Induction of Megakaryocytic Differentiation in Chronic Myelogenous Leukemia Cell K562 by 3-Hydrogenkwadaphnin

Azadeh Meshkini and Razieh Yazdanparast*
Institute of Biochemistry and Biophysics, P. O. Box 13145-1384, University of Tehran, Tehran, Iran

Received 16 April 2007, Accepted 16 July 2007

3-Hydrogenkwadaphnin (3-HK) is a daphnane-type diterpene ester isolated from Dendrostellera lessertii (Thymelaeaceae) with high differentiation and apoptotic potency in leukemic cells without any measurable adverse effects on normal cells (Moosavi et al., 2005b). In this study, we report that 3-HK (12 nM) has the ability to cease proliferation, induce differentiation and apoptosis in chronic myelogenous leukemia (CML) K562 cell line. The treated cells lost erythroid properties and differentiated along the megakaryocytic lineage based on the morphological features apparent after Wright-Giemsa staining, DNA content analysis and the expression of cell surface marker glycoprotein IIb as analyzed by flow cytometry. Moreover, using Hoechst 33258 and Annexin V double staining indicated the occurrence of apoptosis among the treated cells. On the other hand, restoration of the depleted GTP pool size by exogenous addition of guanosine (50 µM) reduced the effect of the drug regarding the extent of differentiation while no further enhancement of 3-HK effect was obtained by addition of exogenous hypoxanthine (100 µM). These interesting results necessitate further investigation regarding the mechanism of action of this unique anti-leukemic agent.

Keywords: Apoptosis, Dendrostellera lessertii, Differentiation, K562 cells, Leukemia

Introduction

Specific gene rearrangements or mutations usually result in the formation of new abnormal cellular products which block final differentiation of cells leading to accumulation of immature cells (Sell, 2006). Chronic myelogenous leukemia (CML) is a clonal disease of stem cell origin that develops when a single pluripotent hematopoietic stem cell acquires the Philadelphia chromosome that result from reciprocal translocation between chromosome 9 and 22 (Copland et al., 2005). At present, CML therapies mostly include chemotherapies, interferon treatments and bone marrow transplantation as well as combination therapies with serious side effects (Sillaber et al., 2003). Thus, therapy with minimal side effects is highly demanded in the clinical field. In that respect, differentiation induction therapy has attracted universal attention (Koefﬂer, 1983; Spira and Carducci, 2003). Differentiation therapy is an alternative approach to leukemia treatments due to its higher speciﬁcity compared to the traditional approaches with cytotoxic drugs (Sell, 2006). The human CML K562 cell line has been established from pleural effusion of a patient in blast crisis (Lozzio and Lozzio, 1977). These leukemic cells are blocked in their normal maturation at an early stage of differentiation and have been extensively used as an in vitro model for studying differentiation potential of many compounds (Koefﬂer and Golde, 1980). Numerous differentiation agents such as hemin (Villeval et al., 1983), duunomycin (Tomii et al., 1987), herbimycin A (Honma et al., 1989) and GTP (Moreau et al., 2000) have been used to induce the erytroid differentiation of K562 cell line. In contrast, treatment of K562 cells with phorbol esters (phorbol dibutyrate and phorbol 12-myristate 13-acetate, PMA) (Tetteroo et al., 1984; Aitio, 1990) and thrombopoietin (TPO) (Rouyer et al., 1997) have induced differentiation along a megakaryocytic lineage. Regardless of these achievements, clinical evaluations of these compounds have not been promising. For instance, clinical use of PMA is not recommended due to its tumor-promoting activity. Thus, universal efforts have been devoted to find new and potent differentiation inducers devoid of general toxicities. In that respect, plants have been the center of focus as a natural source for obtaining novel anti-leukemic agents with differentiation capability (Shu, 1998). Among plant-derived agents, the daphnane-type diterpene esters such as genkwadaphnin (Hall et al., 1982), gnidilatimonoestin...
(Yazdanparast and Sadeghi, 2004), genididin, geniditrin and gnidilatin (Stanoeva et al., 2005), possess significant anti-leukemic activities with major metabolic effects on DNA and protein syntheses.

It has previously been shown that 3-HK (Scheme 1), a daphnane-type diterpene ester from D. desertii (Thymelaeaceae) has differentiating and apoptotic activities in leukemia cell lines without any adverse effect on normal cells (Moosavi et al., 2005b; Yazdanparast et al., 2005). More precisely, it has been found that 3-HK is capable of inhibiting type II inosine-5'-monophosphate dehydrogenase (IMPDH) activity (Moosavi et al., 2005b) which is usually up-regulated in human leukemia cell lines. It is known that IMPDH catalyzes the rate-limiting reaction of de novo purine nucleotide biosynthetic pathway whose altered activity has been linked to the regulation of cellular growth, transformation, differentiation and apoptosis (Inai et al., 2000; Collart and Huberman, 1990). In fact, several IMPDH inhibitors such as mycophenolic acid and tiazofurin have been used as chemotherapeutic drugs for treating leukemic cells (Inai et al., 2000). In addition, it has been established that the anti-proliferative activity of 3-HK in human acute myeloid leukemia HL-60 (Yazdanparast et al., 2005) and NB4 cell lines (Moosavi et al., 2006) significantly affect the differentiation of the treated cells.

This study was designed to evaluate the probable differentiation effect of 3-HK in human CML K562 cell line. Our results indicated that 3-HK through IMPDH inhibition induces megakaryocytic differentiation-dependent apoptosis in this well-known drug-resistant cell line.

**Materials and Methods**

**Materials.** The cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Life technology). Cell line was obtained from Pasteur Institute of Iran. Wright-Giemsa, Hoechst 33258 were purchased from Sigma Chemical Co. Guanosine and hypoxanthine were purchased from Sigma Aldrich. Anti-GPIIb and anti-glycophorin A antibodies were purchased from IQ Products.

**Plant extraction and purification of 3-HK.** The powdered plant material (300 g) was extracted three times with methanol-water (1:1, v/v). The accumulated alcoholic extract was concentrated under reduced pressure and the volume was adjusted to 300 ml. The crude extract was then subjected to CHCl3 extraction for five times. The accumulated chloroform solution was concentrated under reduced pressure to a final volume of 1 ml. The 1 ml residue was fractionated on a silica gel column (1.4 x 50 cm), using diethyl ether as the eluting solvent, into six fractions. The active compound was purified from the sixth fraction using TLC technique. The developing system of TLC was a mixture of chloroform and diethyl ether (1:1, v/v). The relative mobility of the compound of interest in the above mentioned system was around 0.7. The characterization of the active component has been achieved as previously reported (Sadeghi et al., 2005).

**Cell culture.** The human K562 cell line was cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml). The cells were incubated under 5% CO2 humidified atmosphere at 37°C. Cell numbers and viabilities were assessed using a hemocytometer and the abilities of the cells to exclude trypan blue.

**Morphological evaluation of the differentiated cells.** Aliquots of the treated cells were fixed with methanol, stained with Wright-Giemsa and then examined under a light microscope at high magnification (400x). Differentiated cells were identified on the basis of cytoplasmic protrusions and the nuclear patterns.

**Flow cytometric analyses of cell surface markers.** Differentiation was also studied by evaluating the expression of GPIIb (as a marker of megakaryocytic differentiation) and glycophorin A (GPA, as a marker of erythroid differentiation). K562 cells (1 x 10⁶ cells) were treated with 3-HK then the cells were washed two times with PBS and resuspended in PBS containing 1% FBS and 0.1% sodium azide. Ten µL of mouse anti-human FITC-conjugated GPIIb monoclonal antibody was added to 100 µL of the cell suspension, and incubated for 30 min at 4°C. After washing, at least 10⁵ cells were analyzed by flow cytometry (Partec PAS).

**Cell cycle analysis by flow cytometry.** DNA content was determined by propidium iodide (PI) staining using a published procedure with slight modification (Wilhide et al., 1995). The cells (1 x 10⁶ cells/well) were seeded into culture dishes 24 h prior to treatment. After treatment with drug, cells were harvested and washed twice with PBS, fixed in 70% ethanol for at least 2 h at 4°C. The cells were then stained with 20 µg/ml propidium iodide containing 20 µg/ml RNase (DNase free) for 30 min at 37°C. The stained cells were analyzed by flow cytometry.

**Morphological study of the apoptotic cells.** The cells (1 x 10⁶ cells/well) were seeded in 24-well plates and treated with 3-HK for a time course of 96 h. Apoptosis was determined morphologically after staining the cells with Hoechst 33258 using fluorescence...
microscopy. Cells were washed with cold PBS and adjusted to a cell density of $1 \times 10^6$ using PBS. Hoechst 33258 solution (1 mg/1 ml ddH$_2$O) was added to the cell suspension in a final concentration of 100 µg/ml. The cellular morphology was evaluated by Axoscope 2 plus fluorescence microscopy (ZEISS).

**Flow cytometry analyses of the apoptotic cells with FITC-Annexin V and propidium iodide (PI) double staining.** After collecting and washing twice with PBS, the treated and/or untreated cells were resuspended in the binding buffer (100 µl of calcium buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$). FITC-Annexin V (10 µl) was added to the cells followed by the addition of 10 µl PI (50 µg/ml of PBS). The samples were then incubated for 10 min in the dark at 4°C and then subjected to flow cytometry evaluation.

**Statistical analyses.** Data are expressed as mean ± SD of three independent experiments and statistically analyzed using Student's t-test. Values of $p < 0.05$ were considered significant.

**Results**

**Induction of megakaryocytic differentiation by 3-HK.** Treatment of K562 cells with a single dose of the drug (12 nM) inhibited proliferation by almost 50% after 3 days of treatment (data not shown). Phase contrast microscopic examination of the drug-treated K562 cells revealed a distinct alteration in cell morphology that is apparent after 12 h exposure to 3-HK, as reflected by the appearance of rough edges at the cell surface as reported for the same cell line treated with TPA (Herrera et al., 1998). Within 48 to 96 h, the cells found megakaryocytic morphology characterized by a sharp increase in nuclear-to-cytoplasm ratio, while the control cells were morphologically consisted of a homogenous population of immature blast-like cells (Fig. 1A). Megakaryocytic features such as enlargement of nuclear size and cytoplasmic mass are hardly detectable among untreated control samples while these criteria are easily observed among the cells exposed for 3 days to a single dose of the drug, following their staining with Wright-Giemsa (Fig. 1B).

**Effect of 3-HK on the expression of cell surface megakaryocytic marker.** To assess megakaryocytic differentiation in K562 cells, we measured the relative expression of surface antigen, GPIIb which is part of the platelet/megakaryocyte receptor complex GPIIb/IIIa (Lepage et al., 2000; Szalai et al., 2006). As indicated in Fig. 2A, the cell surface content of GPIIb increased by 10, 24 and 36% after exposing the cells to a single dose of the drug for 48, 72 and 96 h, respectively.

In order to confirm the megakaryocytic but not the erythroid differentiation of the treated K562 cells, we measured the glycophorin A content of the cells after 72 h of treatment. Glycophorin A is a sialoglycoprotein which appears on the cell surface of human erythrocyte and erythroblast progenitor cells and therefore, it is considered as a useful differentiation marker toward erythroblast (Robinson et al., 1981). Based on the flow cytometry analyses (Fig. 2B) the cell surface of the

![Fig. 1](image-url)

Fig. 1. Morphological changes of K562 cells treated with a single dose of 3-HK (12 nM). Photomicrographs of the cells were taken by an inverted microscope at 400× magnifications (A). As it is evident, after 48 h the cell size increased and progressively continued over 96 h incubation period (white arrow). Some of the treated cells died after 96 h (black arrow). Wright-Giemsa staining of the drug treated cells (B). Megakaryocytic features such as increase in nuclear size and cytoplasmic mass are clearly evident after 48-72 h of drug exposure relative to the control cells.
Induction of Megakaryocytic Differentiation of K562 Cells

Induction of apoptosis by 3-HK in K562 cells. Treatment of the cells with 3-HK decreases the viability of the cells. Analyses of K562 cells treated with 12 nM 3-HK by Hoechst 33258 staining showed that the drug induces apoptosis in a time-dependent manner (Fig. 4A, B) with major effects after longer treatment times (72 and 96 h). In Fig. 4B, the viable cells are uniformly blue, whereas the apoptotic cells are blue and contain bright blue dots in their nuclei, representing the nuclear fragmentation. These results are consistent with our previous reports concerning the effect of 3-HK on HL-60 (Yazdanparast et al., 2005) and NB4 cells (Moosavi et al., 2006). To further confirm the occurrence of apoptosis, we subjected the drug-treated cells (96 h of drug exposure) to Annexin V/PI double staining followed by flow cytometry analyses. As shown in Fig. 4C, almost 30% of the cells underwent apoptosis after exposing to a single dose of the drug. Similar investigation indicated that the extent of apoptosis was dose dependent (data not shown).

Induction of differentiation by 3-HK in the presence of guanosine and hypoxanthine. As previously stated, IMPDH is the main target of 3-HK (Moosavi et al., 2005b). Inhibition of IMPDH by the drug leads to inhibition of cell growth, as well as differentiation (Moosavi et al., 2006) and induction of apoptosis (Moosavi et al., 2005b). Inhibition of IMPDH activity leads to a profound reduction in guanine nucleotide pool size followed by significant reduction in the extent of nucleic acid synthesis among the treated cells (Moosavi et al., 2005b). We and others have previously reported that guanosine at 25-50 µM through salvage pathway compensate for GTP depletion in various leukemia cell lines (Batiuk et al., 2001; Moosavi et al., 2005a). As shown in Fig. 5, simultaneous addition of 3-HK (12 nM) and guanosine (50 µM) to K562 cells inhibited differentiating effects of the drug which was assessed by evaluating the extent of GPIIb expression after

A. GPIIb
B. Glycophorin A

Fig. 2. Fluorescence histograms of K562 cells stained with FITC-labeled anti-GPIIb (A) and FITC-conjugated anti-GPA (B) antibodies. Cells were incubated for 4 days in the absence or presence of 3-HK and then subjected to flow cytometry analyses. Respective controls are presented as inset in each figure.

Fig. 3. Ploidy analysis of K562 cells treated with 3-HK. Cells were stained with propidium iodide (PI) and then analyzed by flow cytometry. After 72 h of incubation with 3-HK (B), a significant population of 4N cells and some 8N cells became evident.

Polyploidization of K562 is induced by 3-HK. Polyploidization is a unique feature of megakaryocytes in which repeated rounds of DNA replication occur without concomitant cell division (Szalai et al., 2006). The process of polyploidization, also known as endomitosis, is a consequence of abortive mitosis which is characterized by the failure of cells to exit mitosis after completing their DNA syntheses (Vitrat et al., 1998). To further characterize the effect of 3-HK on megakaryocytic differentiation of K562 cells, DNA content analysis by flow cytometry was performed on propidium iodide stained cells (Fig. 3). After 72 h of treatment, a large fraction of cells, with a 2N DNA content, have gained 4N DNA contents and even some cells with DNA content as high as 8N were detected (Fig. 3B). Similar to our observation, erythroleukemia cells such as HEL and K562 that have been exposed to TPA treatments have undergone limited endomitosis resulted in low level of ploidy (Long et al., 1990; Yen et al., 1993). Our results clearly indicated that 3-HK, at 12 nM, had minimal cytotoxic effect and resulted in efficient polyploidization of K562 cells.

Polyploidization and endomitosis are unique features of megakaryocytes in which repeated rounds of DNA replication occur without concomitant cell division (Szalai et al., 2006). The process of polyploidization, also known as endomitosis, is a consequence of abortive mitosis which is characterized by the failure of cells to exit mitosis after completing their DNA syntheses (Vitrat et al., 1998). To further characterize the effect of 3-HK on megakaryocytic differentiation of K562 cells, DNA content analysis by flow cytometry was performed on propidium iodide stained cells (Fig. 3). After 72 h of treatment, a large fraction of cells, with a 2N DNA content, have gained 4N DNA contents and even some cells with DNA content as high as 8N were detected (Fig. 3B). Similar to our observation, erythroleukemia cells such as HEL and K562 that have been exposed to TPA treatments have undergone limited endomitosis resulted in low level of ploidy (Long et al., 1990; Yen et al., 1993). Our results clearly indicated that 3-HK, at 12 nM, had minimal cytotoxic effect and resulted in efficient polyploidization of K562 cells.

Induction of differentiation by 3-HK in the presence of guanosine and hypoxanthine. As previously stated, IMPDH is the main target of 3-HK (Moosavi et al., 2005b). Inhibition of IMPDH by the drug leads to inhibition of cell growth, as well as differentiation (Moosavi et al., 2006) and induction of apoptosis (Moosavi et al., 2005b). Inhibition of IMPDH activity leads to a profound reduction in guanine nucleotide pool size followed by significant reduction in the extent of nucleic acid synthesis among the treated cells (Moosavi et al., 2005b). We and others have previously reported that guanosine at 25-50 µM through salvage pathway compensate for GTP depletion in various leukemia cell lines (Batiuk et al., 2001; Moosavi et al., 2005a). As shown in Fig. 5, simultaneous addition of 3-HK (12 nM) and guanosine (50 µM) to K562 cells inhibited differentiating effects of the drug which was assessed by evaluating the extent of GPIIb expression after

Fig. 2. Fluorescence histograms of K562 cells stained with FITC-labeled anti-GPIIb (A) and FITC-conjugated anti-GPA (B) antibodies. Cells were incubated for 4 days in the absence or presence of 3-HK and then subjected to flow cytometry analyses. Respective controls are presented as inset in each figure.

Fig. 3. Ploidy analysis of K562 cells treated with 3-HK. Cells were stained with propidium iodide (PI) and then analyzed by flow cytometry. After 72 h of incubation with 3-HK (B), a significant population of 4N cells and some 8N cells became evident.

(A) GPIIb
(B) Glycophorin A

Fig. 4. Ploidy analysis of K562 cells treated with 3-HK. Cells were stained with propidium iodide (PI) and then analyzed by flow cytometry. After 72 h of incubation with 3-HK (B), a significant population of 4N cells and some 8N cells became evident.
Fig. 4. Effect of 3-HK on apoptosis of K562 cells. Cells were treated with 12 nM of 3-HK for different times. The percentage of apoptotic cells progressively increased (A). Treated cells were stained with Hoechst 33258 and then evaluated using a fluorescent microscopy after 48 h (B). Apoptosis was confirmed by Annexin V/PI staining after 96 h of drug treatment (C).

Fig. 5. The effect of guanosine and hypoxanthine on the induction of K562 differentiation after 72 h of treatment. The cells treated with 12 nM of 3-HK in the presence or absence of 50 µM guanosine or 100 µM hypoxanthine separately. Differentiation was assessed by evaluating the extent of GPIIb expression using flow cytometry technique.
72 h of treatment (Fig. 5D). Similar observations have been observed using HL-60 (Yazdanparast et al., 2005) and NB4 cell lines (Moosavi et al., 2006).

On the other hand, the alternative pathway for guanine nucleotide biosynthesis is through the salvage of guanine to GMP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). We evaluated the response of the 3-HK-treated cells to exogenous addition of hypoxanthine (100 μM) which is an inhibitor of HGPRT. As shown in Fig. 5 (E and F), hypoxanthine had no measurable effect on the extent of differentiation of the treated cells measured in terms of GPIIb cell surface marker. However, our previous data have indicated that hypoxanthine treatment in the presence of 3-HK potentiated the extent of apoptosis (Moosavi et al., 2005b). This data collectively indicate that differentiating effect of 3-HK on K562 cells is exerted mainly through the de novo rather than the salvage pathway.

Discussion

Therapeutic strategies that focus predominantly on achieving the removal or death of cancer cells such as chemotherapy have a number of predominant disadvantages and toxic side effects. While differentiation-inducing therapy seems to be a promising approach, especially in elderly patients who can not tolerate intensive chemotherapy or bone marrow transplantation (Li et al., 1998), many clinical trials have been based on using either differentiating agents alone or in combination with chemotherapy in myelodysplastic syndrome or CML (Sillaber et al., 2003). However, none of these agents have therapeutic potency comparable to all-trans-retinoic acid (ATRA) in acute promyelocytic leukemia (APL) (Wiernik et al., 1991). Thus, discovery of new agents with differentiation capability and their use in differentiation therapy as a promising strategy in the treatment of CML has been considered in the clinical field.

The data presented here suggested that 3-HK is a novel anti-leukemic agent that can induce differentiation in K562 cell line. According to flow cytometry analyses, induction of megakaryocytic differentiation of K562 cells with nanomolar dose of 3-HK enhanced GPIIb expression in a time-dependent manner and down-regulated GPA expression. This event seems very similar to the effect of TPA on inducing megakaryocytic differentiation of K562 cell line (Murata et al., 1993). We also observed that differentiating efficiency of the drug at concentration above 12 nM decreased which may be due to cytotoxic effects of the drug at higher doses.

Further studies with K562 leukemia cell line revealed that inhibition of IMPDH, and the consequent guanine nucleotide depletion by griddinatoimoneine (Yazdanparast and Sadeghi, 2004) and/or 3-HK (Moosavi et al., 2005b) caused both cell cycle arrest and apoptosis. Inhibition of IMPDH by 3-HK has also been shown to result in the differentiation of leukemia cell lines such as HL-60 (Yazdanparast et al., 2005) and NB4 (Moosavi et al., 2006). Previously, it has been shown that inhibition of IMPDH activity by 3-HK has been associated with GTP depletion and the exogenous addition of guanine or guanosine has diminished the drug effect. Therefore, it can be concluded that the drug-mediated differentiation is linked to a decrease in GTP pool size with the consequent involvement of specific signal transduction pathway(s). Moreover, blocking of the guanine nucleotide salvage pathway by hypoxanthine did not show any detectable effect on the extent of cell differentiation toward megakaryocytic lineage. These data clearly support the strong involvement of 3-HK in the de novo process of guanine nucleotide biosynthetic pathway.

On the other hand, in many similar investigations apoptosis has been the final outcome of cell differentiation (Watson et al., 1997; Yazdanparast et al., 2006). For instance, apoptosis plays an important role in the elimination of activated granulocytes and monocytes (Martin et al., 1990). Our data also clearly indicated that the final fate of the differentiated K562 cells, under the influence of 3-HK was apoptosis.

In summary, these data clearly indicate that 3-HK is a potent and a novel natural substance capable of inhibiting cell proliferation, inducing megakaryocytic differentiation and apoptosis of K562 cells. Based on these criteria, 3-HK might be a good candidate for CML therapy.

Acknowledgments

The authors appreciate the financial support of this investigation by the research council of University of Tehran.

References


Induction of Megakaryocytic Differentiation of K562 Cells


