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Effects of 3-Hydrogenkwadaphnin on intracellular purine nucleotide contents and their link to K562 cell death

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ABSTRACT

Our previous studies have classified 3-Hydrogenkwadaphnin (3-HK), isolated from *Dendrostellera lessertii* (Thymelaeaceae), as a strong anti-proliferative agent that induces apoptosis among the treated leukemia cells mainly through IMP dehydrogenase inhibition. To disclose this point, we measured the intracellular ATP and GTP levels among K562 cells exposed to a single dose of 3-HK (7, 12 and 25 nM). The HPLC analyses of the 3-HK-treated cell lysates indicated a dose-dependent reduction in both GTP and ATP levels. The reduction of purine nucleotide levels was also associated with decreased activity of adenylosuccinate synthetase (AdSS) and enhanced level of cell death through apoptosis. However, the inhibitory effect of 3-HK was reversed by exogenous addition of guanosine with respect to GTP and partially with respect to ATP level. These data suggest that 3-HK targets a delicate regulatory system which governs the life/death fate of the cells partially through modulation of the GTP/ATP pool sizes.

1. Introduction

Leukemia results from incapability of hematopoietic stem cells to differentiate into mature functional cells (Copland, Jorgensen, & Holyoake, 2005). So far, leukemia medical treatments have been based on chemotherapy, interferon treatment and/or bone marrow transplantation as well as combination therapies. All these approaches are usually associated with side effects (Fausel, 2007; Sillaber et al., 2003). Regarding this fact, therapies associated with minimum level of side effects are highly desirable and demanded in clinics. The search for anti-cancer compounds mainly from plant sources have got momentum following the discovery of vinca alkaloids, vinblastine and vincristine (Cragg & Newman, 2005) and these findings have led to the discovery of many new and pharmacologically active drugs in a manner that approximately more than half of the present anti-cancer drugs are from natural and mainly plant sources. Among plant-derived agents, the daphnane-type diterpene esters such as genkwadaphnin (Hall, Kasai, Wu, Tagahara, & Lee, 1982), gnidilatimonoein (Yazdanparast & Sadeghi, 2004), genidin, geniditrin and gnidilatin (Stanoeva, He, & De Kimpe, 2005) have potential anti-leukemia activities with major metabolic effects on DNA and protein syntheses (Hall et al., 1982). Along this line, our lab has characterized a novel daphnane-type diterpene ester (3-Hydrogenkwadaphnin, 3-HK) from *Dendrostellera lessertii* (Thymelaeaceae) with novel differentiation and apoptotic activities among a wide range of human leukemia cell lines without any adverse effects on normal cells (Moosavi, Yazdanparast, Sanati, & Sarraf Nejad, 2005; Sadeghi & Yazdanparast, 2005). It has previously been shown that 3-HK is capable of inhibiting the activity of inosine monophosphate dehydrogenase (IMPDH) (Moosavi et al., 2005) which catalyzes the rate-limiting step of the de novo purine nucleotide biosynthetic pathway. The activity of this enzyme has been linked to the regulation of cellular growth, differentiation and apoptosis (Chen & Pankiewicz, 2007; Floryk & Thompson, 2008). Inhibition of IMPDH activity would then certainly lead to depletion of the GTP pool size. On the other hand, it is well documented that the intracellular levels of purine nucleotides, mainly GTP, are highly up-regulated in many transformed cells relative to their relevant healthy control cells (Traut, 1994). The higher than normal levels of these nucleotides have been mainly attributed to the up-regulation of some of the key enzymes involved in the de novo and/or the salvage biosynthetic pathways such as IMPDH and GMP synthase. This fact has made these enzymes the pharmaceutical targets for treatment of leukemia (Messina et al., 2004). So far, many synthetic and/or natural IMPDH inhibitors have been evaluated against leukemia (Chen & Pankiewicz, 2007). The results of these investigations have shown that IMPDH inhibition usually leads to depletion of intracellular GTP level (Gu et al., 2003), inhibition of DNA synthesis (Laliberte, Yee, Xiong, & Mitchell, 1998), cell cycle arrest in late G1 (Messina et al., 2004),

Abbreviations: 3-HK, 3-Hydrogenkwadaphnin; AdSS, adenylosuccinate synthetase; Guo, guanosine; HPLC, high performance liquid chromatography; IMPDH, inosine 5′- monophosphate dehydrogenase.

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cell differentiation (Floryk & Huberman, 2006) and apoptosis (Floryk & Thompson, 2008; Messina et al., 2004), depending upon the type of cancerous cell line used. The depletion of intracellular GTP content has been considered as the main cause(s) of these observations. Besides, there are couples of studies on the effects of IMPDH inhibitors on the intracellular level of ATP. For instance, it has been shown that leflunomide induces depletion of both GTP and ATP contents in the cells (Ruckemann et al., 1998). On the other hand, mycophenolic acid depletes the GTP, GDP-sugar and ATP pool sizes (Qiu et al., 2000; Vethe, Bremer, Rootwelt, & Bergan, 2008). Regarding the significant and established role of ATP in the life/death fate of a cell, in this study, we were aimed to find out whether 3-HK treatment of K562 cells would modulate their intracellular ATP.

2. Materials and methods

2.1. Materials

The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco BRL (Life Technology, Paisley, UK). Cell line was purchased from Pasteur Institute of Iran (Tehran). The ion-pair reagent tetra-*n*-butyl ammonium bromide was obtained from Merck (Germany). Annexin-V-FITC was purchased from IQ Products (Groningen, The Netherlands). Hoechst 33258, IMP, ATP, GTP, propidium iodide (PI) and guanosine were from Sigma Chem. Co. (Germany). Solvents were HPLC grade, and all other chemicals were reagent grade.

2.2. Plant extraction and purification of 3-HK

The powdered plant material (0.4 kg) was extracted four times with methanol–water (1:1, v/v). The accumulated alcoholic extract was concentrated under reduced pressure, and the volume was adjusted to 400 ml. The crude extract was then subjected to $CHCl_3$ extraction for five times. The accumulated chloroform solution was concentrated under reduced pressure to a final volume of 1 ml. The active compound was purified using thin-layer chromatography (TLC) technique. The developing system for TLC was a mixture of chloroform and diethyl ether (1:1, v/v). The characterization of 3-HK (Fig. 1) has been achieved as previously reported (Sadeghi & Yazdanparast, 2005). The purity (99% pure) of the compound was further confirmed by HPLC technique.

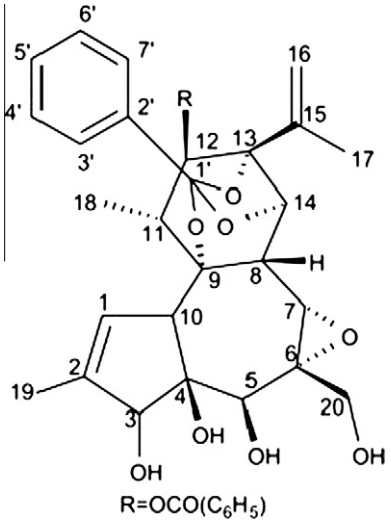

Fig. 1. Structure of 3-Hydrogenkwadaphnin (3-HK).

2.3. Cell culture

The K562 cells, originated from a patient with chronic myelogenous leukemia, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml). Cells were grown at 37 °C in a humidified CO_2 (5%) atmosphere and were normally subcultured every 2–3 days. For all experiments, K562 cells were plated at a density of 2×10^5 cells/ml and used after 24, 48 or 72 h of incubation. The drug, 3-HK, was dissolved in a minimal quantity of dimethyl sulfoxide (DMSO) followed by dilution with RPMI 1640 medium and used at the indicated concentrations. The concentration of DMSO in the culture medium has been kept lower than 0.1% and the control cells have been treated with vehicle containing the same amount of DMSO.

2.4. Cell viability assay

The cells (2×10^5 cells/ml) were seeded, in triplicate, into cell culture plates 24 h prior to treatments. After treatments with 3-HK, the number of cells was established using a hemocytometer and the cell viability was determined by the trypan blue exclusion test.

2.5. Preparation of samples for HPLC analyses

A fixed number (2×10^6) of cells was used for all HPLC experiments. The cells were pelleted at 300g for 5 min at 4 °C, washed twice with ice-cold PBS, The cell pellet was re-suspended in 200 μl of 0.3 M cold perchloric acid and maintained at −80 °C for 10 min. Then, each sample was thawed and the mixture was vortexed for 10 s. The acidic supernatant was collected after centrifugation at 19,000*g* for 10 min at 4 °C, and neutralized with 0.5 M potassium hydroxide followed by centrifugation at 19,000*g* for 10 min at 4 °C to obtain the final supernatant referred to as ASF, acid-soluble fraction (Huang, Zhang, & Chen, 2003). The samples were stored at −80 °C for HPLC analyses.

2.6. Analyses of purine nucleotides by HPLC

Fifty microliters of each sample was analyzed by an HPLC (LC6, Shimadzu, Japan) and the purine nucleotides were resolved using an isocratic approach of 7.5% acetonitrile in 50 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 10 mM ion-pair reagent tetra-*n*-butyl ammonium bromide at a flow rate of 0.8 ml/min with a UV detector set at 254 nm. A reverse-phase column, 4 μm Nova-Pack C_{18} (150 mm × 3.9 mm inside diameter) equipped with a guard-pack pre-column module (Waters, Milfor, MA), was used. The HPLC grade nucleotide standards were used to calibrate the chromatographic signals. The standard solutions of ATP and GTP were prepared at 2 mg/ml and 1 mg/ml, respectively in deionized HPLC water and the serial dilutions of GTP (10–1000 nM) and ATP (10 nM–10 μM) were made using PBS (Baranowska-Bosiacka, Machaliński, & Tarasiuk, 2005).

2.7. Cell treatments

The cells (2×10^5 cells/ml) were seeded in culture plates and incubated at 37 °C in a CO_2 incubator (5%) for 24 h prior to drug treatments. The cells were exposed to a single dose of 3-HK, at various concentrations, for 48 h. Cells were then pelleted by centrifugation. The cells were re-suspended in the ice-cold lysis buffer containing 20 mM Tris/HCl (pH 7.5), 0.5 mM dithiothreitol (DTT), 0.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.2% detergent (Nonidet P-40) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication (3 × 15 s) (Datta, Guicherit, & Kellems,