Apoptosis induced by (di-isopropyloxyphoryl-Trp)$_2$-Lys-OCH$_3$ in K562 and HeLa cells

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According to the method used in our laboratory, our group synthesized (DIPP-Trp)$_2$-Lys-OCH$_3$. It inhibited the proliferation of K562 and HeLa cells in a dose- and time-dependent manner with an IC$_{50}$ of 15.12 and 42.23 μM, respectively. (DIPP-Trp)$_2$-Lys-OCH$_3$ induced a dose-dependent increase of the G2/M cell population in K562 cells, and S cell population in HeLa cells; the sub-G0 population increased dramatically in both cell lines as seen by PI staining experiments using a FACS Calibur Flow cytometer (BeckmanCoulter, USA). Phosphatidylserine could significantly translocate to the surface of the membrane in (DIPP-Trp)$_2$-Lys-OCH$_3$-treated K562 and HeLa cells. The increase of an early apoptotic population was observed in a dose-dependent manner by both annexin-FITC and PI staining. It was concluded that (DIPP-Trp)$_2$-Lys-OCH$_3$ not only induced cells to enter into apoptosis, but also affected the progress of the cell cycle. It may have arrested the K562 and HeLa cells in the G$_2$/M, S phases, respectively. The apoptotic pathway was pulsed at this point, resulting in the treated cells entering into programmed cell death. (DIPP-Trp)$_2$-Lys-OCH$_3$ is a potential anticancer drug that intervenes in the signalling pathway.

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1. Introduction

Rational drug development in cancer therapy appears to concentrate on the discovery of effective pharmaceutical agents that can intervene in diverse signalling pathways (Johnstone *et al* 2002; Sausville *et al* 2003). Apoptosis is a highly regulated cell death process with characteristic biochemical features (Jacobson *et al* 1997; Nagata 1997) and membrane-bond apoptotic bodies (Kidd 1998). It occurs both during normal development and under certain pathological conditions in metazoans, and plays a crucial role in the maintenance of tissue homeostasis by the selective elimination of excessive cells (Thompson 1995; Nayfield *et al* 1991). Evasion of apoptosis is an essential hallmark of cancer (Hanahan and Weinberg 2000). Genetic changes resulting in loss of apoptosis or derangement of apoptosis-signalling pathways in the transformed cells are likely to be critical components of carcinogenesis (Kastan *et al* 1995; Schulte-Hermann *et al* 1997). Killing tumour cells through the induction of apoptosis has been recognized as a novel strategy for the identification of antitumour drugs and a valuable tool for cancer treatment (Smets 1994; Kornblau 1998).

Phosphorus plays a crucial role in life chemistry (Zhao *et al* 1995; Cheng *et al* 2004). Phosphoryl amino acids and phosphoryl amino acid esters, as phosphorus analogues of amino acid, have attracted much attention because they show wide biological activities, such as antibacterial and antitumour activities, and as inhibitors of specific enzyme activity (Cai *et al* 2006; Thiele 1992; Bertenshaw *et al* 1993;

Keywords. Apoptosis; cell cycle; HeLa; K562; N-phosphoryl dipeptide methyl ester

Abbreviations used: Apaf-1, apoptotic protease activating factor-1; (DIPP-Trp)$_2$-Lys-OCH$_3$, (di-isopropyloxyphoryl-Trp)$_2$-Lys-OCH$_3$; FBS, foetal bovine serum; IC$_{50}$, inhibitory concentration 50%; IR%, inhibition rate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PS, phosphatidylserine

Kortylewicz 1990). It has been reported that some peptide methyl esters could induce apoptosis but not effectively (Reeves 1997; Thiele and Lipsky 1985). Based on the above study, the configuration of some dipeptides were modified, a series of N-phosphoryl dipeptide methyl esters was synthesized and their biological activities in cancer cells were studied. In our previous report, we investigated a kind of N-phosphoryl branched dipeptide, which could induce apoptosis in HCT-15 cells (Niu *et al* 2001). This paper focuses on (di-isopropyloxyphoryl-Trp)$_2$-Lys-OCH$_3$ ([DIPP-Trp]$_2$-Lys-OCH$_3$), which has the best biological activities among these compounds, inhibiting cell proliferation and inducing apoptosis as seen by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and flow cytometric analysis in K562 and HeLa cells, respectively.

2. Materials and methods

2.1 *Materials*

A human chronic myelogenous leukaemia cell line (K562) and cervical adenocarcinoma cell line (HeLa) were obtained from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Powdered Dulbecco modified eagle medium, RPMI-1640 medium and trypsin solution were purchased from GIBCO (Grand Island, NY, USA). Foetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from Hyclone Laboratories, Inc. Propidium iodide (PI) and MTT were purchased from Sigma Chemical Co. An annexin V-FITC apoptosis detection kit was purchased from CLONTECH Company. (DIPP-Trp)$_2$-Lys-OCH$_3$ (figure 1) was synthesized using the method previously described (Ji *et al* 1988; Zhao *et al* 2004). It was dissolved in methanol and diluted to different concentrations with PBS.

2.2 *Cell culture and drug treatment*

K562 and HeLa cells were cultured in RPMI-1640 and DMEM medium, respectively, with 10% FBS, 100 UI/ml penicillin and 100 μg/ml streptomycin in humidified air at 37°C with 5% CO_2. Exponentially growing K562 and HeLa cells were collected and re-suspended in fresh medium for 4 h and then exposed to various concentrations of (DIPP-Trp)$_2$-Lys-OCH$_3$.

2.3 *MTT assay*

Survival of cells was evaluated by using a system based on MTT, which was reduced by living cells to yield a soluble formazan product that could be detected colorimetrically. Cells were suspended in 96-well plates of 90 μl medium at a density of 2×10^4 cells/well and 10 μl (DIPP-Trp)$_2$-Lys-OCH$_3$ in different concentrations. These were then incubated in humidified air at 37°C with 5% CO_2 for 24 h, exposed to 10 μl MTT (5 mg/ml) and incubated for another 4 h under the conditions mentioned above. The formazan precipitate was dissolved in 100 μl DMSO. IC_{50} values were tested through the MTT method (Mosmann 1983). Negative and positive control wells were treated with 1% methanol and 50 μg/ml cisplatin, respectively. The inhibition rate (IR%)

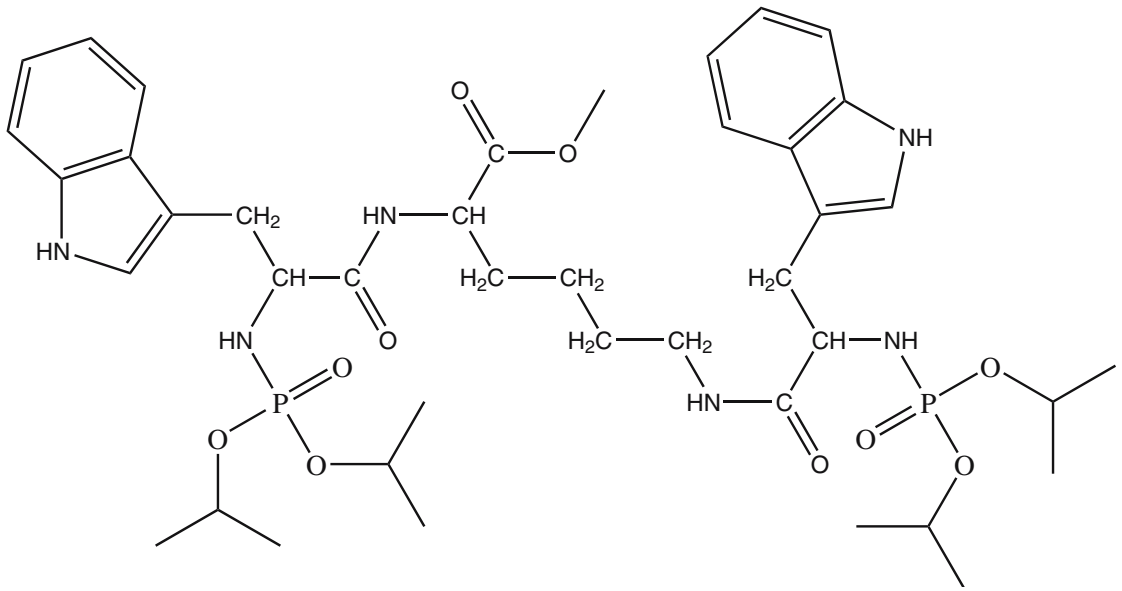

Figure 1. Structure of (DIPP-Trp)$_2$-Lys-OCH$_3$.

was calculated as follows: IR% = (mean control absorbance – mean experimental absorbance)/mean control absorbance × 100%.

2.4 *Flow cytometry analysis*

Cell pellets were fixed in 70% ethanol at –20°C for at least 12 h or overnight. After being washed twice with ice-cold PBS, they were incubated in RNase A/PBS (1 mg/ml) at 37°C for 30 min, and stained with PI (0.5 mg/ml) at room temperature for 15 min. The intracellular DNA was then labelled with PI and the PI fluorescence of individual nuclei determined by a FACSCalibur fluorescence-activated cell sorter at 488 nm excitation. Surface exposure of phosphatidylserine in apoptotic cells was measured by the annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Additional exposure to PI made it possible to differentiate the early apoptotic cells (annexin V-positive/PI-negative) from the late apoptotic cells (annexin V-positive and PI-positive).

3. Results

3.1 *Proliferation of K562 and HeLa cells were inhibited by (DIPP-Trp)$_2$-Lys-OCH$_3$*

The inhibitory effects of (DIPP-Trp)$_2$-Lys-OCH$_3$ on the proliferation of K562 and HeLa cells were tested at different concentrations for 24 h and the IR% was determined (figure 2A). (DIPP-Trp)$_2$-Lys-OCH$_3$ induced a dose-dependent inhibitory effect. Nearly 80%, 85% of K562 and HeLa cells were inhibited by 100 μM (DIPP-Trp)$_2$-Lys-OCH$_3$ (80% of K562 and 85% of HeLa cells). The inhibitory concentration 50% (IC_{50}) was about 15.12 and 42.23 μM, respectively. Inhibition of K562 cell proliferation by 50 μM (DIPP-Trp)$_2$-Lys-OCH$_3$ and of HeLa cells by 100 μM (DIPP-Trp)$_2$-Lys-OCH$_3$ was studied at different time periods (figure 2B). The diminution in cell numbers by (DIPP-Trp)$_2$-Lys-OCH$_3$ was time-dependent. Compared with HeLa cells, inhibition by (DIPP-Trp)$_2$-Lys-OCH$_3$ of K562 cell proliferation was more effective. These results demonstrated that the cell numbers were decreasing on treatment.

After the K562 cells were exposed to (DIPP-Trp)$_2$-Lys-OCH$_3$ (40 μM) for 6 h, the (DIPP-Trp)$_2$-Lys-OCH$_3$ was extracted. There was almost no cell growth after re-feeding with fresh medium in plates free of (DIPP-Trp)$_2$-Lys-OCH$_3$ (figure 3A). The same result was obtained after HeLa cells were treated with (DIPP-Trp)$_2$-Lys-OCH$_3$ (80 μM) for 12 h, followed by removal of (DIPP-Trp)$_2$-Lys-OCH$_3$ (figure 3B). The re-feeding experiments indicated that (DIPP-Trp)$_2$-Lys-OCH$_3$ was more cytotoxic than cytostatic for both the K562 and HeLa cell lines. The cell numbers decreased on treatment.

3.2 *Cell cycle analysis*

To determine whether the K562 and HeLa cells treated with (DIPP-Trp)$_2$-Lys-OCH$_3$ undergo the apoptosis pathway, the cell distribution in the cell cycle was examined by PI

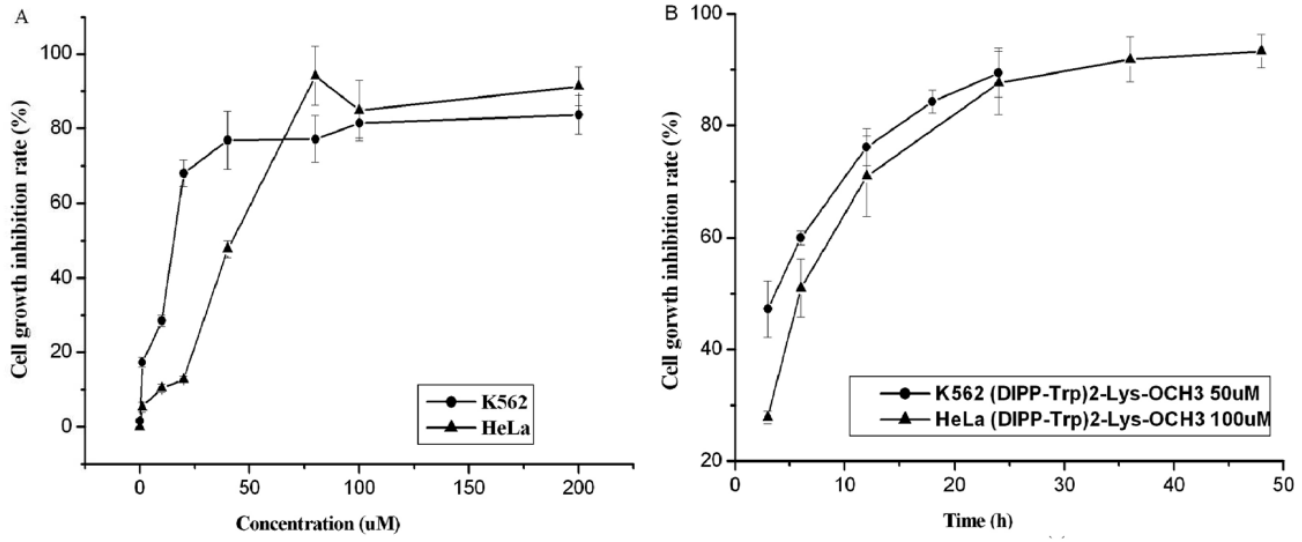

Figure 2. Effect of (DIPP-Trp)$_2$-Lys-OCH$_3$ on cell proliferation. (**A**) K562 and HeLa cells were treated with different concentrations of (DIPP-Trp)$_2$-Lys-OCH$_3$. (**B**) Cells were treated for different time periods. The viability was evaluated by MTT assay. Each IR% represents the mean value of three independent experiments. Values are expressed as mean ± SEM.

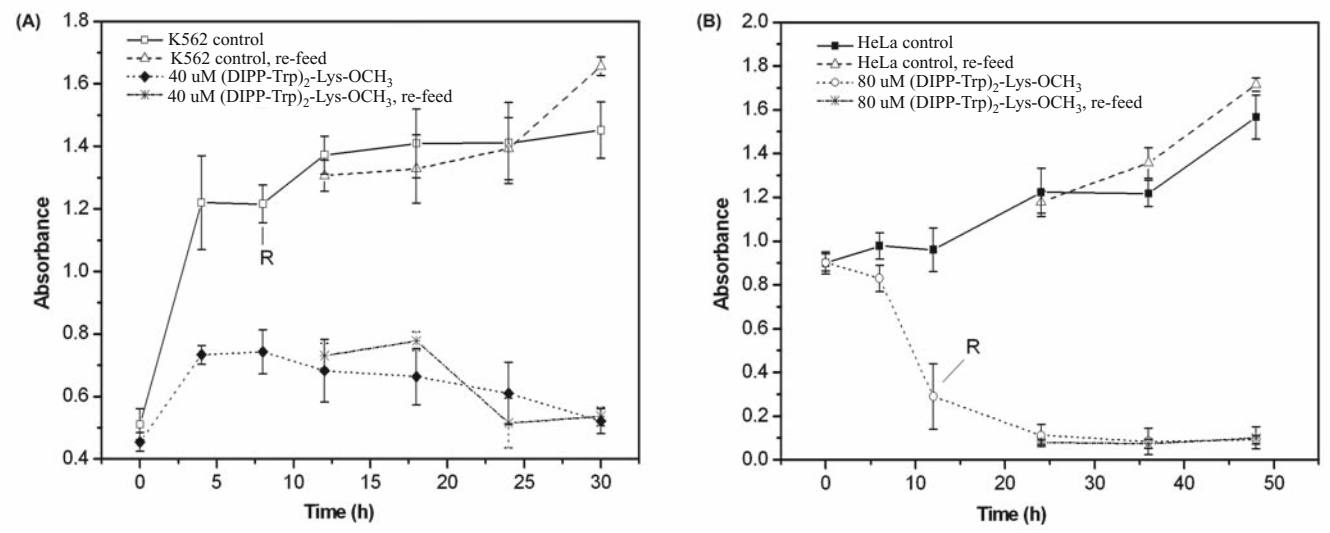

Figure 3. Periodic analysis of (DIPP-Trp)$_2$-Lys-OCH$_3$ (**A**, 40 μM; **B**, 80 μM) against K562 (**A**) and HeLa (**B**) cells with re-feeding of fresh medium in half of the plates. Values are expressed as an average absorbance ± SEM (n=3 for both control and treated cells). The "R" depicts when re-feeding was initiated.

staining. (DIPP-Trp)$_2$-Lys-OCH$_3$ induced a dose-dependent increase of the cell population in the G2/M phase in K562 cells, and S phase cell population in HeLa cells (figure 4). A dramatic hypodiploid (sub-G0) population (24.6%) was also observed after K562 cells were treated with 20 μm of (DIPP-Trp)$_2$-Lys-OCH$_3$ (figure 4A). For HeLa cells, the hypodiploid peak was about 20% after treatment with 10 μm of (DIPP-Trp)$_2$-Lys-OCH$_3$ (figure 4B).

During the rise in concentration from 0 to 20 μM, a decrease in polyploid cells and a reciprocal increase in the sub-G0 population were associated with each other, indicating that the arrested cells entered into apoptosis. The K562 and HeLa cells were arrested in the G2/M and S phases, respectively, during the course of growth inhibition (figure 4C).

3.3 *Flow cytometry analysis of cell apoptosis*

The hallmark of early apoptotic cells is the transverse redistribution of plasma membrane phosphatidylserine (PS) (Van Engeland *et al* 1998); thus, the annexin V binding assay was performed to detect the surface exposure of PS. Figure 5 shows the FACS histogram with dual parameters including annexin V-FITC and PI. In untreated K562 cells, 7.1% of cells were annexin V-positive/PI-negative, 7.1% of cells were both annexin V- and PI-positive. The annexin V-positive/PI-negative and double-positive cells increased to 73.9% and 11.3%, respectively, after being treated with (DIPP-Trp)$_2$-Lys-OCH$_3$ at a concentration of 20 μM for 24 h (figure 5A). For HeLa cells, the annexin V-positive/PI-negative and double-positive cells were 49.07% and 23.26%, respectively, after the cells were treated with (DIPP-Trp)$_2$-Lys-OCH$_3$ at the same concentration for 24 h. Among the control cells, only 13.26% were annexin V-positive/PI-negative and 14.34% double-positive (figure 5B).

4. Discussion

Apoptosis is a highly regulated cell death process with characteristic biochemical features, which help in differentiating apoptotic cells from non-apoptotic ones. Our group synthesized (DIPP-Trp)$_2$-Lys-OCH$_3$ using the method described previously. We then concluded that (DIPP-Trp)$_2$-Lys-OCH$_3$ induces apoptosis in K562 and HeLa cells through biochemical experiments.

The inhibitory effects of (DIPP-Trp)$_2$-Lys-OCH$_3$ on the proliferation of K562 and HeLa cells were tested. It inhibited the proliferation of K562 and HeLa cells in a time- and dose-dependent manner. The IC$_{50}$ was about 15.12 and 42.23 μM, respectively. The differential activity of the compound on both cell lines represented the selectivity of its antitumour action.

As shown in the cell cycle analysis, the sub-G0 population increased dramatically at a concentration of 20 μM of (DIPP-Trp)$_2$-Lys-OCH$_3$, which has been suggested to be the apoptotic DNA, while the population of G$_2$/M increased in a dose-dependent manner in treated K562 cells stained by PI; the population of cells in other phases of the cell cycle remained unaffected. Mitosis in cells takes place in the G2/M phase during the cell cycle, and the results mentioned

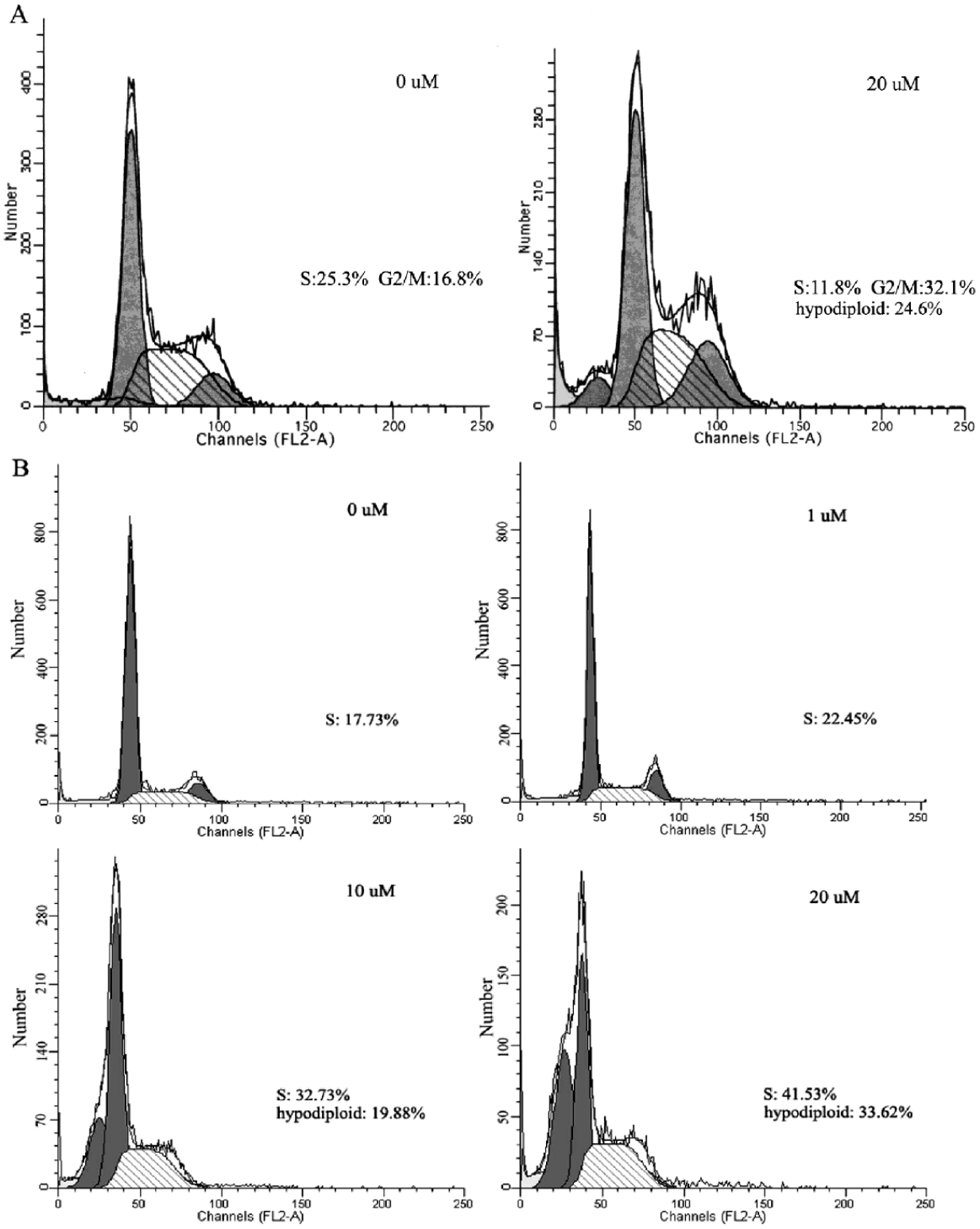

Figure 4A,B. For caption, *see* page No. 60.

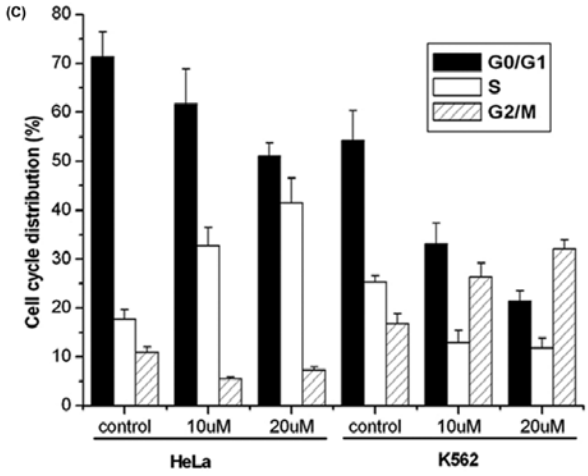

Figure 4. Effect of $(DIPP\text{-}Trp)_2\text{-}Lys\text{-}OCH_3$ on cell cycle distribution in K562 (**A**) and HeLa (**B**) cells. Cells were treated with different concentrations of $(DIPP\text{-}Trp)_2\text{-}Lys\text{-}OCH_3$ (0, 20 μM for K562 [**A**], 0, 1, 10, 20 μM for HeLa [**B**] cells) for 24 h. The percentages of cell cycle distribution were then evaluated by PI staining and flow cytometric analysis as described in the section on Materials and methods. (**C**) Histogram representing the cell cycle distribution of cells after treatment with $(DIPP\text{-}Trp)_2\text{-}Lys\text{-}OCH_3$. Results are representative of three independent experiments. Values are expressed as mean ± SEM.

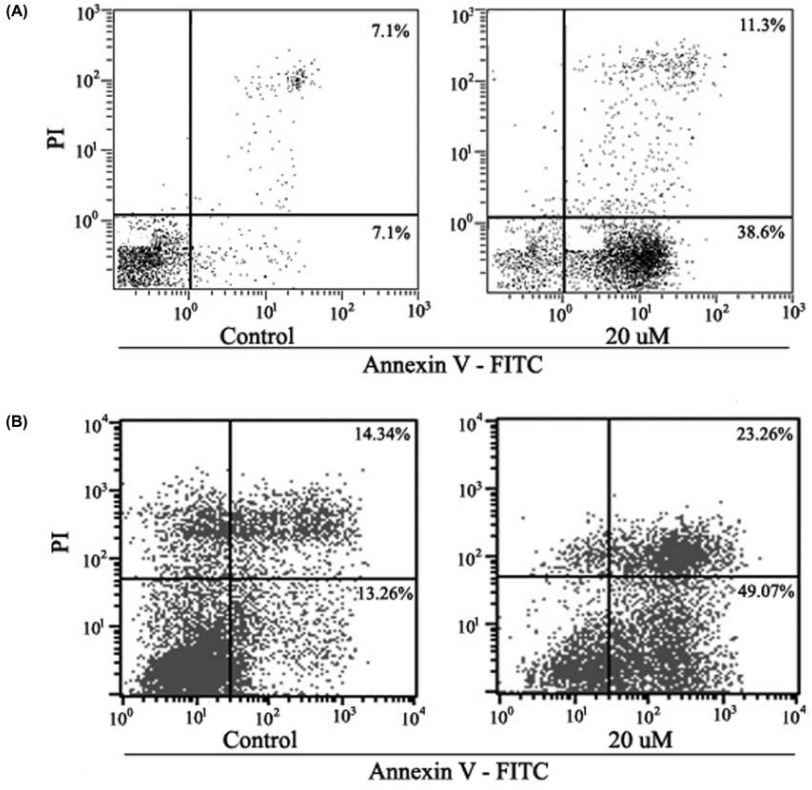

Figure 5. Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining) in K562 (**A**) and HeLa (**B**) cells undergoing apoptosis. The cells were treated with $(DIPP\text{-}Trp)_2\text{-}Lys\text{-}OCH_3$ (20 μM) for 24 h. The dual parametric dot plots combining annexin V-FITC and PI fluorescence show the viable cell population in the lower left quadrant (annexin V^-PI^-), the early apoptotic cells in the lower right quadrant (annexin V^+ PI^-), and the late apoptotic cells in the upper right quadrant (annexin V^+ PI^+). Results are representative of three independent experiments.

above demonstrated that mitosis-related components may be the target of (DIPP-Trp)$_2$-Lys-OCH$_3$ in K562 cells. In HeLa cells, the sub-G0 population also increased dramatically at a concentration of 10 μM of (DIPP-Trp)$_2$-Lys-OCH$_3$, while the S population increased in a dose-dependent manner as seen after staining with PI; the population in the G0/G1, G2/M phases remained unaffected. The S phase checkpoint arrest suggests the possibility that DNA synthesis was blocked by (DIPP-Trp)$_2$-Lys-OCH$_3$ in HeLa cells. It may cross-link with DNA directly or interact with DNA polymerase to inhibit DNA synthesis. (DIPP-Trp)$_2$-Lys-OCH$_3$ arrested the K562 and HeLa cells in the G2/M and S phases, respectively. A previous study described this phenomenon as the development of an aberrant mitotic exit into a G1-like "multinucleate state", which eventually progressed to apoptosis (Jordan *et al* 1996).

The transverse redistribution of plasma membrane PS is the hallmark of early apoptotic cells. Our work demonstrated that PS could significantly translocate to the surface of the membrane in (DIPP-Trp)$_2$-Lys-OCH$_3$-treated K562 and HeLa cells. Early apoptotic cells were observed by both annexin V-FITC and PI staining. The early apoptotic cell population increased in a dose-dependent manner with (DIPP-Trp)$_2$-Lys-OCH$_3$. There are two major pathways in apoptosis that have been elucidated to date. One is the mitochondrial pathway in which cytochrome c is released into the cytosol, where it binds to apoptotic protease activating factor-1 (Apaf-1) to activate procaspase-9 (Li *et al* 1997). Another apoptosis pathway is the ligation of death receptors such as Fas and TNFR1 and subsequent activation of caspase-8 to trigger apoptosis (Nagata 1997). In our previous study, another dipeptide was found to induce apoptosis of K562 cells through the mitochondrial pathway (Yang *et al* 2006). The pathway by which the apoptosis in K562 or HeLa cells was induced by (DIPP-Trp)$_2$-Lys-OCH$_3$ should be investigated in the future. Taken together, we conclude that (DIPP-Trp)$_2$-Lys-OCH$_3$ induces cells into an apoptotic pathway resulting in the inhibition of proliferation of K562 and HeLa cells. It may have promise as a new type of short peptide in cancer therapy.

Acknowledgements

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References

Bertenshaw S R, Rogers R S, Stern M K, Norman B H, Moore W M, Jerome G M, Branson L M, McDonald J F, McMahon E G and Palomo M A 1993 Phosphorus-containing inhibitors of endothelin converting enzyme: effects of the electronic nature of phosphorus on inhibitor potency; *J. Med. Chem*. **36** 173–176

Cai Y M, Gao X, Huang X T, Wang T J and Zhao Y F 2006 Synthesis, spectral characterization and biological activities of phosphoryl amino acid esters; *Chinese J. Org. Chem*. **26** 1677–1681

Cheng C M, Liu X H, Li Y M, Ma Y, Tan B, Wan R and Zhao Y F 2004 N-phosphoryl amino acids and biomolecular origins; *Origins Life Evol. Biosphere* **34** 455–464

Hanahan D and Weinberg R A 2000 The hallmarks of cancer; *Cell* **100** 57–70

Jacobson M D, Weil M and Raff M C 1997 Programmed cell death in animal development**;** *Cell* **88** 347–354

Ji G J, Xue C B, Zeng J N, Li L P, Chai W G and Zhao Y F 1988 Synthesis of N-(diisoproxy phosphoryl) amino acids and peptide; *Synthesis* **6** 444–448

Johnstone R W, Ruefli A A and Lowe S W 2002 Apoptosis: a link between cancer genetics and chemotherapy; *Cell* **108** 153–164

Jordan M A, Wendell K, Gardiner S, Derry W B, Copp H and Wilson L 1996 Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death; *Cancer Res.* **56** 816–825

Kastan M B, Canman C E and Leonard C J 1995 P53, cell cycle control and apoptosis: implications for cancer; *Cancer Metastasis Rev.* **14** 3–15

Kidd V J 1998 Proteolytic activities that mediate apoptosis; *Annu. Rev. Physiol.* **60** 533–573

Kornblau S M 1998 The role of apoptosis in the pathogenesis, prognosis, and therapy of hematologic malignancies; *Leukemia* **12** S41–S46

Kortylewicz Z P 1990 Phosphoramidate peptide inhibition of human skin fibroblast collagenase; *J. Med. Chem*. **33** 263–273

Li P, Nijhawan D, Budihardjo I, Srinivasula S M, Ahmad M, Alnemri E S and Wang X D 1997 Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade; *Cell* **91** 479–489

Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytoxicity assays; *J. Immunol. Methods* **65** 55–63

Nagata S 1997 Apoptosis by death factor; *Cell* **88** 355–365

Nayfield S G, Karp J E, Ford L G, Dorr F A and Kramer B S 1991 Potential role of tamoxifen in prevention of breast cancer; *J. Natl. Cancer Inst.* **83** 1450–1459

Niu Y L, Du W, Jiang Y Y, Cao S L and Zhao Y F 2001 Apoptosis of HCT-15 cell lines induced by a kind of N-phosphoryl branched dipeptide; *J. Tsinghua Univ.* **7** 101–103 (S1)

Reeves J P 1979 Accumulation of amino acids by lysosomes incubated with amino acid methyl esters; *J. Biol. Chem.* **254** 8914–8921

Sausville E A, Elsayed Y, Monga M and Kim G 2003 Signal transduction-directed cancer treatments; *Annu. Rev. Pharmacol. Toxicol.* **43** 199–231

Schulte-Hermann R, Bursch W, Low-Baselli A, Wagner A and Grasl-Kraupp B 1997 Apoptosis in the liver and its role in hepatocarcinogenesis; *Cell. Biol. Toxicol.* **13** 339–348

Smets A 1994 Programmed cell death (apoptosis) and response to anti-cancer drugs; *Anticancer Drugs* **5** 3–9

Thiele D L 1992 Apoptosis is induced in cells with cytolytic potential by L-leucyl-L-leucine methyl ester; *J. Immunol.* **148** 3950–3957

Thiele D L and Lipsky P E 1985 Modulation of human natural killer cell function by L-leucine methyl ester: monocyte-dependent depletion from human peripheral blood mononuclear cells; *J. Immunol.* **134** 786–793

Thompson C B 1995 Apoptosis in the pathogenesis and treatment of disease; *Science* **267** 1456–1462

Van Engeland M, Nieland L J W, Ramaekers F C S, Schutte B and Reutelingsperger C P M 1998 Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure; *Cytometry* **31** 1–9

Yang J, Jiang Y Y, Liu F and Zhao Y F 2006 Mitochondria-regulated death pathway mediates (DIPP-L-Leu)(2)-L-LysOCH(3)-induced K562 cells apoptosis; *Protein Peptide Lett.* **13** 129–134

Zhao Y F, Ju Y, Li Y M, Wang Q, Yin Y W and Tan B 1995 Self-activation of N-phosphoamino acids and N-phosphodipeptide in oligopeptide formation; *Int. J. Peptide Protein Res.* **45** 514–518

Zhao Y F, Zhang J C, Cao S X, Xu J, Rong C L and Qu L B 2004 Synthesis of N-(diisopropyloxyphosphoryl) amino acids; *Chinese J. Org. Chem*. **24** 609–615

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