Cancer Therapy: Preclinical

CPTH6, a Thiazole Derivative, Induces Histone Hypoacetylation and Apoptosis in Human Leukemia Cells

Daniela Trisciuoglio¹, Ylenia Ragazzoni¹, Andrea Pelosi², Marianna Desideri¹, Simone Carradori⁴, Chiara Gabellini¹,³, Giovanna Maresca⁷, Riccardo Nescatelli⁵, Daniela Secci⁴, Adriana Bolasco⁴, Bruna Bizzarri⁴, Chiara Cavaliere⁵, Igea D’Agnano⁷, Patrizia Filetici⁶, Lucia Ricci-Vitiani⁸, Maria Giulia Rizzo², and Donatella Del Bufalo¹

Abstract

Purpose: We previously identified novel thiazole derivatives able to reduce histone acetylation and histone acetyltransferase (HAT) activity in yeast. Among these compounds, 3-methylcyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone (CPTH6) has been selected and used throughout this study.

Experimental Design: The effect of CPTH6 on histone acetylation, cell viability and differentiation, cell-cycle distribution, and apoptosis in a panel of acute myeloid leukemia and solid tumor cell lines has been evaluated.

Results: Here, we showed that CPTH6 leads to an inhibition of Gcn5 and pCAF HAT activity. Moreover, it inhibits H3/H4 histones and α-tubulin acetylation of a panel of leukemia cell lines. Concentration- and time-dependent inhibition of cell viability, paralleled by accumulation of cells in the G0/G1 phase and depletion from the S/G2M phases, was observed. The role of mitochondrial pathway on CPTH6-induced apoptosis was shown, being a decrease of mitochondrial membrane potential and the release of cytochrome c, from mitochondria to cytosol, induced by CPTH6. Also the involvement of Bcl-2 and Bcl-xL on CPTH6-induced apoptosis was found after overexpression of the two proteins in leukemia cells. Solid tumor cell lines from several origins were shown to be differently sensitive to CPTH6 treatment in terms of cell viability, and a correlation between the inhibitory efficacy on H3/H4 histones acetylation and cytotoxicity was found. Differentiating effect on leukemia and neuroblastoma cell lines was also induced by CPTH6.

Conclusions: These results make CPTH6 a suitable tool for discovery of molecular targets of HAT and, potentially, for the development of new anticancer therapies, which warrants further investigations.

Clin Cancer Res; 18(2); 1–12. ©2011 AACR.

Introduction

Epigenetic changes, including histone modifications, often occur in cancer (1). Among the post-translational modifications of histones, one regards the acetylation of specific lysine ε-amino groups in the N-terminal tail of the core chromosomal histones H2A, H2B, H3, and H4. Histone acetylation increases accessibility of several factors to the chromatin at specific genes or over vast regions of the genome (2, 3), and it is involved in the regulation of several cell functions, such as gene transcription (4, 5), DNA repair (6), and replication (7). Histone acetyltransferases (HAT) are the enzymes responsible for histone acetylation, which can be reversed by the activity of histone deacetylases (HDAC), enzymes that hydrolyze the acetyl groups (8). The histone acetylation–deacetylation balance is accurately maintained through an equilibrium of HAT and HDAC enzymatic activities in normal cells. On the contrary, irregular pattern of histone acetylation is often found associated with cancer, and it has been hypothesized to modulate the expression of oncogenes and tumor suppressor genes (9–12). Therefore, both protein acetylation and deacetylation pathways represent attractive targets for cancer therapy. High hyperacetylation of histones in oral squamous and hepatocellular carcinoma patient samples has been reported (13, 14). Translocation of HAT genes (15), mutation of the HAT p300 (16), overexpression of AIB-1, a HAT coactivator of a nuclear hormone receptor (17), represent
**Translational Relevance**

Recent studies strongly support the histone acetylation as a promising therapeutic target with a strong preclinical rationale in hematologic disease and solid tumors. Although a growing body of evidence shows a direct relationship between histone acetyltransferases (HAT) activity and development or progression of cancer disease, only limited progress has been made in the field of HAT inhibitors. Our study identifies the thiazole derivative 3-methylcyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl]hydrazone (CPTH6) as a novel Gcn5 and pCAF HAT inhibitor. CPTH6 treatment in human acute myeloid leukemia (AML) and colon carcinoma cells significantly decreased histones acetylation and cell viability and activated apoptosis. Differentiation of AML and neuroblastoma cells was also evidenced after CPTH6 treatment. Overall, these results are of both biological and clinical significance, and they identify a new inhibitor of histone acetylation, targeting HAT activity and inducing cell death and differentiation in AML that should be further studied for leukemia therapy.

Examples of deregulated HATs in several tumor histotypes. These observations suggest that specific and relatively nontoxic inhibitors of acetyltransferases could be considered as new generation of therapeutic agents for cancer. Although HDAC inhibitors have been extensively studied and several are currently in clinical trials (18), there is little information available on inhibitors of HATs (HATi; ref. 19). Among them, several compounds have been described to elicit antiproliferative, proapoptotic, antitumoral, and antiangiogenic activity toward different tumor histotypes, and they may represent new therapeutic agents for cancer treatment and prevention (20–23). Recently, a set of newly synthesized molecules derived from thiazole has been selected as modulators of the HATs in yeast-based drug screening (24). Among these compounds, the thiazole derivative CPTH6 has been chosen to evaluate its possible antitumoral activity in a large panel of leukemic and solid tumor cell lines.

**Materials and Methods**

**Cell cultures**

Human acute myeloid leukemia (AML) and solid tumor cell lines were obtained from different sources and cultured as reported in Supplementary Materials.

**Generation of Bcl-2, Bcl-xL, and cFLIP stably overexpressing cells**

The HA-cFLIP, bcl-xL, and bcl-2 cDNAs were cloned under the cytomegalovirus promoter of a lentiviral vector, which carried the Enhanced Green Fluorescent Protein reporter gene under the 3-phosphoglycerate kinase promoter. Recombinant lentiviruses were produced as described previously (25). Transduced cells were sorted for green fluorescence (FACS Aria; Becton Dickinson)

**Reagents preparation and treatment**

CPTH6 (3-methylcyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl]hydrazone; Supplementary Fig. S1A) was synthesized as previously reported (24), dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at the concentration of 100 mmol/L and diluted to the final concentrations in complete medium. For all the experiments, cells were treated with 1% DMSO as control. After 24 hours from seeding, exponentially growing AML and solid tumor cells were treated with CPTH6 at concentrations ranging from 1 to 100 μmol/L for 24 to 120 hours. For some experiments, treatment with CPTH6 was followed or preceded by exposure to the HDAC inhibitor trichostatin A (TSA; Sigma-Aldrich), while pan-caspase inhibitors z-VAD-fmk (100 μmol/L; Calbiochem) and qVD (40 μmol/L; Santa Cruz Biotechnology) were added 1 hour before CPTH6 treatment.

Curcumin (Sigma-Aldrich), anacardic acid (Alexis Biochemical), and MB-3 (Alexis Biochemical) were dissolved in DMSO and stored as stock at −20°C. Vitamin D3 and All-Trans-Retinoic Acid (ATRA; Sigma-Aldrich) were dissolved in ethanol and DMSO, respectively, and stored as stock at −80°C. Because of light sensitivity of ATRA, all incubations were done under subdued lighting.

**Measurement of HAT activity**

To evaluate the effect of CPTH6 on in vitro HAT enzymatic activity, 1 μg of Gcn5, pCAF, p300, or CBP recombinant proteins (Enzo Life Science), 2 μg of H3 or H4 histones (New England Biolabs) and 20 μmol/L Acetyl-CoA (Sigma-Aldrich), containing 0.01 μCi/μL [3H]Acetyl-CoA (PerkinElmer) were incubated in HAT assay buffer (50 mmol/L Tris-HCl pH 8.0, 0.1 mmol/L EDTA, 1 mmol/L diethioctrol and 10% glycerol) at 30°C for 1 hour in the presence of 800 μmol/L CPTH6 or 2% DMSO, as control. The reaction mixture was then blotted in triplicate onto P-81 filter papers (Upstate) that were washed, dried, and placed in vials containing scintillation fluid. Radioactive counts were recorded on Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer). To characterize the inhibition kinetics of CPTH6, HAT assays were done using 2 μg of histones with concentrations of [3H]Acetyl-CoA from 20 to 80 μmol/L. To evaluate HAT activity in CPTH6-treated cells, nuclear protein extracts (50 μg) were prepared as previously reported (26) and subjected to colorimetric HAT Activity Assay Kit (Enzo Life Science) following manufacturer’s instructions.

**Measurement of histone deacetylase activity**

The effect of CPTH6 on in vitro HDAC activity and on cellular HDAC activity was analyzed using Epigentek HDAC Activity/Inhibition Assay Colorimetric Kit (Epigentek) following manufacturer’s instructions. Nuclear extracts of U-937 cells, obtained by using Epigentek Nuclear Extraction Kit I (Epigentek), were used as source of HDAC activity.
Assessment of cell viability
Exponentially growing AML and solid tumor cells were seeded in duplicate in 6-well (2 × 10⁵ cells per well) or in sextuplicate in 96-well (3 × 10⁴ cells per well) culture plates (Nunc), respectively, and after 24 hours CPTH6 was added to the medium at concentrations ranging from 5 to 100 μmol/L for 24 to 120 hours. Cell viability of AML and solid tumor cells was evaluated by trypan blue exclusion and by MTT (mitochondrial respiration analysis; Sigma-Aldrich) assays, respectively. AML cells viability was calculated for each concentration of CPTH6 used as "number of CPTH6-treated cells/number of control cells" × 100. MTT was added to each well at the final concentration of 0.5 mg/mL and after 4 hours of incubation at 37°C, the formazan salt was dissolved with 200 μL isopropyl alcohol. The absorbance of each well was measured with ELISA reader (DASIT) at 570 nm wavelength and the viability was calculated for each concentration of CPTH6 used as "OD of CPTH6-treated cells/OD of control cells" × 100. The concentration of CPTH6 that causes 50% of cell viability inhibition (IC₅₀) was also calculated.

Cytoluciferometric analyses of cell-cycle, apoptosis, mitochondrial membrane potential and reactive oxygen species production
To analyze cell-cycle phase distribution, cells were collected by centrifugation, fixed in cold 70% ethanol, and stained in a PBS solution containing propidium iodide (PI; Sigma-Aldrich), and RNase A (1.125 mg/mL; Sigma-Aldrich). Samples were acquired with a FACScan instrument (Becton Dickinson) and the percentage of cells in the different phases of cell-cycle and in sub-G₁ compartment was calculated using ModFit LT software (Becton Dickinson).

Apoptosis was assayed by using Terminal deoxynucleotidyl transferase–mediated fluorescein-dUTP nick-end labeling (TUNEL)-based commercial Kit (Roche Diagnostic) or by staining cells with the Annexin V–fluorescein isothiocyanate apoptosis kit (eBioscience), according to the manufacturer’s instructions. Mitochondrial membrane potential (MMP) was assessed using Mito-ID Detection Kit (Enzo Life Science), according to manufacturer’s instructions. Reactive oxygen species (ROS) production was measured using dihydroethidium staining (DHE; Molecular Probes). For detection of apoptosis, MMP and ROS, at the end of incubations with the respective reagents, about 20,000 events were acquired and gated using forward scatter and side scatter to exclude cell debris, and the percentage of cells relatives to the different analyses was calculated using CellQuest software (Becton Dickinson).

Western blot analysis
Cells were lysed and total extracts were fractionated by SDS-PAGE, transferred to a nitrocellulose filter, and subjected to immunoblot assay, as previously described. Cytochrome c release into the cytosol was detected as previously described (27). Immunodetection was done using the antibodies reported in Supplementary Materials. Horseradish peroxidase–conjugated secondary antibodies binding was visualized by enhanced chemiluminescence according to manufacturer’s specification and recorded on autoradiography film (Amersham Biosciences).

Assessment of cell differentiation
AML cell differentiation was analysed by morphologic and functional analyses [CD11b, CD14, alpha-naphthyl acetate esterase (αNAE)] and neuroblastoma cell differentiation was evaluated by immunofluorescence analysis (NF-200), as reported in Supplementary Materials.

In vivo toxicity and pharmacokinetic analysis
Assessment of in vivo tolerability and pharmacokinetic parameters of CPTH6 was done as reported in Supplementary Materials.

Statistics
Experiments were replicated 3 times and the data were expressed as means ± SD, unless otherwise indicated. Differences between groups were analyzed with a 2-sided paired or unpaired t test, and they were considered to be statistically significant for P < 0.05.

Results
CPTH6 is a specific Gcn5/pCAF inhibitor and reduces histones and α-tubulin acetylation
To identify the specific target(s) of CPTH6, a thiazole derivative (Supplementary Fig. S1A) previously shown to inhibit histone acetylation in yeast (24), we analyzed the effect of the compound on in vitro HAT activity of human recombinant p300, CBP, pCAF, and Gcn5 enzymes. As shown in Fig. 1A, CPTH6 exerted a significant inhibitory effect on HAT activity of both pCAF and Gcn5, whereas it did not affect p300 and CBP HAT activity. As expected, anacardic acid, a well known pan-HAT inhibitor, almost completely inhibited HAT activity of all enzymes analyzed. The inhibitory effect of CPTH6 on Gcn5 activity is quite similar to the one observed for MB-3, a butyrolactone which has been described as a specific Gcn5 HAT inhibitor (28). Using histone H4 as substrate, CPTH6 showed a significant inhibitory effect on the activity of pCAF and Gcn5 (Supplementary Fig. S1B), whose preference for histone H3 has been previously shown (28). We also found that the increase of acetyl-CoA concentration reverted the inhibitory effect of CPTH6 on Gcn5 HAT activity (Supplementary Fig. S1C). Moreover, HDAC activity was not affected by CPTH6 exposure (Supplementary Fig. S1D and E).

By using U-937 cell line, we found that 50 μmol/L CPTH6 decreases HAT activity by about 40% and 75% after 6 and 18 hours of treatment, respectively (Fig. 1B) and reduces acetylation levels of both histones H3 and H4 (Fig. 1C), without affecting their total expression (Fig. 1F). We also evaluated the effect of lower concentrations of CPTH6: reduced acetylation of histone H3 was observed after 72 and 120 hours of exposure to 10/20 and 5 μmol/L CPTH6, respectively.
whereas no effect on H3 acetylation was observed after treatment with 1 μM CPTH6 (Fig. 1D and E).

Besides, CPTH6 downregulation of H3 acetylation was similar to that induced by MB-3 (about 40%), whereas curcumin, a pan-HAT inhibitor, induced higher reduction of H3 acetylation (about 70%; Supplementary Fig. S2A).

The ability of CPTH6 to decrease acetylation of histone H3 specifically at lysine 18 in a dose-dependent fashion was also observed (Fig. 1F), whereas CPTH6 (50 and 100 μM for 24 hours) did not affect H3 histone methylation at lysines 9 and 4 (Fig. 1G).

Tested on α-tubulin, a non-histone substrate, CPTH6 was also able to decrease the acetylation level of the protein, without affecting the expression of total α-tubulin (Fig. 1I).

As reported in Fig. 1H, histone H3 hyperacetylation induced by TSA, a well-known inhibitor of HDACs, was reversed by CPTH6 treatment in a time-dependent manner such that after a 5-hour treatment, the level of acetylated histone H3 was similar to that of control cells. By contrast, pretreatment of cells with CPTH6 was not able to counteract TSA-induced H3 histone and α-tubulin hyperacetylation (data not shown).
Different basal levels of H3 histone acetylation in AML lines, and the ability of CPTH6 to decrease H3 and H4 histones and α-tubulin acetylation were also shown in HL-60, KG1, and HEL97.1.7 leukemia cells (Supplementary Fig. S2B–G).

CPTH6 differently affects in vitro cell viability of human cancer cell lines

Having corroborated the inhibitory effect of CPTH6 on histone acetylation, we evaluated the in vitro viability of a panel 35 established human cancer cell lines of different histotypes exposed to increasing concentrations of CPTH6 up to 100 μmol/L for times ranging from 24 to 72 hours (Fig. 2). As reported in Fig. 2A, 100 μmol/L CPTH6 for 72 hours induced cell viability inhibition higher than 25% in 31 of 35 (about 82%) cell lines. An almost homogeneous response to CPTH6 in terms of cell viability inhibition was observed in glioblastoma (from 21%–43%), melanoma (from 21%–43%), neuroblastoma (from 21%–39%) and breast (from 21%–49%), colon (from 67%–83%), and pancreas (from 41%–49%) carcinomas. On the contrary, a more heterogeneous response was evidenced in AML (from 51%–89%), lung (from 31%–69%), prostate (from 0%–64%), and ovary (from 28%–79%) carcinoma cell lines. As reported in Fig. 2B, a dose- and time-dependent reduction of cell viability by CPTH6 treatment was more evident in some cell lines, such as U-937, HL-60, A2780, and HCT116 than in others (LAN-5 and U-87).

As depicted in Fig. 3A, we observed a decrease of histone H3 acetylation level upon 24 hours of treatment with CPTH6 in several "more sensitive" cells, in terms of reduction of cell viability (HL-60, HCT116, HT-29, H1299, and A2780) but not in other "less sensitive" (M14, MCF7, and U-87) ones. In agreement with these results, whereas longer treatments (72 hours) did not result in significant changes in H3 acetylation level in "less sensitive" cells (M14, MCF7), reduced acetylation levels of histone H3 in the "more sensitive" HCT116 line was already evident after 24 hours CPTH6 treatment (Fig. 3B).

CPTH6 induces cell-cycle perturbation and apoptosis in AML cells

We next evaluated the cell-cycle profile after CPTH6 treatment of U-937 and HL-60 leukemia cell lines. As shown in Fig. 4A and B, CPTH6 had a strong effect on cell-cycle progression, being an accumulation in the G0/G1 phase with depletion of cells from S and G2/M phases already evident after 24 hours of treatment in both cell lines. Consistent with the observed G0/G1 accumulation, CPTH6 modulated the expression of key proteins that regulate cell-cycle progression (Fig. 4C): increased level of cyclin-dependent kinase inhibitor p27 and transcription factor E2F4, together with hypophosphorylation and reduced expression of the tumor suppressor retinoblastoma proteins Rb and Rb2, respectively, were observed after CPTH6 treatment.

The sub-G1 population (about 40% and 20% in U-937 and HL-60, respectively, after 72 hours of treatment) observed in Fig. 4A and B is indicative of apoptosis. As shown in Fig. 4D, a time-dependent increase in the percentage of apoptotic cells was induced by 50 and 100 μmol/L CPTH6 in U-937 cells. The induction of apoptosis was confirmed.
by TUNEL assay (Fig. 4E) being the percentage of TUNEL-positive cells about 15% and 40% after treatment with 20 and 100 μmol/L CPTH6, respectively.

Also CPTH2, a compound closely related to CPTH6 (24), reduced H3 and H4 histones acetylation, induced accumulation of cells in the G0/G1 cell-cycle phase, and activated the apoptotic program in U-937 cells (Supplementary Fig. S2H and I).

As reported in Fig. 5A and B, exposure to CPTH6 at doses ranging from 5 to 100 μmol/L reduced the expression of procaspases 8, 9, and 3 and PARP and induced their cleavage. Moreover, CPTH6 treatment did not modulate procaspase 1 expression and did not induce its cleavage. No effect on PARP protein was observed after treatment with 1 μmol/L CPTH6 (Fig. 5B). PARP and procaspase 9 cleavage was also observed after exposure of HL-60 line to 50 and 100 μmol/L CPTH6 for 48 hours (Fig. 5C).

Next, cells were pretreated for 1 hour with the pan-caspase inhibitors qVD and z-VAD-fmk and then exposed to 100 μmol/L CPTH6 for 72 hours, and the percentage of apoptotic cells was evaluated. Both qVD and z-VAD-fmk did not significantly reduce CPTH6-induced cell death, although as expected, both compounds inhibited staurosporine-induced apoptosis by about 60% and 50%, respectively (data not shown).

To determine the role of the mitochondrial pathway, we checked the release of cytochrome c from mitochondria to cytoplasm, ROS production, and the expression of some Bcl-2 family proteins in U-937 cells. Cytochrome c release was evident after exposure to CPTH6 (Fig. 5E), and it was associated with a depolarization of the mitochondrial membrane (Fig. 5F). By contrast, no modulation of Bcl-2, Bcl-xl, and Mcl-1 proteins (Fig. 5A) and less than 4% of ROS generation were observed after exposure to CPTH6 (100 μmol/L for 72 hours; Fig. 5D).

The role of extrinsic and intrinsic apoptotic pathways on CPTH6-induced apoptosis was analysed by stably overexpressing cFLIP or Bcl-2/Bcl-xl, respectively, in U-937 cells (Fig. 5G, Supplementary Fig. S3A). Stably cFLIP-overexpressing cells treated with 100 μmol/L CPTH6 for 72 hours showed a similar percentage of apoptosis (about 35%) when compared with mock-transfected cells (about 40%), although as expected, cFLIP overexpression prevented TNFα-induced cell death (Supplementary Fig. S3C and D). Induction of apoptosis by CPTH6 after forced expression of both Bcl-2 and Bcl-xl proteins was reduced by about 50% when compared with mock-transfected cells (Fig. 5G).

As reported in Supplementary Fig. S3E and F CPTH6 exposure also induces the processing of PARP and the pan-caspase inhibitor z-VAD-fmk blocked PARP cleavage in HT-29 colon carcinoma. Moreover, overexpression of Bcl-2 partly attenuated the cleavage of PARP induced by CPTH6 treatment. On the contrary, exposure to 100 μmol/L CPTH6 for 72 hours did not result in significant reduction in cell viability (lower than 25%), in cell-cycle perturbation, apoptotic activation and did not reduce H3 histone acetylation in normal primary cells of different origins, such as mouse fibroblast NIH/3T3 and human endothelial HU-VEC. (Supplementary Fig. S3A and B).

CPTH6 induces AML and neuroblastoma cell differentiation

On the basis of the relevance of histone acetylation changes on regulation of cell differentiation (29), we investigated whether CPTH6 was able to induce differentiation in AML and neuroblastoma cells. After exposure to 20 and 50 μmol/L CPTH6, U-937 cells displayed a more mature phenotype (about 50%) relative to control cells (about 2%), based on size reduction, decreased nucleo/cytoplasmic ratio, and condensed chromatin, all parameters indicative of mononcytic terminal differentiation, and similar results were also observed for HL-60 cells (Fig. 6A). According to these results, a significantly increased esterase activity (Fig. 6B) and an increased expression of mononcytic-specific membrane antigen CD14 was observed in cells exposed to CPTH6 in a concentration-dependent manner when compared with control cells, whereas the compound did not modulate the specific differentiation marker of granulocytic differentiation CD11b (Fig. 6C).

Also, SH-SY5Y and LAN-5 neuroblastoma cells treated with 50 μmol/L CPTH6 for 5 days showed the outgrowth of neuritic processes, as evidenced by the expression of NF200,
the characteristic marker of neuronal differentiation. The formation of neurites induced by CPTH6 had significant number of branch points, and they occurred in the majority of the cells showing multiple long and well-defined dendritic processes (Fig. 6D). The length of the primary neurite was similar to that obtained by treating the cells with ATRA (Fig. 6E). Also the percentage of cells with neurites outgrowth increases in SH-SY5Y cells after exposure to CPTH6, ranging from 7.4% in control cells to 18.7% and 32% after treatment with 20 and 50 µmol/L CPTH6, respectively. A slight decrease of H3 acetylation was also observed after exposure of SH-SY5Y to CPTH6 (data not shown). Moreover, 50 µmol/L CPTH6 for 5 days induced a reduction of cell proliferation of about 30% and 50% in SH-SY5Y and LAN5 cells, respectively.

In vivo toxicology and pharmacokinetic parameters of CPTH6

When intraperitoneally injected in mice over 30 days, 100 and 300 mg/kg of CPTH6 did not produce any adverse
health effects as monitored by diet consumption, body weight loss, and postural and behavioral changes.

The in vivo biopharmaceutical profile of CPTH6 was studied by measuring pharmacokinetic parameters in plasma samples, obtained at fixed times after intraperitoneal and intravenous delivery of 300 mg/kg CPTH6 (Supplementary Table SI, Supplementary Fig. S4). Results showed that CPTH6 was absorbed very fast, and maximum plasma concentrations (C_{max} 15.08 ± 3.01 ng/mL) were observed 45 minutes after CPTH6 injection. The compound administration resulted in an AUCip of 13.07 ± 2.57 ng/mL h, compared with an AUCiv of 17.18 ± 2.57 ng/mL h.

Therefore, the absolute bioavailability (AUCip/AUCiv ratio of 0.76) of CPTH6 is very high. The elimination half-life was 1.30 ± 0.4 hours.

Discussion

In this article, we showed that CPTH6 significantly reduces H3 and H4 histones and α-tubulin acetylation, cell viability and HAT activity, and activates the apoptotic and the differentiating programs of several cancer cell lines. The analysis of basal levels of H3 histone acetylation in AML cell lines indicated that this parameter did not correlate with the
sensitivity to CPTH6 in terms of cell viability. In fact, HL-60 and KG1 cells that showed the highest and the lowest level of H3 acetylation, respectively, possess a superimposable IC50 (about 50 μmol/L). By contrast, solid tumor cells were differently sensitive to CPTH6 in terms of cell viability. Even though colon carcinoma lines were more sensitive to CPTH6 than the majority of solid tumor cell lines, and some lines belonging the same histotype, such as pancreas carcinoma, showed a similar response to CPTH6, we exclude that the effect of CPTH6 could be related to the tumor histotype. In fact, lung, prostate, and ovary carcinoma lines showed a heterogeneous response to CPTH6 and both cells with low and high sensitivity were found within the same tumor histotype.

CPTH6 effect on cell viability seems to be correlated to the extent to which the histone hypoacetylation occurs. In fact, reduction of cell viability in response to CPTH6 treatment was only observed in leukemia and solid tumor cell lines that showed a decrease of their acetylation level of H3 and H4 histones after CPTH6 treatment.

Also H3 histone acetylation at lysine 18 (H3K18Ac) was reduced by CPTH6 treatment in a concentration-dependent fashion. Given that changes in histones acetylation at several lysines can be predictive of cancer patients clinical

Figure 6. CPTH6 induces differentiation in AML and neuroblastoma cells. A, analysis of monocyte-like features evaluated by May–Grunwald/Giemsa staining in HL-60 and U-937 cells treated with 20 and 50 μmol/L CPTH6 for 72 hours. Representative fields with the classical features of blastic phenotype such as the irregularly shaped nucleus (##) and monocytic phenotype, such as cells with horseshoe nuclear shape (†), rounded nucleus, and small cytoplasm (‡) are shown. B, percentage of U-937 cells positive to the monocytic specific marker CD14 after treatment with 20 and 50 μmol/L CPTH6 for 72 hours. Data are expressed as means ± SEM from 3 independent experiments in duplicate. P value was calculated between control and treated cells (†, P < 0.05). C, flow cytometric analysis of granulocytic CD11b and monocytic CD14 markers expression in U-937 cells untreated (green) and treated with 20 (fuchsia) or 50 μmol/L (blue) CPTH6, 2 μmol/L ATRA (black) or 50 nmol/L vitamin D3 (Vit D3, orange) for 72 hours. Dark blue areas represent negative control (no addition of primary antibody to the cells). The histograms are representative of 3 different experiments with similar results. D, fluorescent images of SH-SY5Y and LAN-5 cells control or treated with 50 μmol/L CPTH6 or 10 μmol/L ATRA for 5 days. Cells were immunostained with anti-NF200 monoclonal antibody (red) and with the DNA dye DAPI (blue). Images are representative of 3 different experiments with similar results. E, neurite outgrowth of SH-SY5Y and LAN-5 cells after 5 days of treatment with CPTH6. Length of primary neurites was quantified in treated cells as fold increase versus control. ATRA-treated cells were used as positive control of differentiation. Data are the means ± SD of 5 randomly chosen fields in each treated or control samples. The experiments were repeated 3 times with similar results.
outcome (30, 31) and high global levels of H3K18Ac are associated with an increased risk of tumor recurrence (32, 33), these data suggest the potential use of CPTH6 for cancer therapy.

High hyperacetylation of histones has been reported in several cancer patient samples (13, 14). We showed hypoacetylation activity of the compound also on histones hyperacetylated by trichostatin, an HDAC inhibitor. On the contrary, the fact that pretreatment of CPTH6 did not influence TSA-induced H3 histone hyperacetylation could be related to the “de novo” acetylation that occurs in our experimental model after removal of CPTH6 (data not shown).

Within cells, many proteins are acetylated in addition to histones (34). In this context, CPTH6 was also able to decrease the acetylation level of α-tubulin, indicating that the potential activity of HAT inhibition may also have nonhistone molecular targets as reported for other HAT inhibitors (35). Moreover, CPTH6 exerted a significant inhibitory effect on the HAT activity of both pCAF and Gcn5.

The increase of the concentration of acetyl group donor acetyl-CoA reverted the effect of CPTH6 on HAT activity, suggesting that CPTH6 may elicit its inhibitory activity preventing the binding of acetyl-CoA. Nevertheless, CPTH6 did not affect HAT activity of p300 and CBP or the activity of enzymes involved in posttranslational modification of histones, such as HDAC. Consistent with these results, CPTH6-induced HAT activity inhibition and H3 histone hypoacetylation were similar to those induced by MB-3, a specific Gcn5 HAT inhibitor (28).

Focusing our attention on the 2 representative leukemia cell lines U-937 and HL-60, we found that decreased cellular viability observed after CPTH6 treatment was associated at earlier time points to an increase of percentage of cells in the G0/G1 compartment associated with a depletion in the S and G2/M phases. An accumulation of both cyclin–cycle in the G1 phase, were also observed.

CPTH6 also induced apoptosis in AML cells and reduced procaspase 3, 9, and 8 expression and concomitantly increased their cleavage, indicative of enzyme activation. In line with this possibility, the PARP cleavage product (a downstream target of caspase 3) was increased after treatment with CPTH6, suggesting a role for caspase activation in the cytotoxic effect of the compound. However, addition of the irreversible, cell-permeable pan-caspase inhibitors, z-VAD-fmk and qVD, at concentrations that prevent caspase activation, did not significantly reverse cell death after exposure to CPTH6. The lack of an effect of the caspase inhibitors was not due to an inapposite use of the 2 inhibitors because they were able to reduce apoptosis induced by staurosporine. Together, these data suggest that cell death induced by CPTH6 may proceed independently from caspases activation. Our results are in agreement with previous reports showing that caspase-independent pathways may play essential roles in apoptosis induced by histone acetylation–targeted drugs (36, 37). It is possible that proapoptotic mitochondrial factors such as calpain, apoptosis-inducing factor, endonuclease G or Omi/HtrA2, which have been reported to participate in caspase-independent apoptosis (38, 39), may play a role in CPTH6-induced apoptosis.

The inability of caspase 8 inhibitor to affect CPTH6-induced cell death (data not shown), and the observation that cFLIP overexpression did not prevent CPTH6-induced apoptosis, rule out a role of caspase 8 in CPTH6-induced apoptosis. This evidence is in line with data showing a non-apoptotic role of caspase 8, but further a role of this enzyme in the regulation of U-937 differentiation (40), suggesting a model in which CPTH6 induces apoptosis and differentiation through the involvement of different pathways. Activation of procaspase 8 we observed after CPTH6 treatment could be considered as a possible downstream “bystander” event and support the evidence that in our model, the intrinsic pathway is more important in protecting cells from death than the extrinsic pathway.

The role of mitochondrial pathway and Bcl-2/Bcl-xL proteins on CPTH6-induced apoptosis was also shown, being a decrease of MMP and the release of cytochrome c induced by CPTH6 treatment, and being CPTH6-induced apoptosis partially reduced after forced expression of Bcl-2 and Bcl-xL proteins.

Processing of PARP and the ability of Bcl-2 to partly attenuate the cleavage of PARP have also been shown in the HT-29 colon carcinoma cell line.

The ability of CPTH6 to induce monocytic differentiation in AML cells has also been shown by morphologic changes, such as size reduction, decreased nuclear/cytoplasmic ratio and chromatin condensation, and by the increased expression of the monocytic specific marker CD14 induced by CPTH6.

CPTH6 was also able to activate the differentiation program in neuroblastoma, which represents an almost unique tumor cell type, given its capacity to differentiate upon treatment with different agents, upregulating a well-established set of differentiation marker genes, making it possible to monitor the differentiation process (41). Indeed, following CPTH6 treatment, we found a marked expression of the NF200, which represents one of the standard neuronal markers. These results are in agreement with papers reporting the ability of several HATi/HDACi to modulate normal and tumor cell differentiation. Examples are represented by quinoline derivatives that induce granulocytic differentiation in leukemia cells (35), curcumin, which actively suppresses differentiation in astrocytes while promoting differentiation into the neurons (42), and regulators of nucleosomal histones acetylation state that induce neuroblastoma cells differentiation (43–45).

In conclusion, the identification of new compounds possessing HAT inhibitory activity can represent a tool for studying the effects of HATs on target molecules. Moreover, (i) the fact that also CPT2H2, a closely related compound belonging to the same class of CPTH6, inhibits H3...
acetylation and induces cell-cycle perturbation and apoptosis in U-937 cells; (ii) the lack or reduced effect of CPTH6 on viability and its inability to induce cell-cycle perturbation and apoptosis in primary normal cells; (iii) the hypoacetylylating and proapoptotic effect of CPTH6 also at low doses; and (iv) the in vivo data about tolerability and pharmacokinetics, can provide information necessary to design more efficient drugs potentially useful for cancer therapy and, in particular, for leukemia and colon carcinoma, which in our study represent the most responsive to CPTH6.

Disclosure of Potential Conflicts of Interest

Y. Ragazzoni, C. Gabellini, and A. Pelosi are PhD students of “Sapienza” University. Y. Ragazzoni and A. Pelosi are recipients of a fellowship from the Italian Foundation for Cancer Research.

References

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Daniela Trisciuoglio, Ylenia Ragazzoni, Andrea Pelosi, et al.

Clin Cancer Res  Published OnlineFirst November 8, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-0579

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/11/08/1078-0432.CCR-11-0579.DC1

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