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Research paper

Induction of apoptosis in human leukemic U937 cells by tetrandrine

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Tetrandrine, a calcium channel antagonist, is a plant alkaloid possessing various pharmacological activities including anti-tumor activity. We studied tetrandrine to determine whether or not this anti-tumor effect occurs through induction of apoptosis. Tetrandrine inhibited both proliferation and clonogenicity of human leukemic U937 cells at an optimal concentration of 2.5 μg/ml. This growth inhibition was dose and time dependent, and accompanied with evidence of apoptotic changes. The characteristic morphological changes of apoptosis were observed in U937 cells under light microscopy and DNA fragmentation was noted by gel electrophoresis. Moreover, flow cytometric detection of surface phosphatidyl serine expression of U937 cells after treatment with tetrandrine confirmed the induction of apoptosis in these cells. The induction of apoptosis by tetrandrine would appear to proceed via non-Ca^{2+}-dependent pathways. [© 1998 Rapid Science Ltd.]

Key words: Apoptosis, leukemic cells, tetrandrine.

Introduction

Tetrandrine, a bisbenzylisoquinoline alkaloid isolated from the dried root of *Stephania tetrandra S. Moore*,[1] possesses a remarkable pharmacological profile. It has been shown to block calcium channels,[2] inhibit production of inflammatory mediators [interleukin-1, tumor necrosis factor (TNF)-α, TNF-β and NO] by monocytes/macrophages and lymphocytes,[3,4] and relax arterial smooth muscle in order to decrease blood pressure.[5] It is also capable of producing an anti-arrhythmic[6] and anti-ischemic effect,[7] and in the lung, an anti-fibrotic effect.[8].

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The anti-tumor growth effect of tetrandrine has been mentioned by a few investigators and the anti-proliferative effect on pulmonary fibroblasts *in vitro* was reported by Reist *et al.*[9] Some calcium influx inhibitors such as carboxyamido-triazole have been shown to inhibit the proliferation and invasiveness of human prostate tumor cell lines.[10] Collected together, these reports led us to hypothesize that tetrandrine, previously shown to be a calcium influx inhibitor, might possess anti-tumor activity or an anti-proliferative effect. Therefore, we used human leukemic U937 cells as the target of tetrandrine in this study and found that tetrandrine did possess anti-leukemic activity by inducing apoptosis in these cells.

Materials and methods

Preparation of tetrandrine

Tetrandrine ($C_{38}H_{42}O_6N_2$) was purchased from Aldrich (Milwaukee, WI). The powder was dissolved in diluted HCl (1 N) and then adjusted to pH 7.0 with 5 N NaOH, to achieve a concentration of tetrandrine of 2 mg/ml.

Cell culture and treatment

The human leukemia cell, U937, was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT) at 37°C in a humidified 5% CO_2 incubator. For the

study, the cells were incubated initially at 1×10^5/ml in the presence or absence of various concentrations (0.1–10.0 μg/ml) of tetrandrine. Analysis was performed at different times during culture of the cells, as described below.

Cell viability and growth kinetics

After treatment, cells were counted on days 1, 3 and 5. The percentage of viable cells was estimated by a Trypan blue dye exclusion test and cell growth curves were then analyzed based on the above data.

Assay for clonogenicity

Colony-forming activity of U937 cells was assessed in soft-agar culture as described by Wang *et al.*[11] Briefly, 1×10^3 U937 cells were plated in 0.3% agar in McCoy 5A medium (Gibco) containing 15% FCS and serially doubled concentrations of tetrandrine (0.625–10 μg/ml) were added while plating. These cultures were incubated for 14 days at 37 C with humidified 5% CO_2 and then counted for colonies (50 cells or more) under an inverted light microscope. Results of these experiments allowed us to choose an optimal concentration of tetrandrine for further studies.

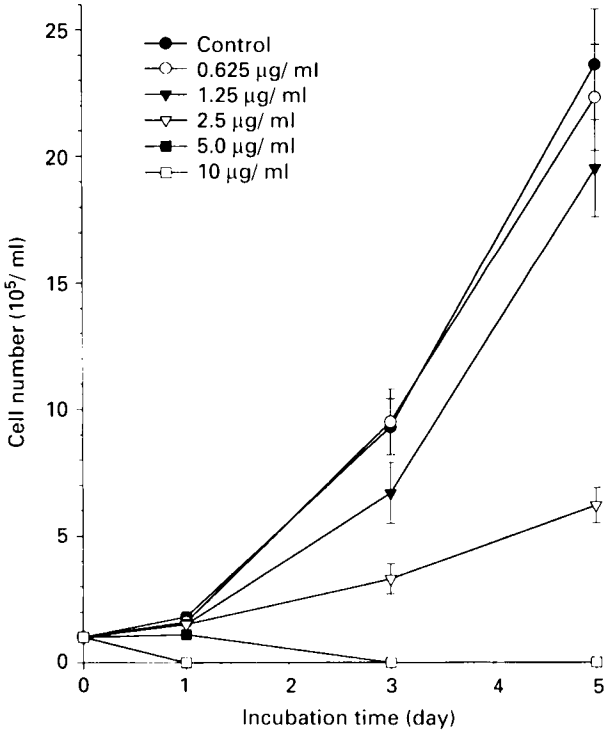

Figure 1. Growth curves of U937 cells with or without treatment. U937 cells (1×10^5/ml initially) were cultured for 5 days in the absence or presence of tetrandrine at various concentrations (0.625–10 μg/ml). Viable cells were counted on days 1, 3 and 5 of incubation. Data are the mean number of cells ± SEM from three separate experiments.

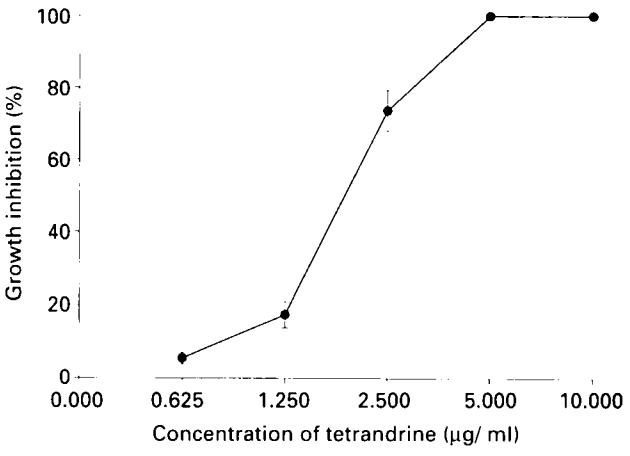

Figure 2. U937 cells were incubated for 5 days in the presence of tetrandrine at various concentrations (0.625–10 μg/ml). Viable cells were counted on day 5 and the growth inhibition rate was calculated. Results from three experiments are expressed as means ± SEM.

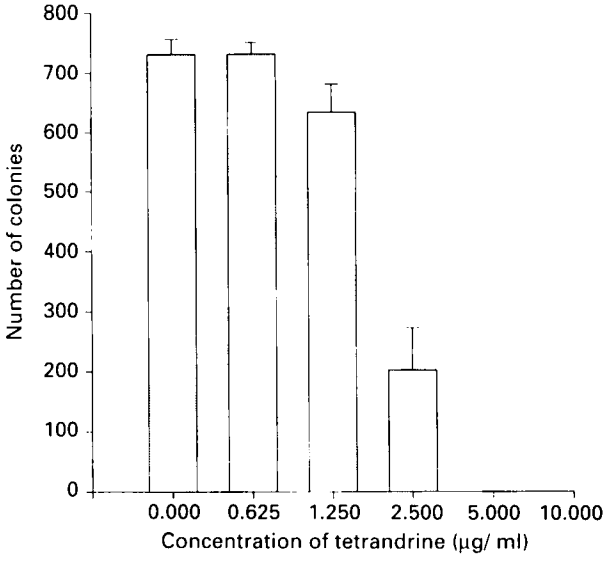

Figure 3. Inhibition of leukemic cell clonogenicity by tetrandrine. U937 cells were plated at 1×10^3 in 1 ml soft-agar culture in the presence of various concentrations of tetrandrine. Cultures were incubated for 14 days at 37 C and then scored for colonies. Data are the means ± SEM of three experiments.

Morphological examination

After 12 h of treatment, the cells were harvested and cytocentrifuged onto a microscope slide using a Cytospine^{2R} (Shandon Southern Instrument) and then stained with Wright's stain.[12] Morphological observation was performed with an Olympus microscopy at a magnification of × 1000.

DNA extraction and gel electrophoresis

DNA was extracted form U937 cells after incubation for 2–24 h in the presence or absence of 2.5 μg/ml tetrandrine using a salting out procedure.[13] Briefly, cells were washed twice in phosphate-buffered saline (PBS) and lysed overnight at 37°C in lysis buffer containing 20 mM Tris-HCl, pH 7.8, 10 mM EDTA, 1% sodium dodecylsulfate and 0.5 mg/ml proteinase K. After complete digestion, saturated NaCl was added to the cell lysates and shaken vigorously. Each sample was then centrifuged at 3000 r.p.m. for 15 min. An equal volume of ethanol was added to the supernatant for DNA precipitation. The DNA pellets were then air dried, dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.1 mg/ml RNase (Sigma, St Louis, MO) for 2 h at 37°C. Electrophoresis was performed on 1.5% agarose gels containing 1 μg/ml ethidium bromide in TBE buffer (90 mM Tris, pH 8.0, 90 mM boric acid and 2 mM EDTA). DNA fragments (200 bp DNA ladder) were visualized by UV fluororescence.

Flow cytometric detection of phosphatidylserine (PS) expression on U937 cells undergoing apoptosis

In addition to DNA laddering, apoptosis was further examined with an ApoAlert Annexin V Apoptosis Kit. This assay is based on the observation that, soon after

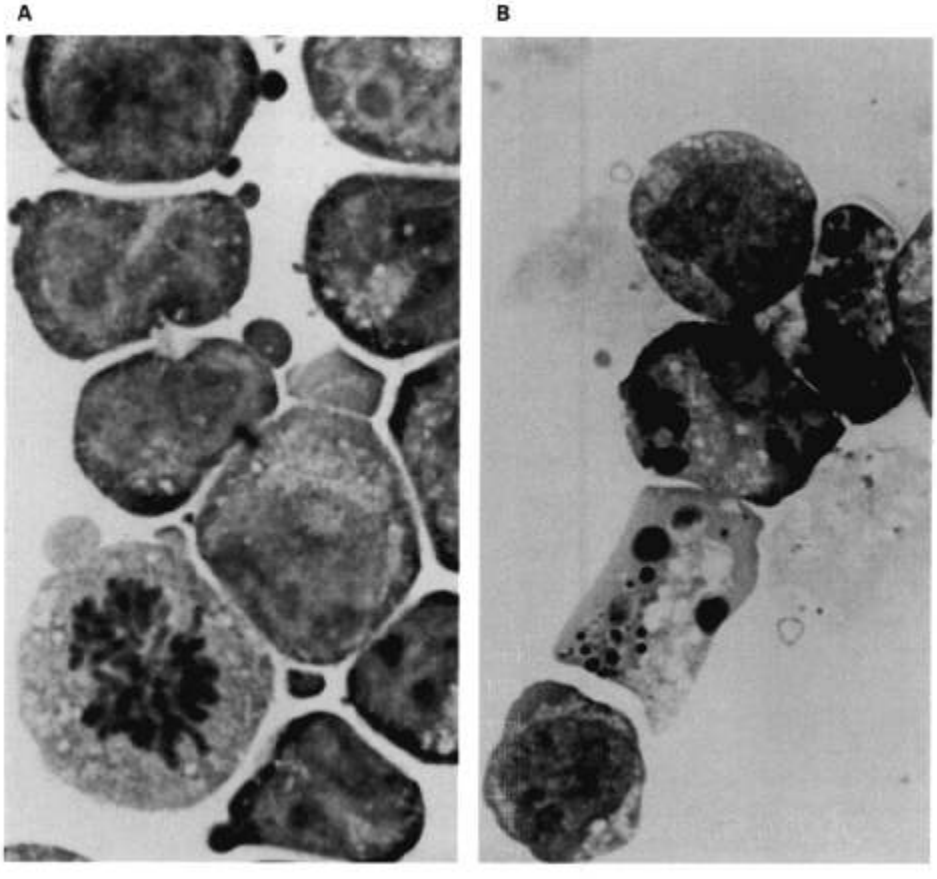

Figure 4. Morphologic features of U937 cells cultured with or without tetrandrine for up to 6 h, illustrating typical apoptotic changes. (A) Untreated U937 cells of slightly varying size show a rounded contour and no changes of cell death. One mitotic cell is noted in the lower left corner of the figure. (B) Cells undergoing apoptosis show a condensed and fragmented nucleus. Magnificiation × 1000.

initiating apoptosis, most cells translocate PS from the inner face of the plasma membrane to the cell surface.[14] This surface-located PS can be detected by FITC-conjugated annexin V.[15] Since externalization of PS occurs earlier than the nuclear changes, this method allowed detection of apoptotic cells earlier than DNA-based assays. For the procedures, the tested cells were collected, washed with PBS and suspended in binding buffer. Cell suspensions were then incubated for 15 min with Annexin V-FITC/propidium iodide (PI) and analyzed by flow cytometry with two-parameter light scatter detection (Becton Dickinson, Mountain View, CA).

Results and discussion

This study demonstrates that tetrandrine is capable of inhibiting the proliferation of human leukemic U937 cells at an optimal concentration of 2.5 μg/ml *in vitro*. This inhibition was accompanied with characteristics of programmed cell death including morphological changes, DNA fragmentation and surface PS expression.

Tetrandrine-induced inhibition of cell growth is shown in Figures 1 and 2. The decrease in viable cells was proportional to the time and dosage. According to the growth curves in Figure 1, 2.5 μg/ml of tetrandrine was chosen as an optimal concentration for further observations. Cell growth was completely inhibited by tetrandrine at higher concentrations (5 and 10 μg/ml).

After 14 days of incubation for clonogenicity, the U937 cells formed colonies with a plating efficiency of 731 ± 26 colonies per 1×10^3 cells in the control agar culture. In the tetrandrine-treated groups, the clonogenicity of U937 cells was inhibited in a dose-dependent manner. The formed colonies decreased from 730 ± 45 colonies per 1×10^3 with 0.625 μg/ml tetrandrine to 633 ± 47 and 202 ± 69 colonies per 1×10^3 cells with 1.25 and 2.5 μg/ml tetrandrine, respectively. Moreover, clonogenicity was completely suppressed by 5 and 10 μg/ml tetrandrine (Figure 3).

Untreated U937 cells are morphologically monoblast-like cells with a round cell contour, scant cytoplasm containing few granules and vacuoles, and an ovoid-shaped nucleus with indentations and some nucleoli (Figure 4A). After 6 h incubation with 5 μg/ml of tetrandrine, the treated U937 cells exhibited marked morphological changes showing the appearance of a large proportion of apoptotic cells which manifested several cytoplasmic blebs, condensed chromatin and apoptotic bodies containing dense micronuclear spheres (Figure 4B).

Treatment with tetrandrine (2.5 μg/ml) induced significant nuclear fragmentation in U937 cells. The DNA ladder shown by gel electrophoresis was noted after 2 h of treatment and became obvious at 6 h of incubation (Figure 5).

Flow cytometry analysis of 10 000 cells for PS expression demonstrated that the number of apoptotic cells noted increased with increasing lengths of exposure to tetrandrine (Figure 6). In untreated control cells, the percentage of apoptotic cells was $1.5 \pm 0.8\%$ and remained below 3% throughout the entire study period (24 h). This percentage increased to 12.9 ± 1.6, 22.2 ± 1.7 and $71.5 \pm 5.8\%$ after 6, 12 and 24 h of cultivation with 2.5 μg/ml tetrandrine, respectively (Table 1).

Ca^{2+} is an important second signal in the regulation of many cellular responses. For example, the increase in $[Ca^{2+}]_i$ with activation of Ca^{2+}-dependent endonuclease is responsible for internucleosomal cleavage of DNA in thymocytes.[16] However, Baffy *et al.* reported that the increase in $[Ca^{2+}]_i$ was not required in all cells.[17] Tetrandrine has previously been shown to be a Ca^{2+} channel blocker[2] and, in our study, was shown to induce apoptosis of U937 cells. These findings imply that the apoptosis-inducing effect of tetrandrine in U937 cells may not be dependent on activation of Ca^{2+}-dependent endonuclease but may target a functional site possibly mediated by a functional target site other than the Ca^{2+} channel. Which pathway tetrandrine affects in order to induce apoptosis remains to

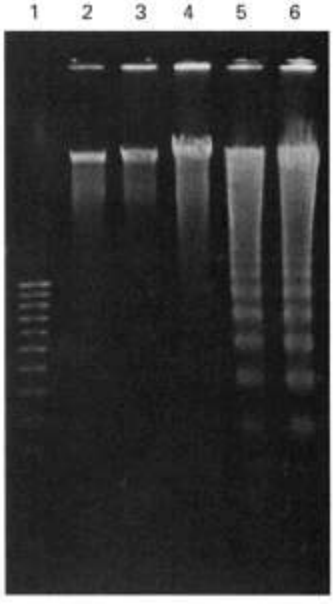

Figure 5. Time-course of tetrandrine-induced DNA fragmentation in U937 cells. Agarose gel electrophoresis of DNA extracted from U937 cells treated with 2.5 μg/ml tetrandrine for 2, 6, 12 and 24 h (lanes 3–6). Lane 1, DNA size marker; lane 2, control.

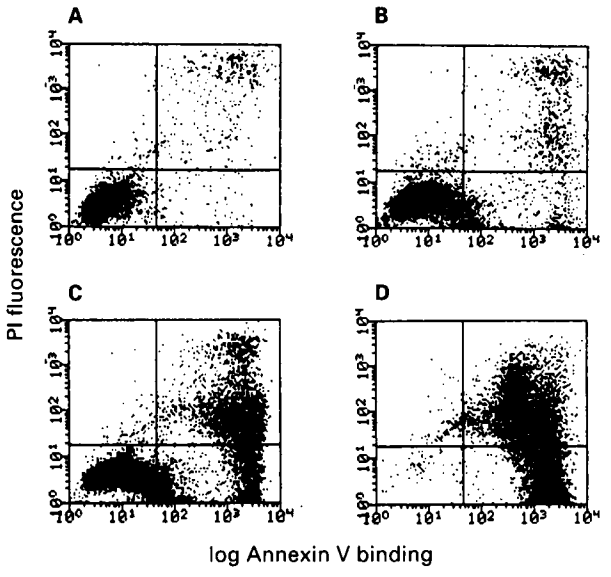

Figure 6. Flow cytometric analysis of apoptosis in U937 cells. U937 cells were treated with 2.5 μg/ml tetrandrine for 0 (A), 6 (B), 12 (C) and 24 (D) h. Cells were then collected and stained with PI and Annexin V–FITC. Apoptotic cells were stained positive for Annexin V–FITC and negative for PI whereas live cells were negative for both.

Table 1. Flow cytometric detection of apoptosis in U937 cells

Incubation time (h)	Control group (%)	Tetrandrine group (%)
0	1.5±0.8	1.6±0.6
6	1.8±0.9	12.9±1.6
12	1.9±0.6	22.2±1.7
24	2.3±1.1	71.5±5.8

U937 cells were incubated with or without 2.5 μg/ml tetrandrine for 0–24 h. Then the percentages of apoptotic cells were analyzed after cytometric detection of surface PS expression. Results from three experiments are expressed as mean percentages±SEM.

be determined—a question we intend to investigate in further studies.

Conclusion

Our findings suggest the following. (i) Tetrandrine inhibits proliferation and clonogenicity of human leukemic U937 cells, and induces them to initiate apoptosis. (ii) The optimal concentration of tetrandrine for both growth inhibition and apoptosis induction is 2.5 μg/ml. (iii) The apoptosis-inducing activity of tetrandrine may not be Ca^{2+} dependent.

References

1. Chen KK, Chen AL. The alkaloids of han-fang-chi. *J Biol Chem* 1935; **109:** 681–5.
2. Zernig D. Clinical future for calcium antagonists looks more promising. *Trends Pharmacol* 1991; **12:** 439–42.
3. Seow WK, Ferrante A, Summors A, Thong YH. Comparative effects of tetrandrine and berbamine on production of the inflammatory cytokines interleukin-1 and tumor necrosis factor. *Life Sci* 1992; **53:** PL453–8.
4. Kondo Y, Takano F, Hojo H. Inhibitory effect of bisbenylisoquinoline alkaloids on nitric oxide production in activated macrophages. *Biochem Pharmacol* 1993; **46:** 1887–92.
5. Zeng FD, Shaw DH, Ogilvie RI. Kinetic disposition and hemodynamic effects of tetrandrine in anesthetized dogs. *J Cardiovasc Pharmacol* 1985; **7:** 1034–9.
6. Dai GZ, Zeng B, Zhang YL, Lu YX. Intravenous tetrandrine in terminating acute episodes of paroxysmal supraventricular tachycardia. *J Cardiovasc Dis* 1986; **14:** 324–6.
7. Yu SL. Cardiothermographic study of the effects of tetrandrine on experimental myocardial ischemia and infarction. *Chinese Med J* 1986; **66:** 29–31.
8. Jin H. A study on the inhibition effect of tetrandrine on the pingyangmycinum induced pulmonary fibrosis. *Chinese J Tuber Respir Dis* 1991; **14:** 359–61.
9. Reist RH, Dey RD, Durham JP, Rojanasakul Y, Castranova V. Inhibition of proliferative activity of pulmonary fibroblasts by tetrandrine. *Toxicol Appl Pharmacol* 1993; **122:** 70–6.
10. William JW, Ann JP, Kenneth DS, *et al.* Effect of calcium influx inhibitor carboxyamido-trizole on the proliferation and invasiveness of human prostate tumor cell lines. *Int J Cancer* 1996; **68:** 259–64.
11. Wang SY, Hsu ML, Hsu HC, *et al.* The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int J Cancer* 1997; **70:** 699–705.
12. McDonald MJ. Modifications of the Wright-Giemsa stain for rapid staining. *Med Lab Sci* 1987; **44:** 88.
13. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16:** 1215.
14. Martin SJ, Reutelinsperger CPM, McGahon AJ, *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; **18:** 1545.
15. Koopman G, Reutelinsperger CPM, Kuitjen GAM, Keehnen RMJ, Pals ST, Van Oers MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; **84:** 1415–20.
16. Cohen JJ, Duke RC. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* 1984; **132:** 38–42.
17. Lennon SV, Klifeather SJ, Hallet MB, Campell TG, Cotter TG. Elevations in cytosolic calcium are not required for apoptosis in human leukemic cells. *Clin Exp Immunol* 1991; **87:** 465–71.

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