) for 1 min and exposed to Hyperfilm-ECL (Amersham).

Tetrandrine (> 98% purity) was supplied by Zhejiang Jinghua Pharmaceutical Co, PR China.

RESULTS

Tetrandrine at 10^{-7} M to 10^{-4} M produced concentration-dependent loss of cell viability when added to cultures of mouse resident peritoneal macrophages for 16 hr (Fig. 1A) or to the mouse macrophage-like J774 cell line for 6 hr (Fig. 1C). The concentration required to kill half the cells was about 5×10^{-6} M in both cases.

The amounts of prostaglandin E_2 released from these macrophages are shown in Figs. 1B and 1D. Basal release during the respective 16-hr and 6-hr periods was low, but it was significantly increased in a biphasic manner by the higher concentrations of tetrandrine. In this set of experiments on peritoneal macrophages and J774 cells, the maximal PGE_2 production was increased about 3-fold in both cases. These results suggest that PGE_2 overproduction occurs in cells whose viability is reduced by the drug treatment, but also that the highest tetrandrine concentrations may directly affect the prostaglandin biosynthetic pathway.

Verapamil also reduced the viability of mouse peritoneal macrophages and J774 cells in a dose-dependent fashion (Fig. 2A,C), but was a little less potent than tetrandrine (concentration required to kill half the cells approximately $1–2 \times 10^{-5}$ M). However, verapamil did not increase the amount of PGE_2 released into the medium except at the two highest concentrations in mouse peritoneal macrophages (Fig. 2B,D).

Preaddition of 10^{-6} M indomethacin to mouse peritoneal macrophages reduced their ability to generate PGE_2, but did not affect the loss of viability caused by tetrandrine or verapamil (Table 1).

There was no evidence for generation of nitric oxide (NO) under these experimental conditions. Following treatment of J774 or peritoneal macrophages with toxic doses of tetrandrine or verapamil, NO levels were less than 1.0 μM, similar to those in control cultures (data not shown). Thus, the ability of dying macrophages to generate prostaglandins is not accompanied by any increase in nitric oxide synthase activity.

The time-course of the toxic response to an intermediate concentration of tetrandrine was studied using both mouse peritoneal macrophages and J774 cells. Figure 3A shows that within 5 hr of adding 10^{-5} M tetrandrine to mouse

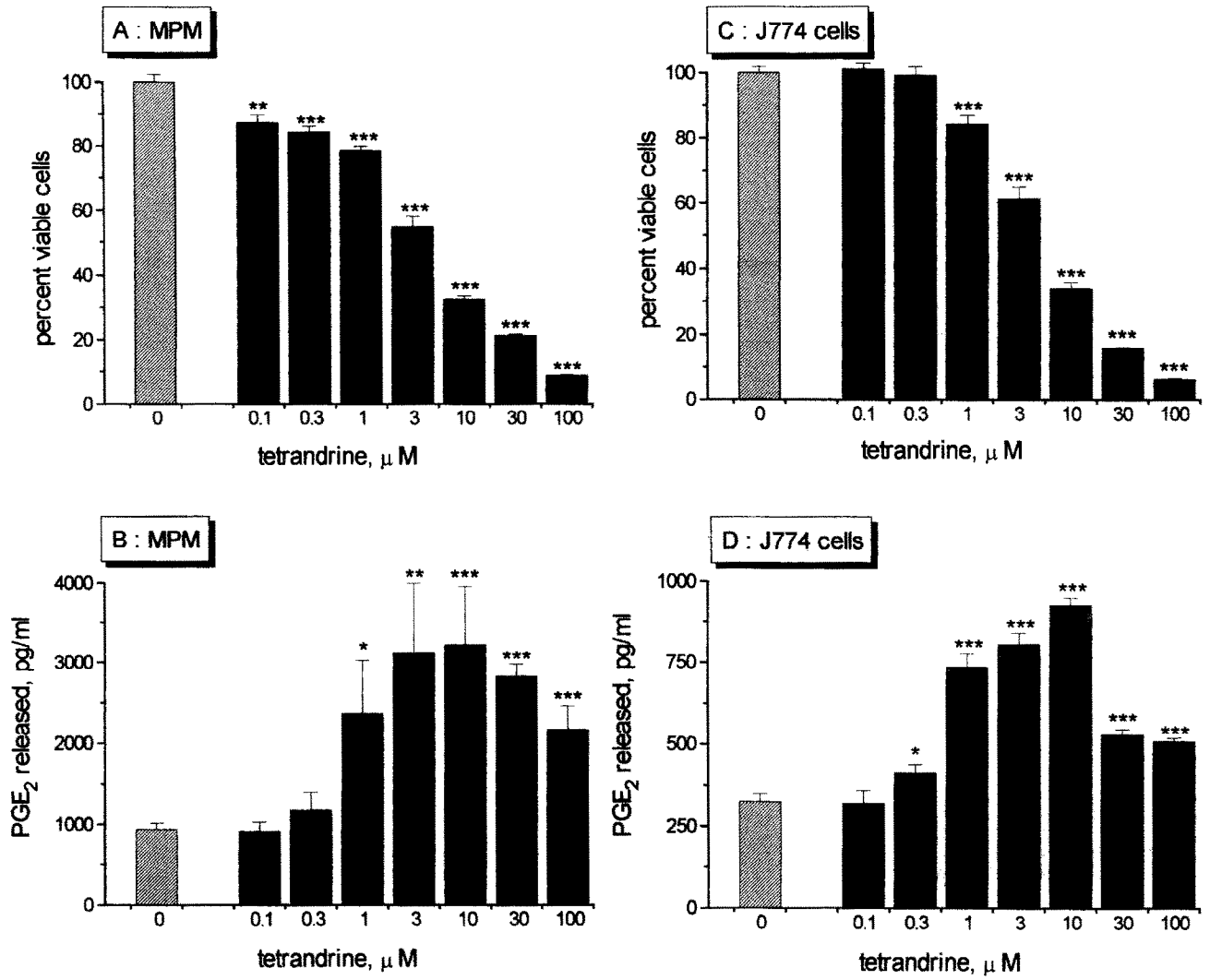

FIG. 1. Effect of tetrandrine on cell viability (A) and PGE_2 release (B) from mouse peritoneal macrophages and (C), (D) J774 mouse macrophage-like cells. Cells were cultured in 96-well plates as described in Materials and Methods and incubated with tetrandrine for 16 hr (peritoneal macrophages) or 6 hr (J774 cells). Medium was removed for PGE_2 analysis, and the wells then processed for cell viability measurements using the MTT assay. Results show mean ± SEM for 4–6 wells per treatment. Statistically significant changes compared to control (to which the tetrandrine vehicle was added), *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, respectively.

peritoneal macrophages, cell viability had fallen to less than half its initial value. At 24 hr, more than 90% of the cells had been killed. The release of PGE_2 showed an inverse profile (Fig. 3B): amounts present in the culture medium increased sharply between 2 hr and 4 hr, to reach a peak by 8 hr. In the case of J774 cells (Fig. 3C,D), there was a similar inverse relationship between cell death (40% within 1 hr) and generation of PGE_2 (already more than doubled within 1 hr).

A similar time profile of tetrandrine toxicity was observed for guinea-pig alveolar macrophages, except that the reduction in cell viability was precipitous, with more than 50% loss of viability within 1 hr (Fig. 4). PGE_2 release was not measured in this experiment.

To investigate the effects of tetrandrine on the generation of PGE_2 and NO caused by cell activation, mouse peritoneal macrophages were cultured in the presence of LPS or IFN-γ. These treatments induce COX-2 and iNOS enzymes. As expected, LPS caused an increase in both PGE_2 and NO release into the medium (Table 2). LPS also increased cell viability according to the MTT assay (Table 2). IFN-γ on its own caused an increased release of NO but did not enhance PGE_2 generation (Table 2), consistent with induction of iNOS, but not COX-2 (LH Pang and JRS Hoult, unpublished experiments). However, IFN-γ was strongly synergistic with LPS, causing greatly enhanced enzyme activities for both PGE_2 and NO release (Table 2).

In the presence of 10^{-5} M tetrandrine, there was a marked loss of cell viability to about one fifth after 16 hr, regardless of the treatment (Table 2), together with complete suppression of NO release. Although direct measurements of iNOS protein could not be made (due to loss of cell contents), it is assumed that the failure to release NO reflects absence of enzyme rather than its inhibition by

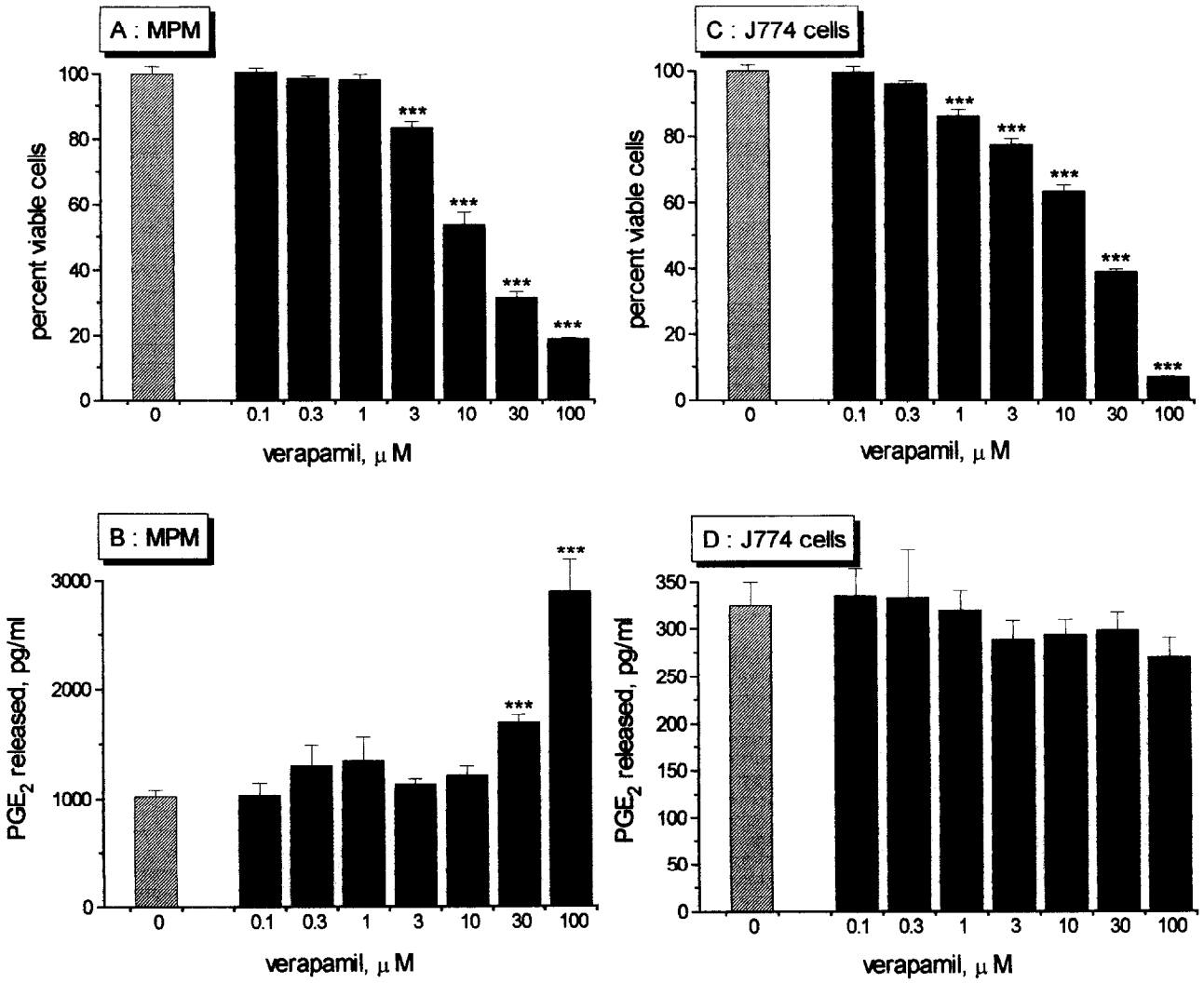

FIG. 2. Effect of verapamil on cell viability (A), (C) and PGE_2 release (B), (D) from mouse peritoneal macrophages and J774 cells. Cells were cultured with tetrandrine for 16 hr (peritoneal macrophages) or 6 hr (J774 cells). Results show mean ± SEM for 4 wells per treatment. Statistically significant changes compared to control, ***$P < 0.001$.

tetrandrine. In contrast, tetrandrine-treated cells released large amounts of PGE_2. The amounts were elevated for those treatments not associated with COX-2 induction (control, IFN-γ), but reduced somewhat for those in which COX-2 induction occurred (LPS + IFN-γ). Taken together, these results again show that tetrandrine has two effects on the prostaglandin system: to enhance prostaglandin output accompanying cell death but to inhibit its generation following cell activation. However, these experiments do not reveal whether the latter action is exerted by preventing the induction of cyclo-oxygenase rather than direct enzyme inhibition (or, indeed, whether the suppression of NO output has a similar explanation).

In an attempt to investigate if tetrandrine does exert any action to prevent the induction of COX-2 and iNOS, J774 macrophage-like cells were treated for a shorter period (8

TABLE 1. Effect of indomethacin on the actions of tetrandrine and verapamil on cell viability and PGE_2 release from mouse peritoneal macrophages

	Cell viability, %		PGE_2 release, pg/mL	
Treatment	**No addition**	**+Indo 10^{-6} M**	**No addition**	**+Indo 10^{-6} M**
Cells alone	100 ± 2.3	109.5 ± 1.2	1024 ± 63	668 ± 166*
+ Tetrandrine 10^{-5} M	32.4 ± 1.1	32.8 ± 1.5	3220 ± 734	1413 ± 72*
+ Verapamil 10^{-5} M	53.5 ± 4.1	55.0 ± 3.7	1700 ± 71	970 ± 31**

Results show mean ± SEM for 4–6 tests. Significantly inhibited compared to control,* $P < 0.05$; ** $P < 0.001$.

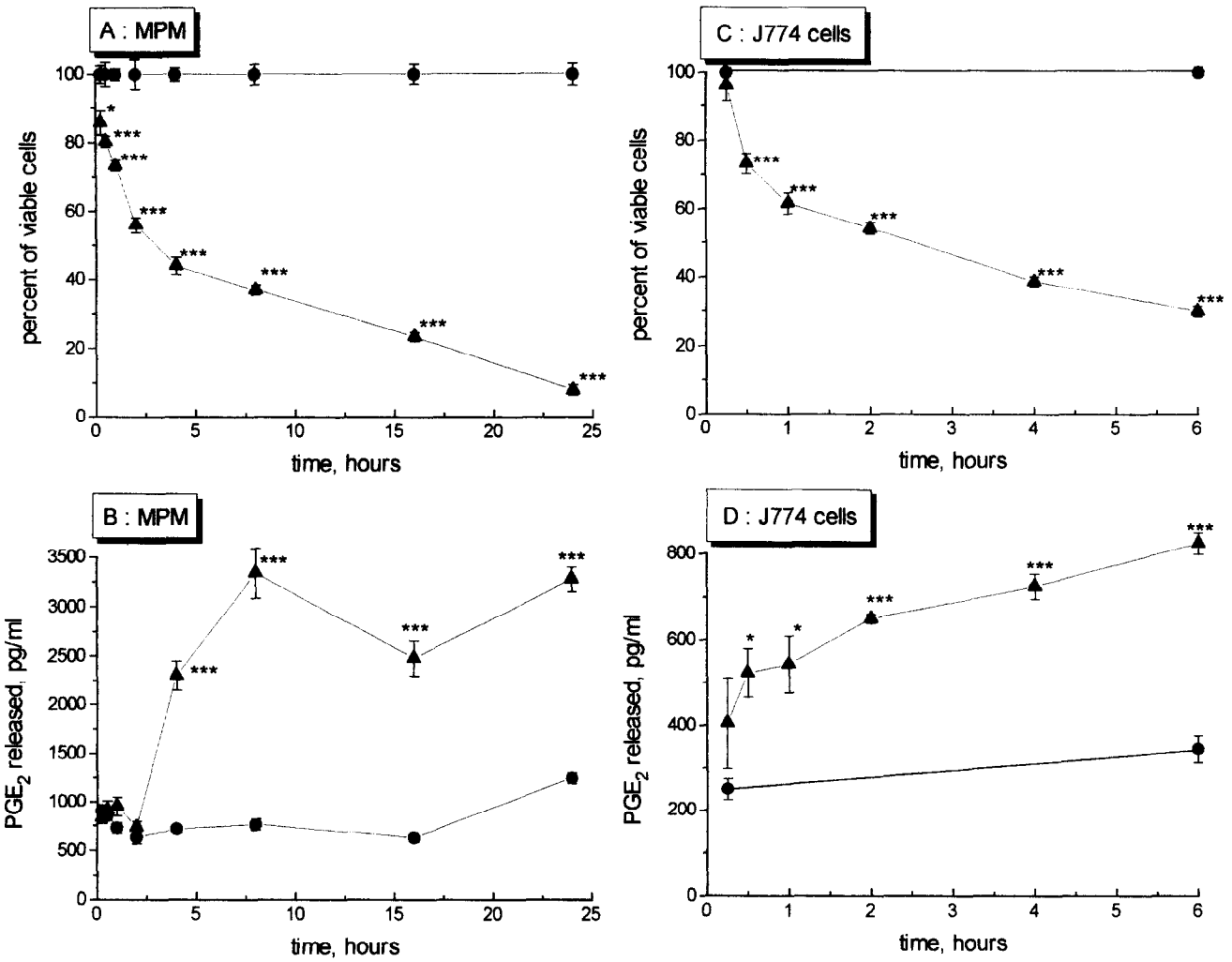

FIG. 3. Time course of cytotoxic effect and release of PGE_2 in response to 10^{-5} M tetrandrine in mouse peritoneal macrophages (A,B) and J774 cells (C,D). Results show mean ± SEM for 4–5 wells per treatment, (●) control cells treated with tetrandrine vehicle, (▲) tetrandrine. Statistically significant changes compared to vehicle-treated wells, *$P < 0.05$; ***$P < 0.001$.

hr) with both tetrandrine and verapamil at intermediate concentrations, while being exposed to LPS and interferon-γ to induce both enzymes (Table 3). As expected, both drugs caused a reduction in NO generation in cells treated with LPS + IFN-γ, but did not cause NO release on their own. Tetrandrine's effects on PGE_2 generation were similar to those observed above (reduction of induced PGE_2 release, biphasic effect when added alone), whereas verapamil produced a weak inhibition of induced release but had little action on its own (Table 3). Western blots of the protein extracts of the cells treated with LPS + IFN-γ showed that there were slight dose-dependent reductions in both iNOS and COX-2 proteins in response to both tetrandrine and verapamil, presumably reflecting the gradual loss of integrity of the cells (Fig. 5). Neither drug induced iNOS or COX-2 (Fig. 5, lanes G,H).

Possible direct effects of tetrandrine on COX activity were also tested in short-term experiments (1 hr), using J774 macrophages stimulated by calcium ionophore A23187, phorbol myristate acetate, and arachidonic acid. Figure 6 shows that 10^{-6} M to 10^{-4} M tetrandrine exerts a weak, but biphasic, effect on PGE_2 generation. Inhibition was maximal at 10^{-5} M, whereas amounts of released PGE_2 were greater than control at 10^{-4} M tetrandrine in 3 of the 4 test systems. A similar profile was evident when tetrandrine was incubated with arachidonic acid in cells exposed previously to LPS to induce COX-2 activity (Fig. 7). In this case, inhibition of COX activity by 10^{-5} M tetrandrine was only 29.0%, whereas that of 10^{-6} M indomethacin was 94.2%. In both sets of experiments, 10^{-4} M tetrandrine clearly caused greater release of PGE_2 than occurred at lower doses: this is most likely attributable to the onset of substantial cell toxicity within the 1-hr incubation, as would be predicted from Figs. 1C and 3C.

DISCUSSION

These experiments show that micromolar concentrations of tetrandrine, an alkaloid widely used to treat silicosis in China, are cytotoxic for macrophages, and that cell death is accompanied by the release of large quantities of PGE_2.

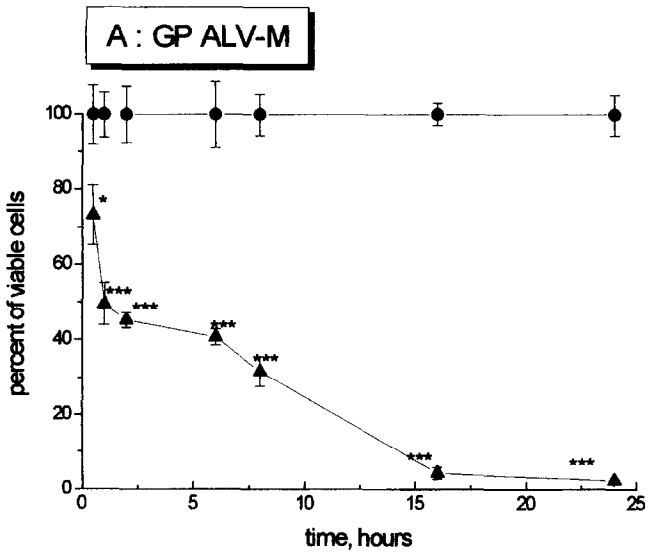

FIG. 4. Time-course of effect of 10^{-5} M tetrandrine on viability of guinea-pig alveolar macrophages. Results show mean ± SEM for 5 wells per treatment, (●) control cells, (▲) tetrandrine. Statistically significant changes compared to vehicle-treated control. *$P < 0.05$; ***$P < 0.001$.

Cytotoxicity has recently been demonstrated for several other plant-derived bisbenzylisoquinoline alkaloids under investigation as potential antiplasmodial and antiamoebic agents [13–15]. In general, they were found to be more toxic to *P. falciparum* than to mammalian cells [15]. However, it is interesting that, in a survey using 11 different mammalian cell lines to test the cytotoxic effects of 13 tetrandrine-like alkaloids from *Stephania erecta*, Likhitwitayawuid *et al.* [13] found that the macrophage-like cell line P388 was the most sensitive (the other cell lines were not of leukocyte lineage). In the companion study [14], the concentration of tetrandrine required to kill 50% of the P388 cells after 3 days was given as 0.8×10^{-6} M, compared to our value of 5×10^{-6} M required to kill 50% of the J774 macrophage-like cells after 16 hr. Taken together, these results suggest that macrophages are more sensitive than other kinds of mammalian cells to the cytotoxic actions of tetrandrine and related alkaloids, but they do not elucidate the mechanism. However, the fact that tetrandrine has been shown to interfere with calcium homeostasis (see Introduction) prompted us to compare its actions on macrophages with those of a classical calcium antagonist.

Verapamil also killed J774 and mouse peritoneal macrophages in dose-dependent fashion with a potency only slightly less than that of tetrandrine. Because verapamil is best known as a phenylalkylamine-type calcium channel antagonist with preferential action on L-type voltage-dependent slow-inactivating channels [16], it is tempting to ascribe its toxicity to this property, especially as it has already been shown that concentrations in the range 10^{-6} M to 10^{-4} M as used here can prevent the influx of calcium into macrophages (e.g. [17–20]). Nevertheless, at the higher concentrations, verapamil possesses other properties apparently unrelated to the reduction of gated calcium entry, such as the ability to inhibit macrophage cholesterol ester deposition [21, 22], antioxidant activity [23], and inhibition of the transporter responsible for multiple drug resistance [24, 25]. Thus, as for tetrandrine, the adverse effects of verapamil on macrophage viability cannot unambiguously be attributed to prevention of calcium access into the cells.

An unexpected finding was that tetrandrine caused a large increase in the release of PGE_2 into the culture medium of both mouse peritoneal macrophages and J774 cells, a property shared by the highest doses of verapamil in mouse peritoneal macrophages. It is clear, from our experiments, that prostaglandin overproduction occurs as a consequence of cell death and does not cause it (and may therefore be regarded as an epiphenomenon). Thus, inhibition of prostaglandin biosynthesis in mouse peritoneal macrophages with indomethacin did not affect drug-induced toxicity (Table 1). A similar separation between cell death and prostaglandin generation was obtained using J774 cells. For example, 1×10^{-7} M actinomycin D caused 80.6 ± 1.2% cell death after 6 hr incubation, and this was unaffected by cotreatment with 10^{-6} M indomethacin, which completely suppressed the PGE_2 overproduction (reduced from 1333 ± 17 pg/mL to 330 ± 23 pg/mL, similar to the basal output).

The mechanism for the prostaglandin overproduction is not known, but it was clearly not due to the induction of COX-2. This enzyme is induced in macrophages by various treatments, such as exposure to TNFα, interleukin-1, and bacterial lipopolysaccharide [26–28], explaining why these agents cause the release of large amounts of eicosanoids over similar time-courses as used in the toxicity experi-

TABLE 2. Effect of tetrandrine on cell viability, NO, and PGE_2 release from mouse peritoneal macrophages

	NO release, μM		Cell viability, %		PGE_2 release, pg/mL	
Treatment	**No addition**	**+Tet 10^{-5} M**	**No addition**	**+Tet 10^{-5} M**	**No addition**	**+Tet 10^{-5} M**
Cells alone	1.6 ± 0.3	nt	100 ± 5.0	22.1 ± 1.8	570 ± 30	5000 ± 200
LPS 1 μg/mL	2.5 ± 0.2	0.1 ± 0.1	175.8 ± 4.4	22.8 ± 1.3	7400 ± 200	10050 ± 450
LPS 10 μg/mL	5.4 ± 0.2	0.0 ± 0.0	167.5 ± 5.4	17.0 ± 4.0	10500 ± 0	8500 ± 500
IFNγ 100 U/mL	5.9 ± 0.1	0.2 ± 0.2	111.0 ± 3.2	24.5 ± 1.3	900 ± 100	5300 ± 500
LPS 1μg/mL + IFNγ	16.1 ± 0.2	0.1 ± 0.2	132.2 ± 10.7	25.2 ± 1.0	17500 ± 1500	7900 ± 170
LPS 10 μg/mL + IFNγ	18.1 ± 0.8	0.1 ± 0.0	120.3 ± 4.1	21.2 ± 3.7	20500 ± 1500	7400 ± 200

Cells were preincubated 2 hr with tetrandrine or vehicle, then incubated for a further 16 hr with stimulants. Values show mean ± SEM, 4 wells per treatment. nt = not tested.

TABLE 3. Effects of tetrandrine and verapamil on NO and PGE_2 release from J774 cells

Treatment	NO release, μM	PGE_2 release, pg/mL
Cells alone	0.7 ± 0.2	740 ± 20
LPS 500 ng/mL + IFNγ 50 units/mL	7.1 ± 0.2†	3900 ± 100†
LPS 500 ng/mL + IFNγ 50 units/mL + tetrandrine 10^{-6} M	5.1 ± 0.2§	4000 ± 100
LPS 500 ng/mL + IFNγ 50 units/mL + tetrandrine 10^{-5} M	4.3 ± 0.1‖	2500 ± 153‖
LPS 500 ng/mL + IFNγ 50 units/mL + verapamil 10^{-6} M	6.4 ± 0.6	3100 ± 208‡
LPS 500 ng/mL + IFNγ 50 units/mL + verapamil 10^{-5} M	3.4 ± 0.1‖	2000 ± 100‖
Tetrandrine 10^{-6} M	1.1 ± 0.4	3167 ± 145†
Tetrandrine 10^{-5} M	0.8 ± 0.3	1483 ± 17†
Verapamil 10^{-6} M	0.8 ± 0.1	693 ± 18
Verapamil 10^{-5} M	0.8 ± 0.1	873 ± 23*

Cells were preincubated 30 min with tetrandrine, verapamil, or vehicle, then incubated for a further 8 hr with LPS+IFNγ. Values show mean ± SEM, 3 wells per treatment. Statistically significant increase compared to cells alone, * $P < 0.05$; † $P < 0.001$. Significant inhibition compared to treatment with LPS+IFNγ, ‡ $P < 0.05$; § $P < 0.01$; ‖ P 0.001 (Students t-test).

ments here. It is supposed that the ongoing expression of new COX-2 enzyme allows the continued generation of large amounts of prostaglandin, the synthesis of which would otherwise be curtailed due to self-inactivation of the synthase enzyme during catalysis [29, 30].

Therefore, some other mechanism is needed to explain why dying macrophages release large amounts of PGE_2. Other observations on the activation of the arachidonate cascade in macrophages associated with drug-induced cytotoxicity [31–33] are consistent with the results shown here. For example, Peters-Golden and Shelly [31] showed that 10 μg/mL auranofin caused 67% toxicity (Trypan Blue staining) after just 90-min incubation with alveolar macrophages and this was associated with an 18-fold increase in PGE_2 release. In subsequent studies by other authors using mouse macrophages, it was shown that, although auranofin at low doses may inhibit the expression of cytokines that activate the arachidonate cascade, at higher toxic doses there is mobilization of arachidonic acid that may, thus, permit prostaglandin biosynthesis [32, 33]. Thus, it may be assumed that a consequence of drug-induced cytotoxicity in macrophages is activation of a phospholipase A_2 (PLA_2) enzyme within the cells leading to the release of arachidonic acid.

Both the cytosolic and secretory forms of PLA_2 are present in macrophages [34–39], and other PLA_2 isoforms may also be found within lysozomes [40]. However, it is not known how such enzyme(s) might become activated during a nonspecific process like cytolysis, or if it might be secondary to an event such as calcium influx into leaky cells (if, indeed, this can occur with drugs, such as tetrandrine and verapamil, that interfere with calcium mobilization).

Nevertheless, the implication is that the released arachidonic acid must be efficiently coupled to a constitutive form of cyclo-oxygenase that is not subjected to autodestruction. Efficient coupling of arachidonate to cyclo-oxygenase is not necessarily always the case because large amounts of exogenous arachidonic acid must be added to generate similar amounts of PGE_2 by macrophages in the absence of COX-2 induction (for example, Fig. 6 shows that the yield of PGE_2 following addition of 2×10^{-6} M arachidonic acid is approximately 350 pg/mL or less than 0.1%). Furthermore, treatment of macrophages with secretory types I, II, and III phospholipases A_2 does not lead to the generation of large amounts of PGE_2, even though there must be release of substantial amounts of arachidonic acid within the membrane (JRS Hoult, unpublished experiments).

We also investigated whether or not tetrandrine affects the L-arginine: NO system in macrophages, and found that it does not cause NO release *per se*, but strongly inhibits NO generation induced by LPS, especially in mouse peritoneal macrophages (Tables 2,3). Kondo *et al.* [12] also found that tetrandrine and other related alkaloids inhibit the generation of NO by LPS-stimulated mouse peritoneal macrophages, but claimed that this was due to suppression of NOS induction. Although it was stated that doses similar to those used here were not cytotoxic, no direct data was provided and Western blots of iNOS were not obtained. Thus, their interpretation of the effects of tetrandrine on NO generation must remain provisional, in view of our evidence that tetrandrine has a strong cytotoxic effect on macrophages without specifically suppressing iNOS induction.

Finally, it is relevant to consider these results in relation to the use of tetrandrine for the treatment of silicosis. It is believed that macrophage-derived TNF is a "decisive factor" in fibrotic lung diseases such as silicosis [41]. Thus, any agent that diminishes alveolar macrophage viability might

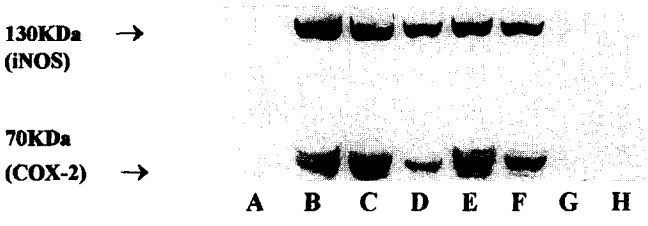

FIG. 5. Effects of tetrandrine and verapamil on the expression of iNOS and COX-2 in J774 cells. Lane A: control cells cultured for 8 hr; Lane B: 500 ng/mL LPS + 50 U/mL IFNγ for 8 hr; Lanes C,D: as B, but pretreated 30 min with tetrandrine at 10^{-6} M, 10^{-5} M; Lanes E, F as C,D but verapamil; Lanes G, H: 10^{-5} M tetrandrine or verapamil alone (without LPS + IFNγ). The positions and molecular weights of iNOS and COX-2 were validated by reference to molecular weight markers (Rainbow™, Amersham). These blots are representative of similar results obtained in independent experiments.

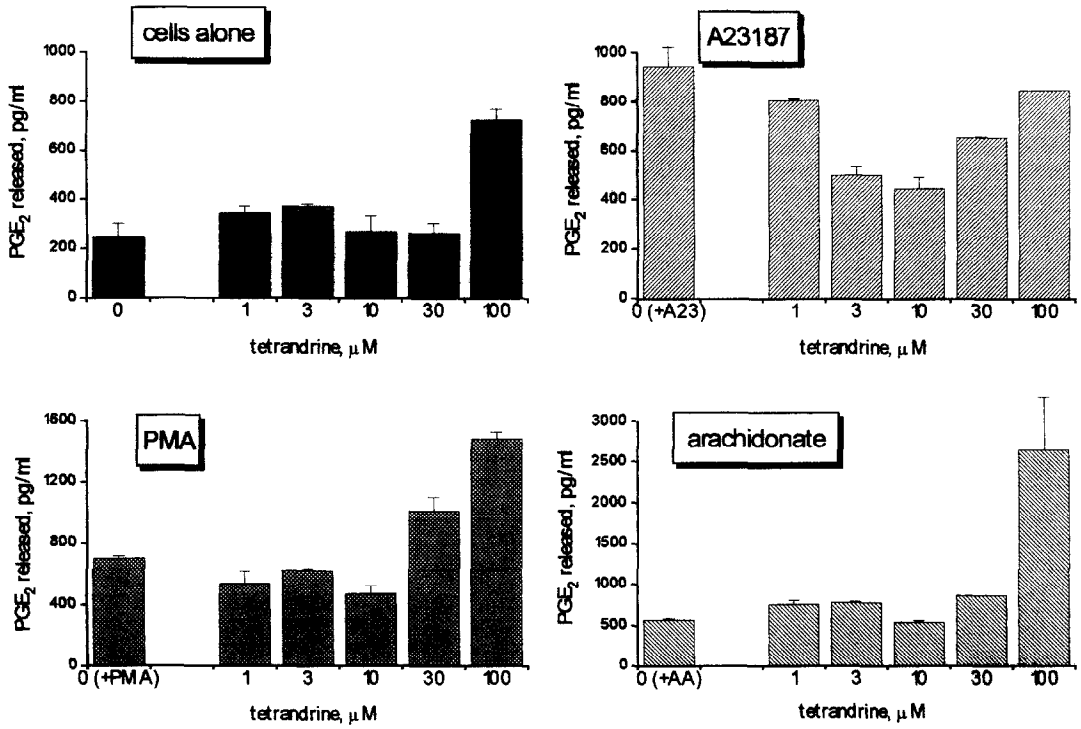

FIG. 6. Effect of tetrandrine on ability of J774 macrophages to generate eicosanoids when treated alone or with A23187 (10^{-5} M), PMA (5×10^{-6} M) or arachidonic acid (2×10^{-6} M). Cells were pretreated 30 min with tetrandrine, then 60 min with the stimulants.

indirectly improve the pathological progression of fibrosis by reducing cytokine-driven growth of fibroblasts and collagen deposition. It should also be noted that PGE_2 is well recognised for its ability to downregulate TNF production in macrophages [42–44]. Thus, tetrandrine might protect the lung from the damaging effects of silica particulates by effectively removing macrophages and the perpetuation of cell proliferation, both directly and *via* prostaglandins. It is therefore appropriate that efforts are being made to increase the specificity of the pharmaceutical delivery of tetrandrine to the lung to minimise its side effects on other organs [45] and to improve its activity against the destructive effects of phagocytic cells within the lung [46–48].

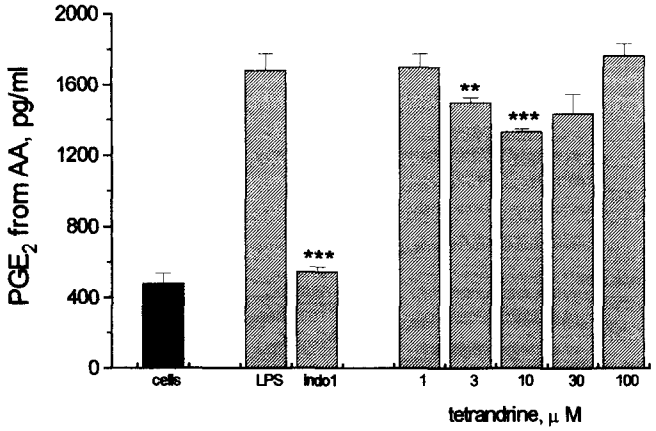

FIG. 7. Effect of tetrandrine on COX-2 activity induced in J774 macrophages. Cells were pretreated for 16 hr with 500 ng/mL LPS (or vehicle control), then washed 3 times in PBS before 30-min exposure to tetrandrine, indomethacin, or vehicle (concentrations are micromolar). Arachidonic acid was added at 10^{-6}M for 60 min and the resulting PGE_2 measured by radioimmunoassay. Results are mean ± SEM for 4 wells per treatment.

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