

The Cytotoxic and Anti-proliferative Effects of 3-Hydrogenkwadaphnin in K562 and Jurkat Cells Is Reduced by Guanosine

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3-hydrogenwadaphnin (3-HK) is a new daphnane-type diterpene ester isolated from *Dendrostellera lessertii* with strong anti-tumoral activity in animal models and in cultures. Here, prolonged effects of this new agent on proliferation and viability of several different cancerous cell lines were evaluated. Using [³H]thymidine incorporation, it was found that the drug inhibited cell proliferation and induced G1/S cell cycle arrest in leukemic cells 24 h after a single dose treatment. The cell viability of Jurkat cells was also decreased by almost 10%, 31% and 40% after a single dose treatment (7.5 nM) at 24, 48 and 72 h, respectively. The drug-treated cells were stained with acridine orange/ethidium bromide to document the chromatin condensation and DNA fragmentation. These observations were further confirmed by detection of DNA laddering pattern in the agarose gel electrophoresis of the extracted DNA from the treated cells. Treatment of K562 cells with the drug at 7.5, 15 and 30 nM caused apoptosis in 25%, 45% and 65% of the cells, respectively. Exogenous addition of 25-50 μM guanosine and/or deoxyguanosine to the cell culture of the drug-treated cells restored DNA synthesis, released cell arrest at G1/S checkpoint and decreased the apoptotic cell death caused by the drug. These observations were not made using adenosine. However, the drug effects on K562 cells were potentiated by hypoxanthine. Based on these observations, perturbation of GTP metabolism is considered as one of the main reasons for apoptotic cell death by 3-HK.

Keywords: Apoptosis, Cell cycle arrest, *Dendrostellera lessertii*, DNA synthesis, Guanylate metabolism, 3-Hydrogenkwadaphnin

Introduction

The search for new pharmacologically active drugs from natural sources has led to the discovery of many useful drugs and approximately 60% of the anti-tumor agents which are clinically used, or they are in the late stages of development, are from natural sources (Shu, 1998). Indeed, today's medical world is highly dependent on several natural products such as taxol, vincristin and complithecine in fighting different kinds of cancer (Pezzuto, 1997). Along this line, plant-derived compounds have great potential to be developed into anti-cancer drugs because of their multiple mechanisms and low side effects (Yoshida *et al.*, 1996). It has become increasingly evident that apoptosis is an important mode of action for many anti-tumor drugs so that modulation of cell apoptosis seems to be a reliable approach for the evaluation of the potency of newly discovered compounds as effective anti-cancer agents, particularly when it is specifically achieved (Yoshida *et al.*, 1996; Taraphdar *et al.*, 2001). Apoptosis is a form of cell death that allows for elimination of damaged or unwanted cells without damage to the organisms. It has been originally defined by morphological criteria such as cellular shrinkage, chromatin condensation and fragmentation of the cells into apoptotic bodies. A central role in apoptosis is played by caspases, a family of proteases responsible for many of morphological as well as biochemical changes observed in apoptotic cells (Earnshaw *et al.*, 1999).

In this respect, our lab has initiated screening programs on new anti-cancer agents from Iranian medicinal plants with emphasis on Thymelaeaceae family. Various species of Thymelaeaceae family have been used to treat cancer since 200 A.D. We observed that methanol extracts of two species: *Dendrostellera lessertii* and *Daphne mucronata*, have potent anti-cancer property in different human leukemic cell lines and in animal models (Hedayati *et al.*, 2002; Sadeghi and Yazdanparast, 2003; Yazdanparast *et al.*, 2003). These cytotoxicity effects were shown to be related to their active compounds: 3-hydrogenkwadaphnin (Fig. 1) and gnidilatimonoein,

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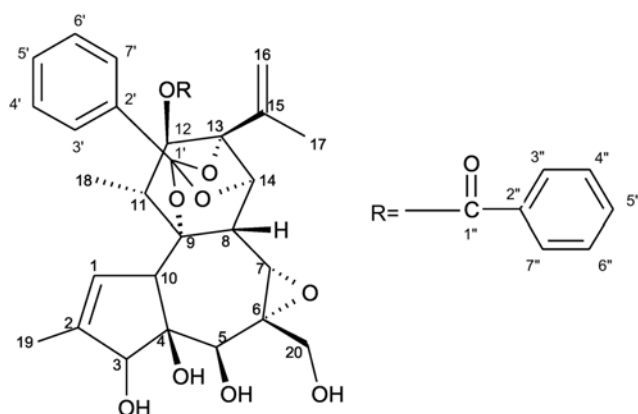


Fig. 1. Structures of 3-hydrogenkwadaphnin (3-HK).

respectively (Mianabadi *et al.*, 2003; Yazdanparast *et al.*, 2003; Mianabadi and Yazdanparast, 2004; Yazdanparast and Mianabadi, 2004). Both compounds are ortho-esters bearing daphnane-type diterpenes with an isopropylene side chain at C₁₃. Indeed, daphnane-type diterpene esters which possess an isopropylene side chain at C₁₃ have previously been reported to have anti-leukemic activity (Hall *et al.*, 1982). Further studies with K562 leukemic cell line revealed that gnidilatinone (Sadeghi and Yazdanparast, 2004) and 3-HK (submitted) induce G1/S cell cycle arrest and inhibited nucleic acid synthesis. In addition, the activity of inosine monophosphate dehydrogenase (IMPDH; EC1.1.1.205), the rate-limiting enzyme in the biosynthetic pathway of guanine nucleotides from IMP, was significantly reduced by gnidilatinone (Sadeghi and Yazdanparast, 2004) and 3-HK (submitted). The effects of IMPDH inhibition and consequent guanine nucleotide depletion have been investigated in many cell culture systems using different drugs (Ahmed and Weidemann, 1995) which induce cell-cycle arrest in late G1, inhibition of nucleic acid synthesis, differentiation and recently induction of apoptosis (Jayaram *et al.*, 1992; Vitale *et al.*, 1997; Laliberte *et al.*, 1998; Jayaram *et al.*, 1999; Metz *et al.*, 2001). The cause(s) of all of the above effects seems to be GTP depletion (Li *et al.*, 1998; Zimmerman *et al.*, 1998; Gu *et al.*, 2003).

According to literature, other daphnane-type diterpene esters such as genkwadaphnin, yuanhuacine and genidilatin, with similar structures, have potent anti-leukemic activities similar to our isolated compounds. In addition their inhibitory effects against IMPDH have also been reported (Hall *et al.*, 1982; Liou *et al.*, 1982). However, the exact molecular mechanism(s) of their anti-leukemic effects have not been studied. Therefore, it is interesting to investigate the mechanism(s) of anti-leukemic effects of the diterpene esters with focus on their relationship with IMPDH inhibition.

To get a better understanding about the mechanism of action of 3-HK, and to elucidate the relation of DNA synthesis, G1 cell cycle arrest and apoptosis to the drug-induced IMPDH inhibition, we examined the involvement of

guanine nucleotides and showed that the effects of the drug on the cells are mainly related to GTP depletion.

Materials and Methods

Materials The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Life technologies, Paisley, Scotland). The culture plates were obtained from Nunc (Roskilde, Denmark). Cell lines were obtained from Pasteur Institute of Iran (Tehran, Iran). MTT [3-(4,5'-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and actinomycin D were purchased from Sigma. Chem. Co. (Munich, Germany). Chloroform, isoamyl alcohol, isopropanol and phenol were obtained from Merck (Darmstadt, Germany). [³H]thymidine was purchased from Amersham Pharmacia Biotech (Bucks, UK). Guanosine, deoxyguanosine and adenosine were purchased from Aldrich Chemical Co. Ltd (Gillingham, England). RNase A (DNase free), ethidium bromide and acridine orange were obtained from Pharmacia LKB Biotechnology AB Uppsala, Sweden.

Cell culture Molt4, HL-60, HepG2, MCF7, K562 and Jurkat cell lines were cultured in RPMI 1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml), and penicillin (100 U/ml). Cell numbers were assessed using a hemocytometer and the ability of the cells to exclude trypan blue.

Cell viability assay The number of viable cells was estimated by MTT assay (Vistica *et al.*, 1991; Zhang *et al.*, 2000). Briefly, the cells (5 × 10⁴ cells/well) were seeded in flat-bottom 96-well plates. After culturing for 24 h, different concentrations of 3-HK were added to each well for various time intervals and 10 µl MTT (5 mg/ml) was added to each well 4 h before harvesting. The reaction was stopped by adding 100 µl of 0.04 N HCl in isopropanol and the absorbance values at 570 nm were determined on a multiwell plate reader (Elx 800 Microplate Reader, Bio-TEK).

DNA synthesis DNA synthesis was estimated by measuring [³H]thymidine incorporation (Nio *et al.*, 1990; Yamada *et al.*, 2002). Briefly, 2 × 10⁴ cells were seeded per well and different concentrations of 3-HK were added to each well and incubated for various time intervals. [³H]thymidine (1 µCi/well) was included during the last 3 h. After drying at room temperature, the glass fiber filters were placed into counting vials with 5 ml of the scintillation fluid and their radioactivity was measured in a liquid scintillation counter (Rackbeta, LKB) as a measure of thymidine incorporation into DNA. The extent of inhibition of DNA synthesis was determined by the following equation: %inhibition = [1 - test (cpm)] / [control (cpm)] × 100.

Flow cytometry analyses DNA content was analyzed on an EPICS II flow cytometer (Beckman counter, France) according to the established procedure (Papazisis *et al.*, 2000). Jurkat cells (1 × 10⁶ cells/well) were seeded into culture dishes 24 h prior to treatments. The cells were harvested after 24 h of treatment with 3-HK (7.5 nM) in presence and absence of 25 µM guanosine. Cells were washed twice with PBS (Phosphate Buffer Saline), fixed in

70% ethanol, and kept at -20°C until analysis. The cells were then stained with $20\ \mu\text{g/ml}$ propidium iodide containing $20\ \mu\text{g/ml}$ RNase (DNase free) for 2 h. The stained cells were analyzed by flow cytometry. The population of G0/G1, S, and G2/M were determined using Mulicycle Cell Cycle Software. The results are expressed as percentage of the cells in each phase.

DNA fragmentation assay DNA fragmentation in K562 cells was measured after extraction of DNA from a constant number of cells. After treatment of K562 cells for 72 h, 1×10^6 cells were collected and washed twice with cold PBS, resuspended in $100\ \mu\text{l}$ lytic solution (10 mM EDTA, 50 mM Tris, pH 8, 0.5% SDS, 100 mM NaCl) and incubated with proteinase K ($200\ \mu\text{g/ml}$) at 50°C overnight. DNA was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1), washed with ethanol, resuspend in TE (Tris-EDTA) buffer, and separated by electrophoresis in a 2% agarose gel containing ethidium bromide ($0.5\ \mu\text{g/ml}$). The gel was photographed under UV transillumination. DNA from Jurkat cells was measured by Quick Apoptotic DNA Ladder Detection Kit from Biosource (USA) according to manufacturer procedures. Briefly, soluble DNA fragments were extracted from the treated Jurkat cells and loaded onto a 1% agarose gel containing $0.5\ \mu\text{g/ml}$ ethidium bromide in both gel and the running buffer ($1 \times \text{TBE}$). The gel was run at 5 V/cm for 2 hours.

Morphological study of the apoptotic cells Apoptosis was determined morphologically after staining with acridine orange/ethidium bromide by fluorescence microscopy (Saydam *et al.*, 2003). Cells were washed in cold PBS and adjusted to a cell density of 1×10^6 cell/ml of PBS. Acridine orange/ethidium bromide solution (1 : 1, v/v) was added to the cell suspension in a final concentration of $100\ \mu\text{g/ml}$. The cellular morphology was evaluated by Axoscope 2 plus fluorescence microscopy from ZEISS (Oberkochen, Germany).

Results

The cytotoxic effects of 3-HK in different tumor cell lines

The results presented in Table 1, clearly indicate that the new diterpene ester had potent cytotoxic activity toward a panel of six human cancerous cell lines used in this investigation. The IC_{50} , determined 48 h after a single dose treatment of each cell type, ranges between 10-25 nM. Apparently, the potency of the compound is not related to p53 status of cells since K562, HL-60 and HepG2 cells lacks active p53 protein (Pipano *et al.*, 2002). Also the activity of the new diterpene ester is very noticeable in K562 cells which are believed to be resistant to many anti-cancer drugs (McGahon *et al.*, 1994). Regarding the IC_{50} values, the rest of our investigation was mostly done using the most sensitive (Jurkat) and the so called drug resistant K562 cells.

The acute and long-term effects of 3-HK on viability of K562 and Jurkat cells

Treatment of K562 and Jurkat cells with different concentrations of 3-HK for long times resulted in cell death. The cytotoxic evaluation by MTT assay

Table 1. Cytotoxic effects of 3-HK in six different cancer cell lines. The IC_{50} s have been established 48 h after the drug treatment as explained in materials and methods. Each measurement represents the average of triplicate experiment \pm SEM ($p < 0.05$)

Cell type	IC_{50} (nM)
HepG2	25.0 ± 0.1
MCF7	20.0 ± 0.2
K562	15.0 ± 0.4
Molt4	13.0 ± 0.8
HL-60	12.0 ± 0.7
Jurkat	10.0 ± 0.5

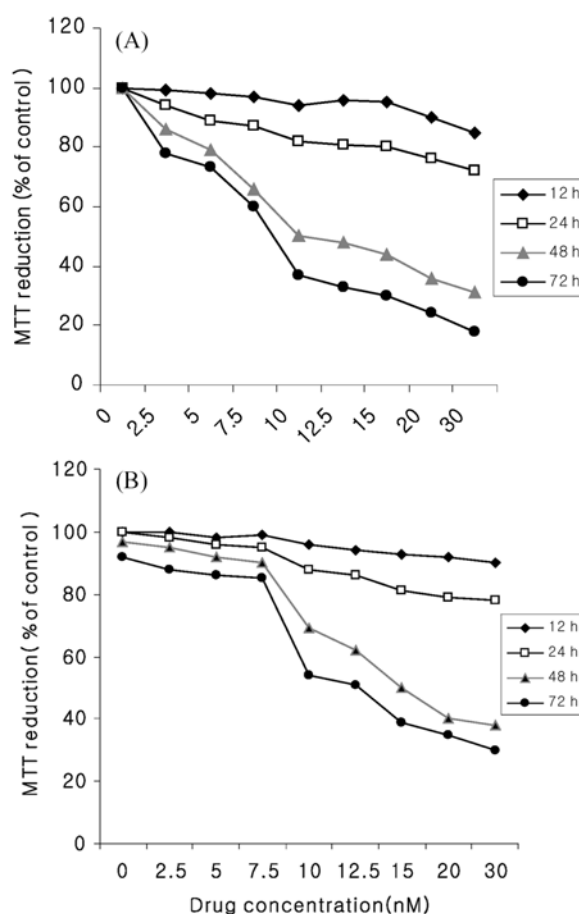


Fig. 2. Prolonged 3-HK treatment induces cell death. Exponentially growing Jurkat (A) and K562 (B) cells in the 96-well plates were treated with a single dose of the drug at different concentrations for various time intervals and cell viability was determined by MTT assays. The results represent those of a single typical experiment.

indicated that no significant changes in the extent of formazan production occurred in K562 and Jurkat cells 12 h after treatment with 2.5-30 nM of the drug (Fig. 2) and a slight decrease occurs after 24 h. However, after 48 h of treatment, the cell viability was reduced by 50% at 15 nM and 10 nM of the drug in K562 and Jurkat cells, respectively. Treatment for

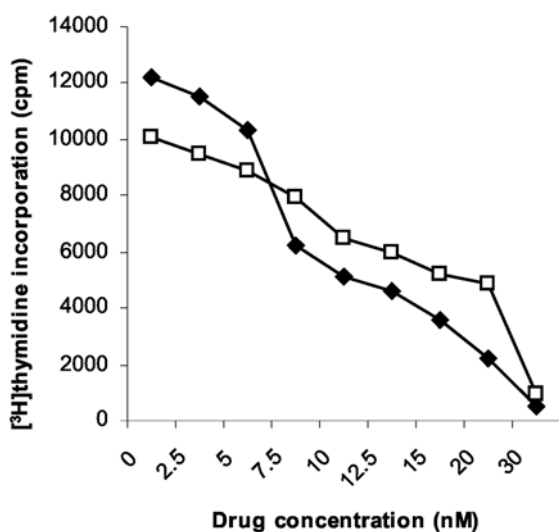


Fig. 3. Effects of different concentrations of 3-HK on $[^3\text{H}]$ thymidine incorporation into DNA of Jurkat (\blacklozenge) and K562 (\square) cells. The cells were treated with different concentrations of the drug for 24 h followed by the addition of $[^3\text{H}]$ thymidine ($1 \mu\text{Ci}/\text{well}$) three hours before the cell harvesting. The results represent those of a single typical experiment.

longer periods of time resulted in a massive cell death even at low concentrations of 3-HK.

Effects of 3-HK on DNA synthesis in K562 and Jurkat cells

The anti-proliferative activity of the 3-HK was evaluated by measuring its effects on the extent of DNA synthesis in K562 and Jurkat cell lines. As shown in Fig. 3, the extent of $[^3\text{H}]$ thymidine incorporation into DNA decreases in a dose dependent manner. In other words, at 7.5, 10.0, 15.0 and 30.0 nM concentrations, DNA synthesis is inhibited by 21%, 35%, 48% and 90% in K562 cells and by 43%, 57%, 70% and 95% in Jurkat cells. Indeed these cells are very sensitive to DNA synthesis inhibition, so that in Jurkat cells DNA synthesis inhibition occur as early as 3 h after drug treatment and reduced nearly up to 50% after 24 h at 7.5 nM drug concentration (data not shown).

Effects of 3-HK on cell cycle progression of Jurkat cells

Based on Fig. 3 the drug, even at low concentrations, had potent effect on DNA synthesis after 24 h of treatment with a single dose. However, the cells viability did not significantly varied under the same experimental conditions (Fig. 2A). In order to get a better understanding of the anti-proliferative activity of the drug, the growth pattern of the treated and untreated Jurkat cells were investigated using flow cytometry. Twenty-four hours after seeding, the Jurkat cells were treated with a single dose (7.5 nM) of 3-HK. The cells were incubated for 24 h at 37°C followed by cell cycle analysis with respect to the corresponding control cells. The cell growth distribution in the control sample was 28% in G1 phase, 43% in S phase and 29% in G2 phase. This distribution changed upon treatment and resulted in the accumulation of 41% of cells in G1 phase which accompanied by a slight compensatory decrease in the proportion of S and G2/M cells. (Fig. 4, A and B). However, after this time point (24 h) the cells at G1 phase decreased and a sub-G1 peak was appeared (data not shown).

Effects of guanine nucleotides on DNA synthesis, cell cycle progression and cell viability

According to the literature, both guanosine and deoxyguanosine at $25 \mu\text{M}$ are capable of increasing the pool size of guanine nucleotides with the lowest anti-proliferative effects in Jurkat cells (Batiuk *et al.*, 2001). In our investigation, addition of guanosine ($25 \mu\text{M}$) to the 3-HK-treated Jurkat cells removed the G1/S arrest and resulted in cell accumulation in S phase of the progression cycle (Fig. 4C). These results are similar to the effect of guanosine on cell cycle progression in human T lymphocytes (Laliberte *et al.*, 1998). Inhibition of DNA synthesis and cytotoxicity (Table 2) in the drug-treated cells were also impeded upon guanosine addition. According to these data, both guanosine and deoxyguanosine, at $25 \mu\text{M}$ concentrations, restored the DNA synthesis in the drug treated cells by 97% and 64%, respectively. However, adenosine at various concentrations ($25\text{-}50 \mu\text{M}$) did not show such a preventive effect (Table 2). At higher concentrations both guanosine and deoxyguanosine were also capable of restoring DNA synthesis in the drug

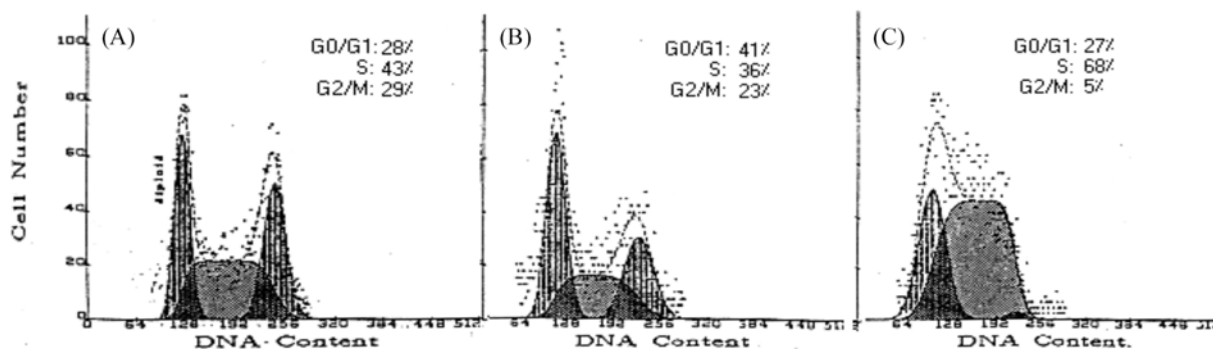


Fig. 4. Cell cycle progression in the absence (A) and the presence of 3-HK (B and C). Jurkat cells were treated with 7.5 nM 3-hydrogenkwadaphnin for 24 h. Simultaneous addition of the drug and guanosine ($25 \mu\text{M}$, C) result in elimination of G1/S cell cycle arrest and accumulation of the treated cells in the S phase of the progression cycle.

Table 2. Effects of nucleosides on metabolic viability and DNA synthesis of Jurkat cells. The Jurkat cells (5×10^4 /well) were culture in 96-well plates and were exposed to 7.5 nM of the drug alone or combined with other agents in the culture medium for 24 h (DNA synthesis) or 48 h (MTT assay) as explained in materials and methods. Each measurement represents the average of three analyses \pm SEM ($p < 0.05$)

Addition	Formazan production (% of control)		$[^3\text{H}]$ thymidine incorporation (% of control)	
	Control	Drug-treated	Control	Drug-treated
None	100.00 \pm 0.00	50.00 \pm 0.30	100.00 \pm 0.00	57.00 \pm 0.90
Guanosine (25 μM)	104.00 \pm 1.30	90.00 \pm 0.60	109.00 \pm 0.50	97.00 \pm 0.20
Deoxyguanosine (25 μM)	89.00 \pm 0.09	60.00 \pm 0.20	91.00 \pm 0.80	64.00 \pm 0.10
Adenosine (25 μM)	92.00 \pm 0.70	48.00 \pm 0.06	93.00 \pm 1.30	44.00 \pm 1.50
Hypoxanthine (100 μM)	94.00 \pm 0.80	31.00 \pm 0.30	ND	ND
Actinomycine D (0.05 μM)	96.00 \pm 0.12	69.00 \pm 0.88	97.00 \pm .003	79.00 \pm .040

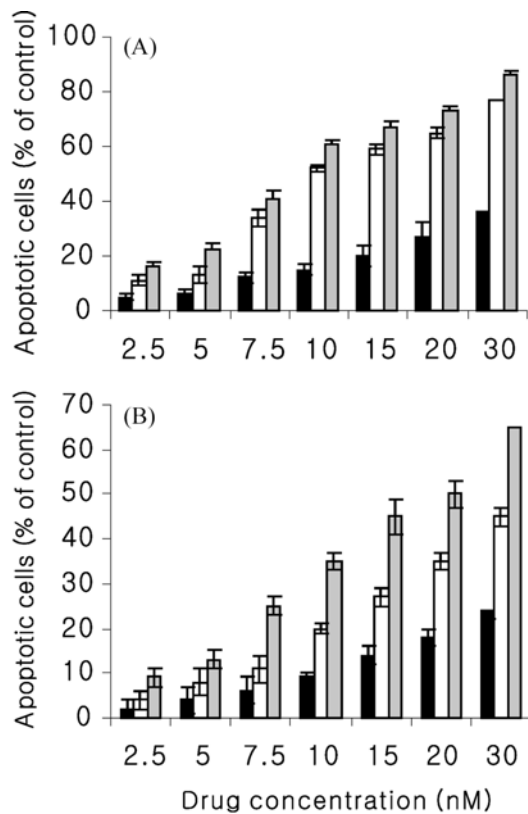


Fig. 5. Effect of different concentrations of 3-HK on the induction of apoptosis at various time intervals. The Jurkat cells (1×10^6 /well, A) and K562 cells (1×10^6 /well, B) treated with a single dose of the drug ranging between 2.5-30 nM. At different time intervals, the cells were harvested and stained with Acridine orange/Ethidium bromide. At least 200 cells were counted in each experiment by fluorescence microscopy. The columns show the treated cells after 24 h (■), 48 h (□) and 72 h (▨). The results present the average of triplicate counting \pm S.E.M ($p < 0.05$).

treated K562 cells. In this cell line, guanosine and deoxyguanosine at 50 μM restored DNA synthesis by 88% and 55% of the control cells, respectively (data not shown). These results indicate that in both cell lines guanosine almost completely and deoxyguanosine partially restored DNA

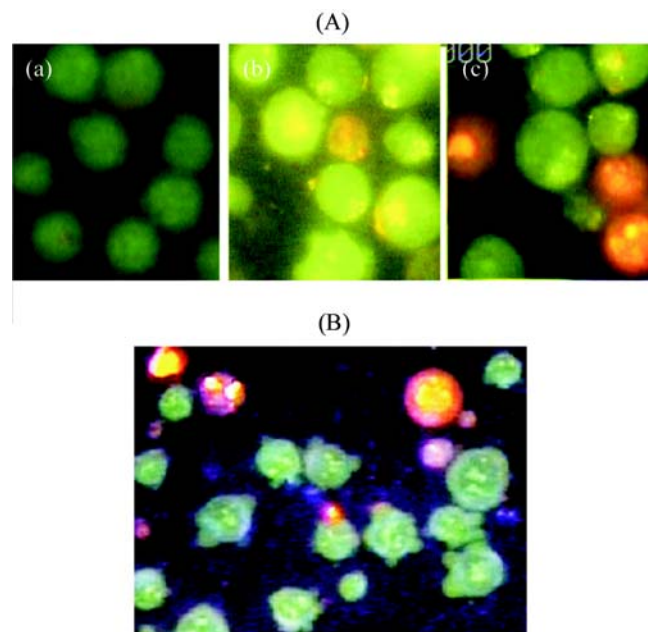


Fig. 6. Fluorescence microscopic analyses of the drug treated K562 and Jurkat cells. K562 (A) and Jurkat (B) cells were incubated with 10 and 7.5 nM of the drug, respectively, along with membrane permeable acridine orange and ethidium bromide which is impermeable to the normal membrane but stain the nuclei of necrotic and late apoptotic cells (orange color). Chromatin condensation and margination as well as ruffling of nuclear membrane are observed. Control K562 cells (a) and drug treated cells after 24 h (b) and 72 h (c). B: drug-treated Jurkat cells after 72 h. Magnification 100 \times .

synthesis whereas adenosine did not play any role in this respect. However, the addition of hypoxanthine, which prevents the salvage of guanosine to GMP (Vitale *et al.*, 1997), enhanced the drug effects (Table 2). It has been reported that hypoxanthine alone (100 μM) dose not have any effects on apoptosis or DNA synthesis of K562 cells (Vitale *et al.*, 1997). These results clearly indicate a role for 3-HK in GTP metabolism. In order to found a link between GTP pool and DNA synthesis, the drug-affected Jurkat cells were treated

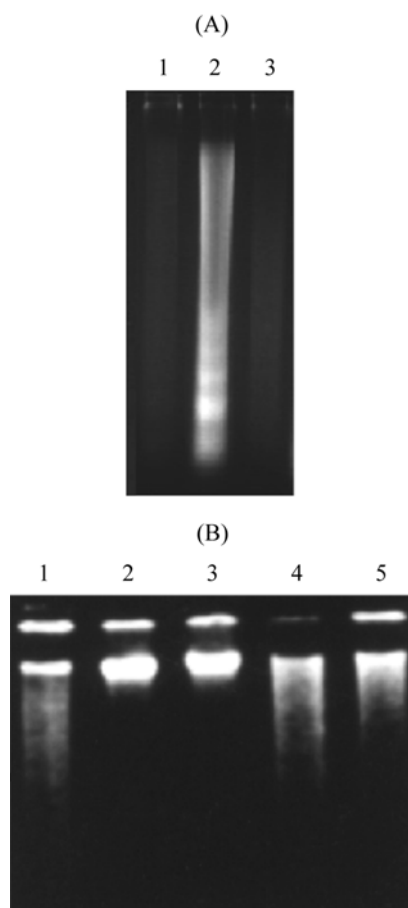


Fig. 7. The effect of 3-HK on DNA fragmentation. The Jurkat cells (5×10^5 cell/well, A) and K562 (1×10^6 cell/well, B) were seeded in microwell plates and treated with 7.5 and 10 nM of the drug, respectively, for 72 h unless specified. DNA was extracted as explained in materials and methods. A: Lan1, control cell; Lan2, treated cells; Lan3, treated cells plus 25 μ M guanosine. B: Lan1, 48 h-treated cells; Lan2, control cells; Lan3, 72 h-treated cells plus 50 μ M guanosine; Lan4, 72 h-treated cells; Lan5, 72 h-treated cells plus 50 μ M adenosine.

with actinomycine D (0.05 μ M) which is believed to block RNA synthesis without affecting DNA synthesis (Cohen *et al.*, 1981). The results showed the DNA synthesis was restored by 79% (Table 2).

Induction of apoptosis by 3-HK in K562 and Jurkat cells

Analyses of the drug treated K562 and Jurkat cells by acridine orange/ethidium bromide double staining showed that the new drug induces apoptosis in the cells in a time and dose-dependent manner (Fig. 5). Exposure of Jurkat cells to a 7.5 nM concentration of the drug induced apoptosis in 14%, 34% and 40% of the cells after 24, 48 and 72 h, respectively. The K562 cells, despite their resistance to many anti-cancer agents, were very sensitive to 3-HK and apoptosis was easily observed in these cells, too (Fig. 6A). The appearance of chromatin condensation, nuclear fragmentation and shift from

early to late apoptosis are evident in Fig. 6. In this figure, viable cells are uniformly green, early apoptotic cells are green and contained bright green dots in their nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporate ethidium bromide and therefore stain orange and in contrast to necrotic cells, they show condense and oftenly fragmented nuclei. DNA fragmentation to oligonucleosomes is clearly observed in Jurkat cells 72 h after a single dose (7.5 nM) treatment (Fig. 7A). However, oligonucleosomes were not detected in the treated K562 cells, although DNA degradation was documented in these cells (Fig. 7B). In both cells, the DNA fragmentation is blocked by guanosine but not by adenosine (Fig. 7A and B).

Discussion

Many species of Thymelaeaceae family are known to contain toxic and biologically active ingredients of chemically related diterpene esters. Compounds belonging to the daphnane diterpene esters have been shown to possess anti-leukemic activity as apposed to tigliane diterpene esters which are known to be carcinogenic (Kupchan *et al.*, 1976; Evans and Soper, 1978). In that respect, daphnane diterpene esters such as genkwadaphnin, yuanhuacine (Hall *et al.*, 1982; Liou *et al.*, 1982), genididin, giniditrin and genidilatin (Evans and Soper, 1978; Stanoeve *et al.*, 2005) possess significant anti-leukemic activities both in vivo and in vitro with major metabolic effects on the DNA and protein syntheses (Hall *et al.*, 1982; Liou *et al.*, 1982). Recently, a new diterpene ester has been characterized from *Dendrostellera lessertii* which shows high anti-leukemic activity, in nanomolar ranges (Table 1) compared to micromolar doses reported for some other diterpene esters (Kupchan *et al.*, 1976; Hall *et al.*, 1982; Yazdanparast and Sadeghi, 2004). Concerning this biological potency, a detailed understanding of the mode of action of this active compound should provide useful information concerning its further use in cancer therapy.

The anti-leukemic activity of many diterpene esters is mainly attributed to their inhibitory action toward different enzymes particularly IMPDH (Hall *et al.*, 1982; Liou *et al.*, 1982; Hall *et al.*, 1986). The new diterpene ester, 3-HK, shares similar activity toward IMPDH (Yazdanparast and Sadeghi, 2004). According to our results, the drug at short treatment times (less than 24 h) potently inhibited cell proliferation followed by the cell cycle arrest in G1 phase, without significant cytotoxic effects. These means that the drug at low concentrations and/or at low treatment times has cytostatic property rather than cytotoxic effects. However, longer treatment times (2 to 3 days) or higher concentrations were required for cell death occurrence. Indeed, post-G1 arrest apoptosis was occurred under drug administration. This matter was further confirmed by morphological and biochemical observations. Condensation and fragmentation of nuclear DNA were demonstrated morphologically by fluorescent

staining with DNA-binding dyes (acridine orange/ethidium bromide). The biochemical evidence of apoptosis was provided by detecting the DNA fragments which are hallmark of apoptosis.

Reduction of DNA synthesis (Fig. 3) and inhibition of IMPDH activity as reported previously (Yazdanparast and Sadeghi, 2004) under the effect of 3-HK may be responsible for the cell apoptosis as shown for mycophenolic acid and tiazofurin which both inhibit DNA synthesis and induce apoptosis (Jayaram *et al.*, 1992; Vitale *et al.*, 1997; Laliberte *et al.*, 1998). Reduction in GTP and/or dGTP pool size, as a consequence of IMPDH inhibition, might be the main cause of our observation (Jayaram *et al.*, 1999). Restoration of DNA synthesis and the progression of cells through the G1/S checkpoint after guanine nucleotide addition to the drug-treated cells confirm this prediction. It has been shown by Cohen group that exogenous guanosine is salvaged to GTP via guanine by sequential action of several enzymes and further converted to dGTP by ribonucleotide reductase (Cohen *et al.*, 1981). Deoxyguanosine is also salvaged to GTP and dGTP by the same enzymes as is guanosine. However, deoxyguanosine is also directly converted to dGTP (Cohen *et al.*, 1981). Based on this knowledge, if dGTP depletion was responsible for 3-HK toxicity and inhibition of DNA synthesis, then exogenous deoxyguanosine seems to be a more effective rescue agent than guanosine. The higher potency of exogenous guanosine in reducing the drug toxicity and enhancing the extent of DNA synthesis (Table 2) suggest that probably the salvage to GTP rather than dGTP is responsible for alleviating the drug effects. The effects of deoxyguanosine on reducing the drug toxicity and enhancing DNA synthesis (Table 2) might be related to its salvage into GTP rather than dGTP. The specific action of the drug on GTP pool was further evident concerning the effect of hypoxanthine on the drug-treated cells which potentiates the drug effects through depletion of GTP pool size (Vitale *et al.*, 1997). Final decision concerning the effect of the drug on the pool size of GTP and dGTP awaits determination of their exact concentrations in the treated cells. Further evidence concerning the manipulations of GTP pool size by 3-HK is provided by the following observation.

Inhibition of RNA synthesis in drug-treated Jurkat cells by 0.05 M actinomycin D enhanced DNA synthesis by almost 79% compared to the drug-treated cells. In fact, inhibition of the RNA biosynthetic pathway will reduce the demand for GTP (used in RNA syntheses) and consequently more GTP will be available to be used in DNA synthesis (Catapano *et al.*, 1995).

In conclusion, the results of the present investigation showed that, upon inhibition of de novo synthesis of guanine nucleotides, mainly GTP, in Jurkat and K562 cells by 3-HK, primarily DNA synthesis is inhibited and the cell cycle progression ceased at G1/S checkpoint. These changes finally led to high apoptotic cell death and marginal necrosis at longer treatment times or at higher drug concentrations. In

addition, exogenous addition of guanosine to the drug-treated cells, restored DNA synthesis and allowed the cell entry into S phase of their progressing cycle. This observations confirm the effects of the drug on the guanine nucleotide metabolic pathways.

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