Semisynthetic homoharringtonine induces apoptosis via inhibition of protein synthesis and triggers rapid myeloid cell leukemia-1 down-regulation in myeloid leukemia cells

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Abstract

Semisynthetic homoharringtonine (ssHHT) is now being evaluated in phase II clinical trials for the treatment of chronic myelogenous leukemia and acute myelogenous leukemia patients. Here, we examined the mechanism of the apoptosis induced by ssHHT in myeloid leukemia cells. First, we have shown that ssHHT induces apoptosis in HL60 and HL60/MRP cell lines in a time- and dose-dependent manner, and independently of the expression of Bax. The decrease of mitochondrial membrane potential and the release of cytochrome *c* were observed in the apoptotic cells induced by ssHHT. To unveil the relationship between ssHHT and the mitochondrial disruption, we have shown that ssHHT decreased myeloid cell leukemia-1 (Mcl-1) expression and induced Bcl-2 cleavage in HL60 and HL60/MRP cell lines. The Bcl-2 cleavage could be inhibited by the Z-VAD.fmk caspase inhibitor. However, Mcl-1 turnover was very rapid and occurred before caspase activation. The Mcl-1 turnover was only induced by ssHHT and cycloheximide, but not by daunorubicin and cytosine arabinoside, and could be restored by proteasome inhibitors. Second, we confirmed that ssHHT rapidly induced massive apoptosis in acute myelogenous leukemia patient cells. We have also confirmed the release of cytochrome *c* and a rapid turnover of Mcl-1 in these patient cells, taking place only in apoptotic cells induced by ssHHT but not in cells undergoing spontaneous apoptosis. Finally, we have shown that ssHHT inhibits protein synthesis in both cell line and patient cells. We suggest that the inhibition of protein synthesis and resulting Mcl-1 turnover play a key role in the apoptosis induced by ssHHT. Our results encourage further clinical trials for the use of ssHHT in acute myelogenous leukemia. [Mol Cancer Ther 2006; 5(3):723–31]

Introduction

Homoharringtonine (HHT) is a cephalotaxine ester initially extracted from the bark of *Cephalotaxus* (natural HHT). Natural HHT has been shown to inhibit cell growth and induce cell differentiation and apoptosis. Still, little is known about the mechanism of its action. Natural HHT seems to inhibit protein synthesis by acting on early peptide elongation (1), and the apoptosis induced by natural HHT seems to happen via the translocation and up-regulation of the proapoptotic protein Bax (2). Natural HHT, both alone and in combination with IFN-α and low-dose cytarabine, has been investigated in chronic myeloid leukemia patients in late and early chronic phase of the disease. Both the complete hematologic remission and the cytogenetic response rates in patients treated with natural HHT and INF-α were significantly higher than those seen in historical control patients receiving only INF-α therapy (3). Triple therapy with natural HHT, INF-α, and cytarabine, followed by imatinib, resulted in an estimated 5-year survival rate of 88% (4). Some encouraging results were also obtained in refractory or relapsed acute myelogenous leukemia (AML; ref. 5).

J-P. Robin (6) was the first to semisynthesize HHT from cephalotaxine extracted from dry leaves of *Cephalotaxus* (the natural HHT is extracted from the bark of *Cephalotaxus*). For 1 kg of the natural HHT, ~70 times more *Cephalotaxus* is needed than for the semisynthetic HHT (ssHHT). Furthermore, the ssHHT has a higher purity (99.7%) than the natural HHT. The action of the ssHHT (Stragen Pharma, Geneva, Switzerland) is now being evaluated in chronic myelogenous leukemia and AML patients (phase II clinical trials). Recently, a phase I/II study in chronic myelogenous leukemia patients showed that the addition of ssHHT could reduce the level of residual disease in patients with Ph-positive chronic myelogenous leukemia who seemed to had achieved a suboptimal response to imatinib (7).

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Activation of apoptosis pathways is a key mechanism by which anticancer drugs kill tumor cells. The two known major apoptosis pathways are the intrinsic pathway initiated by the mitochondria and the extrinsic pathway initiated by cell surface receptors. The Bcl-2 family proteins regulate mitochondrial changes during both apoptosis and necrosis (8, 9). Proapoptotic members of this family, including Bax, Bak, Bid, and Bad promote apoptosis, whereas antiapoptotic members, including Bcl-2, Mcl-1, and Bcl-x_L inhibit apoptosis (10). Antiapoptotic members are present in the mitochondrial outer membrane and they oppose the channels formed by proapoptotic members (11). Thus, the investigation of these targets could be extremely important.

In our study, we examined the mechanism of the apoptosis induced by the ssHHT in myeloid leukemia cells. We showed that ssHHT promptly induces apoptosis in AML patient cells, and we were able to discern the apoptotic pathways induced by ssHHT. We also showed that ssHHT inhibits the protein synthesis and triggers the turnover of myeloid cell leukemia-1 (Mcl-1), one of the antiapoptotic proteins of the Bcl-2 family.

Materials and Methods

Cell Cultures

The studies were carried out with the myeloid leukemia cells (HL60) and its derivation, the HL60/MRP cell line (12), expressing MRP1 and lacking the expression of Bax. The cells were cultured in RPMI 1640 containing 10% FCS, penicillin 50 units/mL, and streptomycin 50 μg/mL and incubated in a humidified atmosphere containing 5% CO_2. ssHHT was provided by Stragen Pharma. Z-VAD.fmk (caspase inhibitor) was purchased from Biomol (Plymouth Meeting, PA) and Alexis Biochemicals (Coger, France), respectively. MG-132, lactacystin (proteasome inhibitors), and cycloheximide (protein synthesis inhibitor) were purchased from Sigma (St. Quentin Fallavier, France).

Patients

Peripheral blood samples from 16 AML patients were collected after their informed consent had been obtained. Mononuclear cells were isolated using Ficoll-Hypaque density gradient. The percentage of blasts was >80%. The fresh leukemia cells were cultured in the same general conditions as the cell lines.

Apoptosis Assay

Cells (10^6) were stained with Annexin V–FITC and propidium iodide in calcium-HEPES buffer for 15 minutes, as instructed by the manufacturer (Roche, Meylan, France). The percentage of apoptotic cells was determined by flow cytometry.

Western Blotting Analysis

Whole cell extracts were prepared by lysis with radioimmunoprecipitation assay buffer [Tris-HCl 10 mmol/L (pH 7.5), NaCl 150 mmol/L, SDS 0.1%, sodium deoxycholate 1%, NP40 1%, β-flycerophosphate 10 mmol/L, Na-orthovanate 1 mmol/L, NaF 10 mmol/L, leupeptin 10 μg/mL, aproptinin 10 μg/mL, and phenylmethylsulfonyl fluoride 100 μg/mL]. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Fifty micrograms of total protein were put on 7.5%,12%, or 15% SDS-PAGE gel for separation. The proteins in the gel were transferred to Immobilon-P transfer membranes (Millipore, St. Quentin en Yvelines, France). The blots were blocked with 5% milk in TBS-Tween (0.05%) for 1 hour. Membranes were incubated with primary antibodies for 2 hours at room temperature, and then washed thrice for 10 minutes in TBS-T. The membranes were blotted with an appropriate horseradish peroxidase–linked secondary antibody for 1 hour at room temperature. The proteins were then visualized with a chemiluminescence assay system (ECL Detection, Amersham, Orsay, France). Mcl-1: clone S-19, a polyclonal antibody raised against a peptide mapped within an internal region of human original Mcl-1, Bcl-xL, and Bax antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-2, cytochrome *c*, caspase-3, and poly(ADP)ribose polymerase antibodies were from PharMingen (San Diego, CA); and actin antibody was from ICN Biomedicals (Vannes, France).

Mitochondria and Cytosol Fractionation

Collected cells (10^7) were suspended in cytosol extraction buffer containing 400 μg/mL digitonin, 80 mmol/L KCl, 250 mmol/L sucrose, and protease inhibitors. The cells were homogenized with a pipette and left on ice for 10 minutes, then centrifuged at 700 × *g* for 10 minutes at 4°C. The supernatant was collected and the pellet was discarded. The supernatant was again centrifuged at 10,000 × *g* for 60 minutes at 4°C. The supernatant now obtained was used as the cytosolic fraction, and the pellet was suspended in radioimmunoprecipitation assay buffer as described above and used as the mitochondrial fraction.

Mitochondrial Membrane Potential

Changes in the inner mitochondrial transmembrane potential were determined by $DiOC_6(3)$ (Molecular Probes Cergy Pontoise, France; ref. 13).

Caspase-3, Caspase-8, and Caspase-9 Enzymatic Activity

A fluorimetric assay kit (R&D Systems, Minneapolis, MN) was used to determine the enzymatic activity of caspase-3, caspase-8, and caspase-9 according to the instructions of the manufacturer. The enzymatic reaction was done on a 96-well microplate. The cells were first lysed and 200 μg total protein in 50 μL cell lysate were mixed with 50 μL reaction buffer. The cell lysate was then tested for protease activity by addition of a caspase-specific peptide that was conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (for caspase-3: DEVD-AFC, for caspase-8: IETD-AFC, for caspase-9: LEHD-AFC). The cleavage of the respective peptide by the appropriate caspase released the fluorochrome that, when excited by light at the wavelength of 400 nm, emitted fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate was directly proportional to the fluorescence signal detected with a fluorescent micro plate reader (Wallac Victor, Perkin-Elmer, Boston, MA).

Analysis of Protein Synthesis

The effect of ssHHT on protein synthesis was studied as previously described (14). Cell line cells were seeded into 12-well plates at 4×10^5 per well under standard culture conditions for 24 hours. The cells were in their potential growth phase and the different concentrations of ssHHT were added to the medium. The patient cells were seeded into 12-well plates at 4×10^6 per well and ssHHT was added directly. Following 15 minutes of incubation at 37°C, 1 μCi/well of L-[^{35}S]methionine (1,175.0 Ci/mmol, Perkin-Elmer, Boston, MA) was added to the medium. After additional incubation of 1, 2, 4, or 5 hours, the cells were washed once by PBS and fixed with 5% trichloroacetic acid then washed thrice with ethanol. Then, the cells were dried, dissolved in 200 μL of 0.1 mol/L NaOH containing 0.1% SDS, and the radioactivity was measured on a LKB 1209 RACKBETA liquid scintillation counter. Results were expressed as percentage of the radioactivity incorporated in the control cells.

Results

ssHHT Induces Apoptosis in HL60 Cells Independently of Bax

Our studies were carried out on the HL60 cell line and its derivative, the HL60/MRP cell line, which has a defective Bax expression. The ssHHT was used in the concentration of ≤90 ng/mL because the pharmacokinetic studies (15) had previously shown the mean plasma concentration of ssHHT to be 10.5 ± 8.8, 78.0 ± 18.2, and 96.1 ± 20.3 ng/mL following administration of ssHHT in the dose of 0.5, 3, and 5 mg/m^2/d, respectively. ssHHT showed strong induction of apoptosis in both cell lines in a dose- and time-dependent manner (Fig. 1), irrespective of the fact that the HL60/MRP cells do not express the Bax protein. In addition, ssHHT induced apoptosis more rapidly in HL60/MRP cells than in the HL60 cells.

ssHHT Triggers Decrease of Mitochondrial Membrane Potential and Cytochrome *c* Release

To evaluate the involvement of the mitochondria, we investigated the mitochondrial membrane potential and the release of cytochrome *c* after exposure of HL60 and HL60/MRP cell lines to ssHHT. HL60 and HL60/MRP cells were treated with 30, 60, and 90 ng/mL of ssHHT. On day 2, both the control and the treated cells were labeled with $DiOC_6(3)$, and the $DiOC_6(3)$ fluorescence intensity was shown to be decreased in cells treated with the ssHHT (Fig. 2A), indicating mitochondrial disruption. Western blotting was done to monitor the expression of cytochrome *c* in both the cytosol and mitochondrial fractions. Although cytochrome *c* expression increased in the cytosol fraction, it decreased in the mitochondrial fraction. In both cell lines, the ssHHT was shown to provoke the release of the proapoptotic mitochondrial protein, cytochrome *c*, in a dose-dependent manner (Fig. 2B).

ssHHT Triggers Caspase-3 and Caspase-9 but not Caspase-8 Activity

Mitochondrial disruption and cytochrome *c* release trigger caspase-dependent apoptosis. In the caspase-dependent pathway, caspase-3 serves as an effector molecule, cleaving proteins, including the poly(ADP-ribose) polymerase (PARP). We detected caspase-3 activation in ssHHT-treated cells and showed the increase of caspase-3 activity (Fig. 3). We confirmed the results with immunoblotting, which showed the cleavage of both caspase-3 with appearance of the 11 kDa active protease and the downstream substrate PARP (data not shown).

Caspase-3 activity can be initiated through a variety of signaling cascades, including the tumor necrosis factor receptor (caspase-8) and the mitochondria (caspase-9) apoptosis pathway. To characterize further the apoptotic pathway of caspase-3 activation initiated by ssHHT, we examined if there was an increase of the activity of either caspase-8 or caspase-9 following treatment with ssHHT. The two cell lines were exposed to ssHHT at 60 ng/mL for 4,

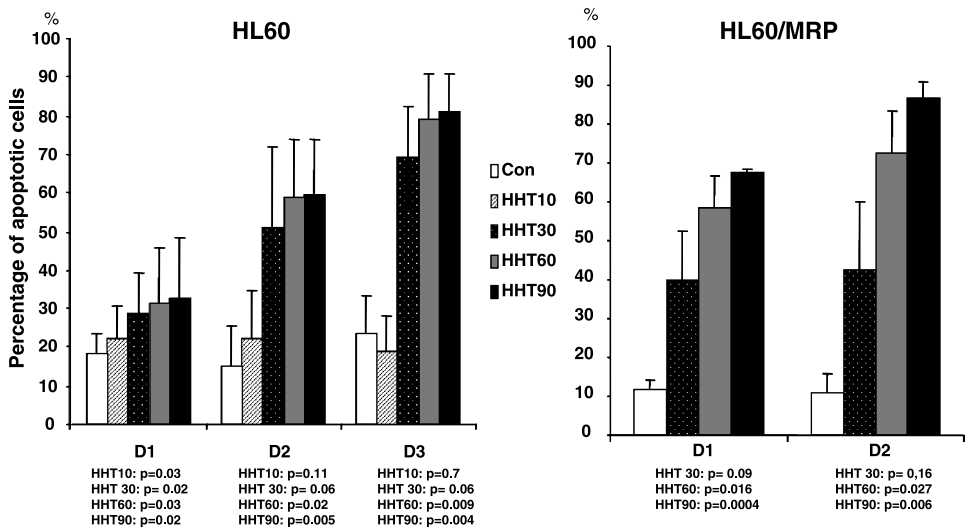

Figure 1. Apoptosis induced by ssHHT in HL60 and its derivative, the HL60/MRP, cells. HL60 and HL60/MRP cells were treated with ssHHT at different concentrations: 10, 30, 60, and 90 ng/mL for 1, 2, and 3 d, and the Annexin V/propidium iodide staining for apoptosis was done. For HL60/MRP cells, we did not perform the test following the 3rd day because of too many cells that died. *Columns,* percentages of apoptotic cells under different conditions. All the experiments were done in triplicate. Paired *t* test was used for statistic analysis. At 60 and 90 ng/mL of ssHHT, all the *P* values were significant ($P < 0.05$).

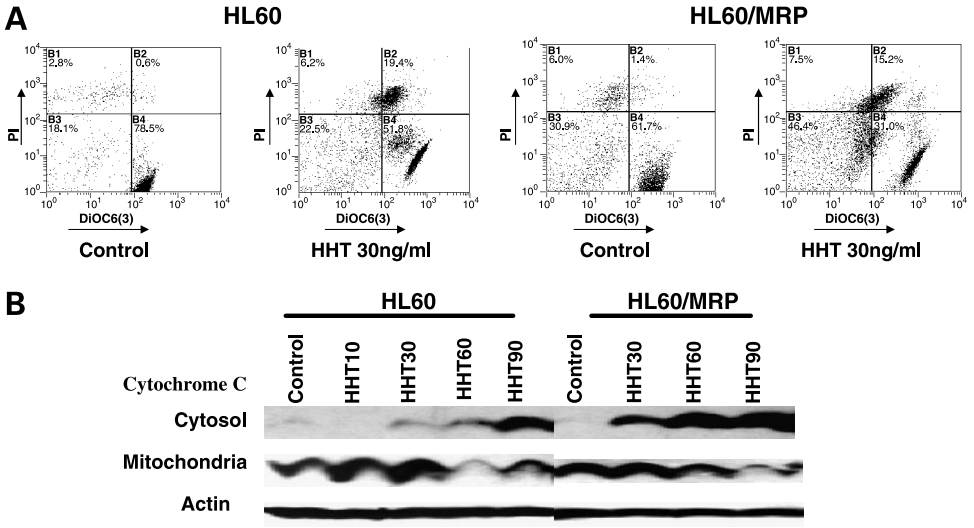

Figure 2. Decrease of mitochondrial membrane potential (*MMP*) and release of cytochrome *c* in the cells treated with ssHHT. **A,** flow cytometry was used to measure the change in $DiOC_6(3)$ and propidium iodide fluorescence intensity. Compared with controls, $DiOC_6(3)$ fluorescence intensity decreased and propidium iodide fluorescence intensity increased in the HL60 and HL60/MRP cells treated with ssHHT. Control cells and cells treated with ssHHT at 30 ng/mL. **B,** HL60 and HL60/MRP cells were treated with ssHHT at 10, 30, 60, and 90 ng/mL. Cytosol and mitochondrial extractions of treated cells were prepared, and cytochrome *c* expression was examined by Western blotting.

8, 16, and 24 hours, and subsequently analyzed for caspase-3, caspase-8, and caspase-9 activity. HL60 and HL60/MRP cells exposed to ssHHT displayed maximal caspase activity at 16 and 8 hours, respectively. These data, depicted in Fig. 3, show that the activity of caspase-3 and caspase-9 increased 16 and 8 hours following treatment, whereas the caspase-8 activity changed only minimally. We confirmed these findings by immunoblotting, showing no decrease in procaspase-8 and no cleaved active caspase-8 (data not shown). Because caspase-8 can cross-activate caspase-9 through cleavage of Bid, using immunoblotting, we also checked for the cleaved Bid, but it showed no Bid cleavage (data not shown). Therefore, we concluded that the apoptosis induced by ssHHT was caspase-8 independent and caspase-9 dependent: The increased activity of caspase-9 seemed to be induced by the mitochondrial disruption and cytochrome *c* release observed following the administration of ssHHT.

ssHHT Decreases Mcl-1 Expression in a Caspase-Independent Manner and Leads to Caspase-Dependent Bcl-2 Cleavage

To unveil the relationship between ssHHT and the mitochondrial disruption, we investigated the expression of the Bcl-2 family proteins. We showed ssHHT to decrease Mcl-1 expression and induce Bcl-2 cleavage in both HL60 and HL60/MRP cells (Fig. 4A). However, there was no modification of Bcl-x_L expression in either HL60 or HL60/MRP cells (Fig. 4A), and there also was no modification of Bax expression in HL60 cells, whereas it is faulty in HL60/MRP cells (Fig. 4A).

To further disclose if the decrease in Mcl-1 expression and Bcl-2 cleavage was directly linked to ssHHT, or if it was the consequence of mitochondrial disruption and caspase activation, we used the pan-caspase inhibitor Z-VAD.fmk for the inhibition of the caspases. We added Z-VAD.fmk (20 μmol/L) to cell cultures 1 hour before ssHHT, and then examined the apoptosis, caspase-3, and caspase-9 activity and Mcl-1 and Bcl-2 expression. We observed the inhibition of activity of the caspase-3 and caspase-9 (Fig. 4B, *a*), and Bcl-2 cleavage by Z-VAD.fmk (Fig. 4B, *c*). However, the apoptosis was only partially inhibited (Fig. 4B, *b*) and Mcl-1 turnover was not prevented (Fig. 4B, *c*). Thus, Mcl-1 down-regulation was independent from caspase activity.

Mcl-1 Down-Regulation by ssHHT Is a Very Early Event Not Seen in Cells Treated with Daunorubicin and Cytosine Arabinoside and Can Be Restored by Proteasome Inhibitors

To further understand the role of Mcl-1 in apoptosis induced by ssHHT, we studied chronologically Mcl-1

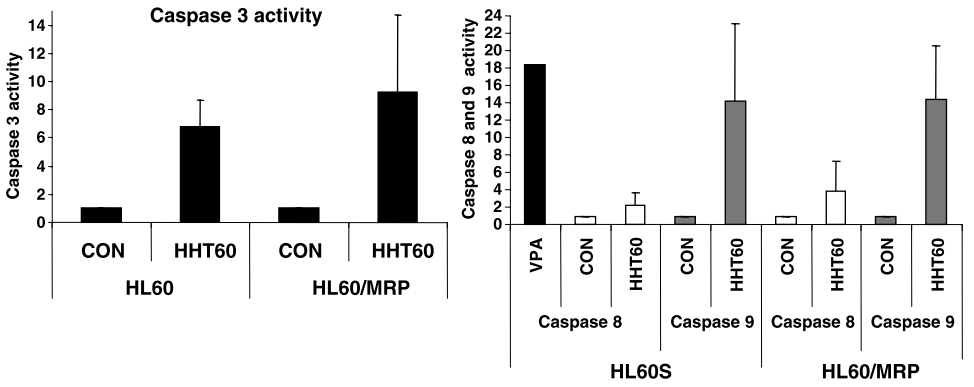

Figure 3. Caspase enzymatic activity assay. The enzymatic activities of caspase-3, caspase-8, and caspase-9 were measured after 16 h of exposure to ssHHT at 60 ng/mL for HL60 and 8 h for HL60/MRP cells, and compared with controls. HL60 cells were treated with valproic acid (*VPA*) for caspase-8-positive control (*CON*). The experiments were done in triplicate.

expression, caspase-3, and PARP cleavage. We treated the HL60 and Hl60/MRP cells with ssHHT at 60 ng/mL from 1 hour to 8 hours. As early as 2 hours, Mcl-1 expression started to down-regulate, whereas caspase-3 activation and PARP cleavage appeared after 4 hours (Fig. 5A). This suggested that Mcl-1 turnover was an initial event for the apoptosis. The down-regulation of all other proteins examined, Bax, Bcl-2, Bcl-x_L, AKT, and mitogen-activated protein kinase, as well as the emergence of Bcl-2 cleavage, could not be seen earlier than 8 hours following ssHHT administration.

Then, we wanted to verify whether this rapid Mcl-1 down-regulation was specific for ssHHT or if it could also be induced by other protein synthesis inhibitors or other chemotherapeutic drugs used in AML treatment. HL60 and HL60/MRP cells were exposed to ssHHT, cycloheximide (protein synthesis inhibitor), daunorubicin, and cytosine arabinoside (Ara-C), drugs currently used in AML treatments. Mcl-1 expression at 6 hours and apoptosis at 24 hours were examined. Mcl-1 expression was down-regulated in the cells treated with ssHHT and cycloheximide, but not in cells treated with daunorubicin and Ara-C (Fig. 5B). However, daunorubicin and Ara-C did induce massive apoptosis in these cells. The apoptosis induced by ssHHT, cycloheximide, daunorubicin, and Ara-C were, respectively, ~23%, 23%, 30%, and 41% for HL60 cells, and 61%, 52%, 58%, and 55% for HL60/MRP cells. It seems that Mcl-1 turnover is a target for ssHHT and also for cycloheximide, but not for daunorubicin and Ara-C.

Mcl-1 protein has a very short half-life due to the presence of a PEST sequence favoring its proteolysis by the caspases (16, 17), and also its degradation mediated through the proteasome. In different models, proteasome inhibitors could restore Mcl-1 expression (18, 19). We wanted to see whether Mcl-1 degradation triggered by ssHHT could be inhibited by proteasome inhibitors. We treated HL60 and HL60/MRP cells with MG-132 and lactacystin at 10 μmol/L 1 hour before adding ssHHT, and then we used Western blotting to examine the Mcl-1 expression. We could observe that both MG-132 and lactacystin could restore Mcl-1 expression, blocking the Mcl-1 degradation induced by ssHHT (Fig. 5C), but we

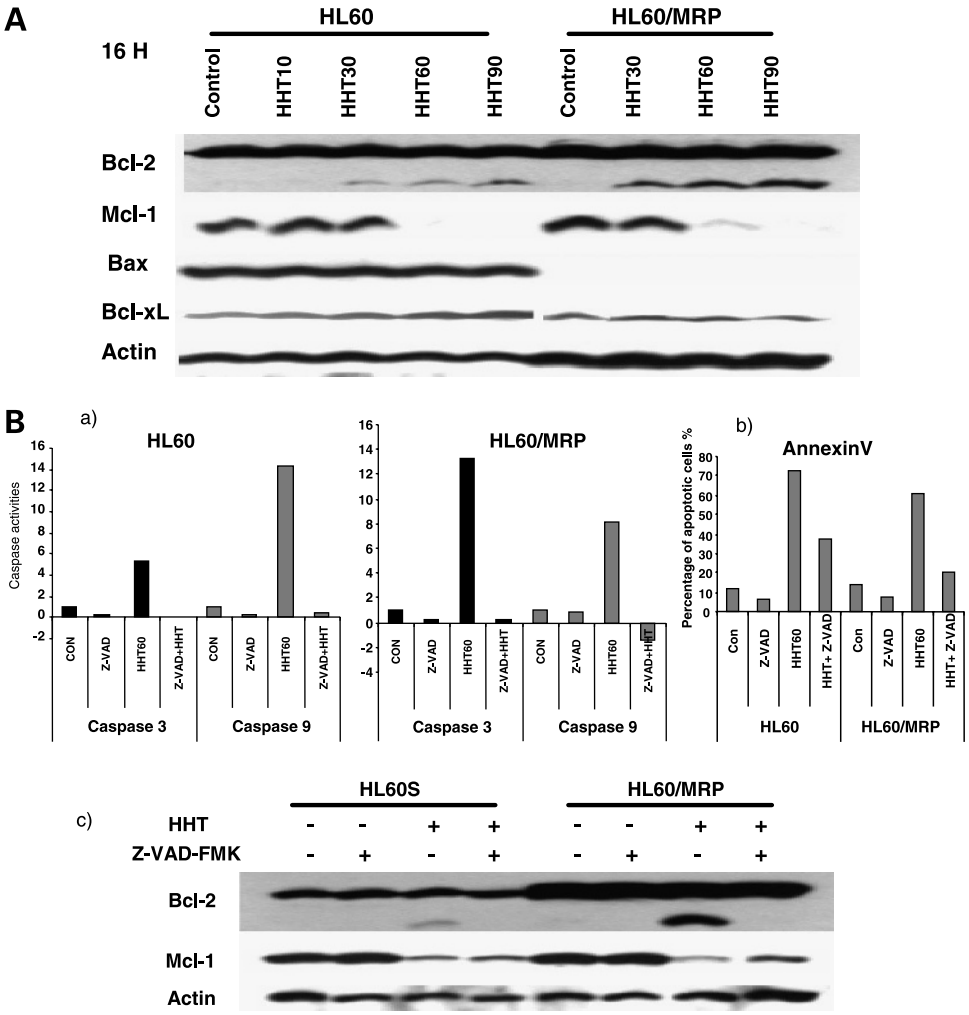

Figure 4. **A,** expression of Bcl-2 family proteins. The cells were treated with 10, 30, 60, and 90 ng/mL ssHHT for 16 h, and the protein expression was analyzed by Western blotting using Bcl-2-, Mcl-1-, Bcl-xL, and Bax-specific antibodies. Actin expression was used as the control. **B,** Z-VAD.fmk inhibits the activity of the caspases and prevents Bcl-2 cleavage, without down-regulation of Mcl-1. The cells were treated with Z-VAD.fmk at 20 μmol/L or ssHHT at 60 ng/mL, and with Z-VAD.fmk (20 μmol/L) and ssHHT (60 ng/mL). *a,* caspase-3 and caspase-9 activity was measured in HL60 and HL60/MRP cells after exposure to Z-VAD.fmk, ssHHT, or both for 16 h. *b,* percentage of apoptotic cells was determined by Annexin V/propidium iodide staining after 24 h following the treatment. *c,* Bcl-2 cleavage and Mcl-1 expression were analyzed after 16 h following the treatment as caspase activity by Western blotting. Actin expression was used as the control.

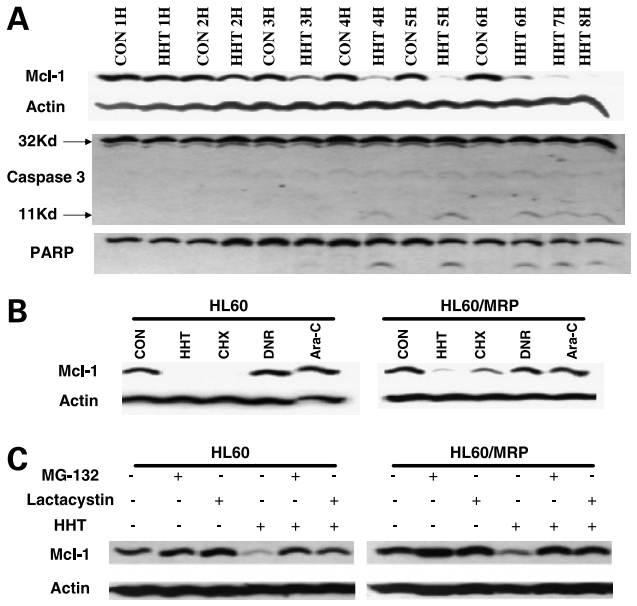

Figure 5. **A,** rapid Mcl-1 down-regulation followed by caspase-3 activation and PARP cleavage. The cells were treated with ssHHT at 60 ng/mL for 1 to 8 h. Mcl-1 expression and the activated form of caspase-3 and PARP cleavage were determined by Western blotting. Actin expression was used as the control. **B,** Mcl-1 expression was down-regulated by ssHHT and cycloheximide, but not by daunorubicin and Ara-C. The HL60 and HL60/MRP cells were treated with ssHHT (60 ng/mL), cycloheximide (10 μmol/L), daunorubicin (1 μmol/L), and Ara-C (1 μmol/L) for 6 h, and the expression of Mcl-1 was analyzed by Western blotting. **C,** Mcl-1 expression was restored by proteasome inhibitors. The HL60 and HL60/MRP cells were treated with MG-132 (10 μmol/L), lactacystin (10 μmol/L), or ssHHT (60 ng/mL) alone; and MG-132 or lactacystin combined with ssHHT for 6 h. The expression of Mcl-1 was analyzed by Western blotting.

were unable to establish if they could also prevent the apoptosis induced by ssHHT in these cells because MG-132 and lactacystin themselves strongly induced apoptosis in these cells.

ssHHT Promptly Induces Apoptosis in AML Patient Cells and Also Cytochrome *c* Release and Mcl-1 Degradation

We did *in vitro* ssHHT studies in AML patient samples with different concentrations of ssHHT up to 90 ng/mL within the range of plasma concentration of patients receiving ssHHT. Apoptosis was substantially and rapidly induced by ssHHT in AML patient cells. Massive apoptosis was already observed at 6 hours (Table 1) and it progressed in a time- and dose-dependent manner. Later on (at 24 hours), the apoptosis ceased to follow the dose of ssHHT, almost reaching the maximum (Table 1). Also, ssHHT induced much less apoptosis in normal lymphocyte cells compared with the patient cells (Table 1), and the apoptosis did not progress significantly in a time- and dose-dependent manner (Table 1). The cells of 16 AML patients (Table 2) treated with ssHHT were examined after 48 hours of culture by the Annexin V staining. The median apoptosis percentage was ~80%, ranging from 46% to 90% at the concentration of 15 ng/mL ssHHT. Ten of those 16 patients were clinically resistant to treatment (idarubicin or daunorubicin/AraC), whereas 8 of 10 resistant patient cells were sensitive to ssHHT *in vitro* (>70% apoptosis).

We also tested cytochrome *c* release, Mcl-1, and Bcl-2 expression in these AML cells. Western blotting showed cytochrome *c* release (Fig. 6A) in the cytosol fraction of the cells treated with ssHHT, as well as Mcl-1 degradation and Bcl-2 cleavage (Fig. 6B). When we treated the cells with Z-VAD.fmk (20 μmol/L) before adding ssHHT, we observed partial inhibition of Bcl-2 cleavage and apoptosis; however, Mcl-1 was again completely degraded (Fig. 6C).

In addition, we also observed Bcl-2 cleavage in the culture of control cells with spontaneous apoptosis but without Mcl-1 degradation (Fig. 6B and C). This strengthened our hypothesis that ssHHT was somehow involved in Mcl-1 down-regulation.

ssHHT Inhibits Protein Synthesis in Both Cell Line and Patient Cells

We have investigated whether ssHHT also inhibits protein synthesis. HL60, HL60/MRP, and patient cells were treated by different concentrations of ssHHT (15, 30, 60, and 90 ng/mL), cycloheximide (10 mmol/L), and daunorubicin (1 mmol/L). Cycloheximide and daunorubicin were used for comparative purposes because their action on protein synthesis is already known: Cycloheximide inhibits protein synthesis by blocking the peptidyl transferase reaction in the ribosome, whereas daunorubicin provokes the DNA disruption and has no effect on protein synthesis. The [^{35}S]methionine incorporation was measured after 1- and 5-hour incubation for cell lines, and 1- and 4-hour incubation for patient cells. All the experiments for the cell lines were done in triplicate. The results have shown that ssHHT was also inhibiting the protein synthesis in a dose- and time-dependent manner (Table 3). At 1 hour, the inhibition of [^{35}S]methionine incorporation by ssHHT at the concentration of 90 ng/mL arrived already at 89 ± 2%, 90 ± 1%, and 74% for HL60, HL60/MRP, and patient cells, respectively. These results were comparable with those of cycloheximide at 88 ± 1%, 93 ± 2%, and

Table 1. Apoptosis induced by ssHHT in patient and normal cells

	Control	ssHHT15	ssHHT30	ssHHT60	ssHHT90
A. Patient cells: percentage of apoptosis cells					
6H	6.7	26.2	42.6	55.5	73.5
16H	14.1	29.7	51.2	63.7	71.2
24H	18.3	70.1	77.6	80.4	79.5
B. Normal cells: percentage of apoptosis cells					
6H	12.3		18.4	14.6	20.4
16H	14.1		33.5	37.5	38.8
24H	10.9		35.1	42.1	44.6

Table 2. Clinical data of AML patients and apoptosis induced *in vitro* by ssHHT

No.	Age (y)	Morphology (FAB)	Leucocytes ($\times 10^9$/L)	Response	Control	ssHHT (15 ng/mL)
1	60	AML	5.1	RE	20.7	71.1
2	71	AML	204	NT	20.5	77.6
3	63	M2	15.2	RE	46.1	80.9
4	55	M1	35	RE	64.1	88.1
5	36	M1	15	CR	19.7	87.1
6	35	M4	81	CR	53.5	86.7
7	33	M5a	10	RE	15.7	53.6
8	55	AML	3.4	RE	38.5	94
9	74	M2	2.4	RE	11.3	70.3
10	35	M1	74	CR	52.5	84
11	22	M2	17.6	RE	23.8	76.1
12	42	M1	93	CR	24.5	90.1
13	35	M5	21	RE	61.5	87.8
14	67	M4	78	RE	24.9	53
15	76	M1	27.4	CR	18.3	70.1
16	70	M6	0.7	RE	17.8	82.5

NOTE: Three AML patients were not classified for French-American-British classification. Responses are for conventional treatments (idarubicin or daunorubicin/Ara C).
Abbreviations: FAB, French-American-British classification for AML; CR, complete remission; RE, resistance to treatments; NT, nontreated.

73% for HL60, HL60/MRP, and patient cells, respectively. At 1 hour with daunorubicin, there was no inhibition of [^{35}S]methionine incorporation. However, following 5 hours of incubation, daunorubicin inhibited 11% and 32% incorporation of [^{35}S]methionine for HL60 and HL60/MRP cells, respectively. We suggested that this decreased incorporation of [^{35}S]methionine could be the consequence of the induced apoptosis.

Discussion

Little is known about the mechanism of the action of natural HHT, and almost nothing is known about the action of ssHHT. According to the few publications available, apoptosis induced by natural HHT seemed to be mediated through the up-regulation and/or translocation of Bax (2). However, one of the first results obtained in our study was that ssHHT also induced apoptosis in HL60/MRP cells lacking Bax. Also, we did not observe any up-regulation of the expression of Bax in either HL60 or patient cells. Therefore, the expression of Bax at the outer mitochondrial membrane seems not to be necessary for the induction of apoptosis by ssHHT. We suggest that the change in Bax is only an event of cell signaling, after the cells have received the apoptotic signal.

Then, we have shown that the apoptosis induced by ssHHT was involved in mitochondrial disruption and the release of cytochrome *c*, resulting in the activation of the caspase-9 and caspase-3. In contrast, ssHHT did not trigger the activation of caspase-8 nor the Bid cleavage. Thus, ssHHT induced apoptosis via caspase-9 but not caspase-8.

Bcl-2 family plays a pivotal role in the mitochondrial membrane permeability regulation (20). We were interested to find out whether ssHHT was directly involved in the regulation of Bcl-2 family proteins, like the Bcl-2 antagonist HA14-1 (21). At the protein level, we observed Bcl-2 cleavage and strong Mcl-1 down-regulation. The pan-caspase inhibitor Z-VAD.fmk could prevent Bcl-2 cleavage, but not Mcl-1 down-regulation. This suggested that ssHHT was not directly responsible for Bcl-2 cleavage, and that Bcl-2 cleavage was just a consequence of caspase activation.

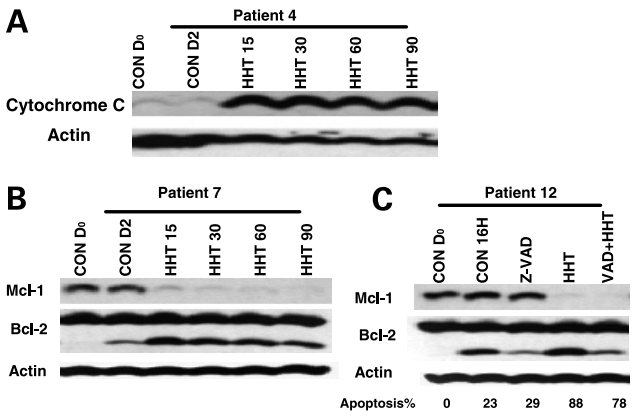

Figure 6. Induction of apoptosis, cytochrome *c* release, and Mcl-1 down-regulation by ssHHT in the cells of AML patients. **A,** patient cells were put in culture and treated with ssHHT at 15, 30, 60, and 90 ng/mL for 2 d. Cytosol extractions of the treated cells and the control were prepared, and cytochrome *c* expression was examined by Western blotting. **B,** analysis of Mcl-1 and Bcl-2 expression using Western blotting. **C,** patient cells were put in culture and treated with ssHHT (60 ng/mL) or Z-VAD.fmk (20 μmol/L) alone, as well as with ssHHT and Z-VAD.fmk for 16 h. The percentage of apoptotic cells was determined by Annexin V/propidium iodide staining, and the expression of Mcl-1 and Bcl-2 were analyzed using Western blotting.

Table 3. Percentage of protein synthesis inhibition measured by the incorporation of [^{35}S]methionine

	HHT15	HHT30	HHT60	HHT90	CHX10	DNR
HL60S						
1H	50 ± 6	68 ± 3	86 ± 2	89 ± 2	88 ± 1	0
5H	54 ± 15	78 ± 4	93 ± 4	97 ± 1	82 ± 8	11
HL60/MRP						
1H	26 ± 8	48 ± 6	77 ± 3	90 ± 1	93 ± 2	0
5H	7 ± 7	58 ± 3	86 ± 11	97 ± 2	92 ± 1	32
Patient cells						
1H	43	57	74	74	73	7
2H	58	76	84	88	70	3
4H	62	80	96	91	60	35

Abbreviation: CHX, cycloheximide.

The early event triggering the apoptosis by ssHHT was Mcl-1 down-regulation. In our study, we observed that ssHHT had triggered a rapid Mcl-1 turnover at 2 hours before the activation of the caspases, whereas no other protein studied—Bcl-2, Bax, Bcl-x_L, mitogen-activated protein kinase, and AKT—was down-regulated before 8 hours. In addition, Mcl-1 turnover could only be set off by ssHHT and cycloheximide, another protein synthesis inhibitor, but not by daunorubicin and Ara-C. Playing a key role in the initiation of the apoptosis induced by ssHHT, we suggest that this Mcl-1 turnover is specific for ssHHT or other protein synthesis inhibitors. Mcl-1 was originally identified as an antiapoptotic Bcl-2 family protein during differentiation of myeloid cells (16). It was shown that Mcl-1 played a critical role in the survival of leukemia cells, because antisense depletion of Mcl-1 triggered apoptosis in leukemia cells (22, 23). Also, granulocyte-macrophage colony-stimulating factor signaling and proteasome inhibition delayed the apoptosis of neutrophils through stabilization of Mcl-1 (19). Mcl-1 down-regulation induced by ssHHT was restored by proteasome inhibitors, but we were unable to confirm protection against apoptosis induced by ssHHT, because the proteasome inhibitors per se were inducing apoptosis. The permeabilization of the inner mitochondrial membrane is also primordial. Using two specific inhibitors of the permeabilization of the inner mitochondrial membrane, bongkrekic acid and cyclosporin A, we failed to inhibit the apoptosis induced by ssHHT, suggesting that inner mitochondrial permeability transition pores were not involved in the induction of apoptosis by ssHHT.

Second, ssHHT induced massive apoptosis in patient cells *in vitro* at 15 ng/mL, which is within the range of plasma concentration of patients receiving ssHHT, as early as 6 hours following the administration of the drug. However, ssHHT induced much less apoptosis in normal lymphocyte cells. From this, we concluded that ssHHT seems to have a selected cytotoxicity to blast cells. In addition, despite the fact that 10 of those 16 patients were clinically resistant to treatment (idarubicin or daunorubicin/AraC), 8 of 10 resistant patient cells were sensitive to ssHHT *in vitro* (>70% apoptosis). The apoptosis induced by ssHHT in patient cells was also mediated by Mcl-1 down-regulation, through the mitochondria, and the release of cytochrome *c*.

Partial inhibition of apoptosis by Z-VAD.fmk suggested that Mcl-1 down-regulation and release of cytochrome *c* were important but were not the only events. We have also shown that ssHHT rapidly inhibits protein synthesis. At 1 hour, the inhibition of incorporation of [^{35}S]methionine reached 89 ± 2%, 90 ± 1%, and 74% for HL60, HL60/MRP, and patient cells, respectively. Mcl-1 protein has a very short half-life. We have shown that its degradation was mediated through the proteasome in presence of ssHHT by restoring its expression using proteasome inhibitors. We suggest that this rapid Mcl-1 down-regulation is due to the inhibition of protein synthesis by ssHHT.

The results obtained provide us with a better understanding of the mechanism of the apoptosis induced by ssHHT in myeloid leukemia cells. The rapid and massive apoptosis induced by ssHHT in AML patient cells, and also the mechanisms of its action on protein synthesis, Mcl-1 down-regulation via the proteasome, and cytochrome *c* release, strongly encourage further clinical trials of ssHHT in AML.

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