Delivery of 5-Azacytidine to Human Cancer Cells by Elaidic Acid Esterification Increases Therapeutic Drug Efficacy

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Abstract

Azacytidine is an established nucleoside drug that is well known for its ability to modulate epigenetic gene regulation by inhibition of DNA methylation. Despite recent advances in the clinical development of azacytidine, the use of the drug is limited by its low bioavailability and dependence on variably expressed nucleoside transporters for cellular uptake. We show here that CP-4200, an elaidic acid derivative of azacytidine, has strong epigenetic modulatory potency in human cancer cell lines, as evidenced by efficient depletion of DNA methyltransferase protein, genome-wide DNA demethylation, and robust reactivation of epigenetically silenced tumor suppressor genes. Importantly, however, the cellular uptake of CP-4200 was substantially less dependent on the nucleoside transporters that are known to be involved in azacytidine uptake. In agreement with this notion, CP-4200 showed a significantly higher antitumoral activity than azacytidine in an orthotopic mouse tumor model for acute lymphocytic leukemia. Together, these data represent a detailed characterization of the CP-4200 mode of action and suggest that elaidic acid modification improves the therapeutic efficacy of azacytidine. Mol Cancer Ther; 9(5); 1256-64. ©2010 AACR.

Introduction

Azacytidine (5-azacytidine) and decitabine (2′-deoxy-5-azacytidine) are two closely related cytidine analogues that have found increasing clinical use for cancer therapy (1). Both compounds are prodrugs that need to be metabolized to the corresponding nucleoside triphosphates before they can be incorporated into DNA and/or RNA (2). After incorporation into DNA, the fraudulent base acts as a suicide substrate for DNA methyltransferase (DNMT) enzymes. The formation of a covalent reaction intermediate between DNA and enzymes triggers the depletion of DNMTs from drug-treated cells. Continuing replication in the absence of functional DNMT enzymes causes progressive loss of methylation and thus provides the mechanistic foundation for the prominent demethylating activity of azanucleoside drugs.

Drug-induced DNA demethylation represents a central aspect of epigenetic cancer therapy (3). Human cancers are characterized by a substantial number of epigenetic mutations, including hypermethylated and epigenetically silenced tumor suppressor genes (4). Because of the close association between DNA demethylation and gene reactivation (5), it is thus generally assumed that azacytidine has the ability to revert epigenetic mutations and thereby restore normal gene expression patterns. As such, azacytidine (like decitabine) represents an archetypical epigenetic drug.

Azacytidine is approved for the treatment of myelodysplastic syndrome and has also been used successfully for the clinical management of acute myeloid leukemia (6, 7). Substantial clinical responses are usually observed at comparably low doses, roughly an order of magnitude below the maximum tolerated doses (1). These doses are generally believed to minimize the toxicity of the drug and, at the same time, maximize demethylation responses. Indeed, it has been shown recently that azacytidine can induce pronounced DNA demethylation in patients when administered under the standard prescription schedule (7–9).

The clinical response rates to azanucleosides are usually in the range of 30% to 50%. The underlying reasons for clinical azacytidine resistances are only beginning to be investigated (10) and the complex mode of action suggests that a variety of cellular pathways can modulate the efficacy of these drugs (2). One pathway that has received particular attention is the deactivation of the compound, both by chemical hydrolysis and concomitant ring opening (11) and by enzymatic deamination (12), resulting in low plasma stability. A second pathway that is being investigated is the dependency on specific and variably expressed nucleoside transporters for cellular uptake (13). Factors associated with these pathways might be important mediators of clinical drug responses.
Recently, two independent strategies have been used to improve the efficacy of azanucleosides. S110, an azadeoxycytidine-deoxyguanidine dinucleotide, showed cellular demethylation activity that was similar to that of azacytidine (14). However, the dinucleotide was largely resistant to deamination by cytidine deaminase (14), a process that has been tightly linked to azacytidine instability (12). In an independent approach, cytidine analogues have been modified by esterification with a fatty acid. In this context, it has been shown that elaidic acid esterification of cytarabine, a cytosine analogue closely related to azacytidine, modified the cellular uptake mechanisms of the drug and rendered it more active in human solid tumor models (15–17). Because the drug must enter into cells to interact with its target, the knowledge of the transport mechanisms is a key aspect for understanding its extended mode of action. Two major families of human nucleoside transporters have been identified: the concentrative and the equilibrative nucleoside transporters (hCNTs/SLC28 and hENTs/SLC29 proteins). The members of these families play a major role in the cellular uptake and therapeutic efficacy of nucleoside analogues (18).

In this study, we have characterized the effects of CP-4200, an elaidic acid ester derivative of azacytidine (Fig. 1A). Our results show that CP-4200 effectively depletes DNMT1 in all human cancer cell lines analyzed. Drug-induced DNA demethylation was observed both at the whole-genome level and at specific genes and was accompanied by robust reactivation of epigenetically silenced tumor suppressor genes. Whereas these results underscored profound similarities of CP-4200 with the parent drug azacytidine, the cellular uptake mechanisms of these two drugs seemed fundamentally different. In addition, CP-4200 showed a significantly improved in vivo efficacy when compared with azacytidine. Taken together, these results show a potent epigenetic regulatory activity for CP-4200 and suggest that elaidic acid esterification of azacytidine increases the therapeutic efficacy of the drug.

Materials and Methods

**Cell culture.** HCT116 (from Bert Vogelstein, Johns Hopkins University, Baltimore, MD), MCF-7 (American Type Culture Collection), and HL60 (American Type Culture Collection) cells were cultured under standard conditions in McCoy’s 5a medium, DMEM, and RPMI 1640, respectively, supplemented with 5% L-glutamine and 10% FCS (Invitrogen). To analyze the effect of CP-4200 and 5-azacytidine, cells were cultivated in media supplemented with the compounds, as indicated. To determine IC₅₀ concentrations for CP-4200 and 5-azacytidine, cell viability was analyzed after 72 hours of drug incubation by using the CellTiter-Glo luminescent viability assay (Promega) according to the manufacturer’s protocol. Madin-Darby strain II canine kidney (MDCK; from Kai Simons, European Molecular Biology Laboratory, Heidelberg, Germany) cells and MDCK cells stably expressing recombinant hCNT1 (MDCK-CNT1) or hCNT3 (MDCK-CNT3) were grown in MEM (Sigma) supplemented with 10% (v/v) fetal bovine serum and 100 units/mL penicillin/streptomycin, as described previously (13).

**DNMT trapping assay.** Cells were treated with cell line–specific IC₅₀ concentrations for the indicated time periods. Proteins were extracted using lysis buffer [PBS (pH 7.5), 1% Igepal 630, 0.1% SDS, and 0.5% sodium deoxycholate] containing 1× complete protease inhibitor cocktail (Roche) and incubated on ice for 40 minutes. The lysate was centrifuged at 4°C for 25 minutes at

![Figure 1. CP-4200 causes DNMT1 depletion. A, structure of CP-4200. B, DNMT1 trapping assay. Cells were treated with cell line–specific IC₅₀ concentrations of CP-4200 or azacytidine (AZA) for 1 to 72 h. Protein extracts were probed for the presence of DNMT1 and GAPDH by Western blotting. co, nontreated control cells.](https://www.aacjournals.org/molcanther)
14,000 rpm. Cleared supernatants were frozen in liquid nitrogen and stored at −80°C. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and analyzed by Western blotting using standard procedures. The primary antibodies used were anti-DNMT1 (C17, 1:500, Santa Cruz Biotechnology) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5, 1:1,000, Santa Cruz Biotechnology). Primary antibodies were visualized by enhanced chemiluminescence (Perkin-Elmer) according to the manufacturer's protocol.

DNA methylation analysis. Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen). Global DNA methylation levels were determined by capillary electrophoresis as described previously (19). HumanMethylation27 DNA Analysis BeadChip arrays (Illumina) were used for genome-wide methylation pattern analysis (20) according to the manufacturer's instructions. Briefly, bisulfite-converted DNA was hybridized to methylation-specific oligonucleotides and washed. The hybridized oligonucleotides were then minisequenced with hapten-labeled dideoxynucleotides. After staining, the BeadChip was scanned. Results were analyzed with BeadStudio software (Illumina); histograms and heatmaps were drawn with R (http://www.R-project.org).

RNA isolation and reverse transcription-PCR. RNA was isolated using the RNeasy RNA Isolation kit (Qiagen). cDNA was prepared with the QuantiTect Reverse Transcription kit (Qiagen). For quantitative reverse transcription-PCR (RT-PCR), 1 μg of RNA was reverse transcribed according to the manufacturer's protocol. Each cDNA sample was analyzed in triplicate using the QuantiFast SYBR Green PCR kit (Qiagen). A QuantiFast Primer Assay (Qiagen) was used for tissue inhibitor of metalloproteinase-3 (TIMP-3; TIMP-3; His_TIMP3_1_SG, QT00046382). The DAPK1 gene was amplified using the primers DAPK1_fwd (TCCAAAGAAGAGAATGATTCAATTCGA) and DAPK1_rev (TGCCCTTGTGGATCTCTTATGG). The housekeeping gene GAPDH was amplified using the primers GAPDH_fwd (ATTCCACCATGGCAAATTC) and GAPDH_rev (TCTCGCTCCTGGAGATGGT). Using GAPDH as a reference, relative quantification was done using Q-Gene software (21). Primer-specific efficiencies were calculated from serial cDNA dilutions using the dilution method of quantification on the LightCycler (Roche) instrument.

tRNA methylation analysis. RNA bisulfite sequencing of tRNAAsp was done as described previously (22). For 454 sequencing, bisulfite sequence-specific PCR amplions were gel purified and reapplied using primer pairs containing 454 linker sequences. 454 deep sequencing of tRNA-derived cDNAs was done by Agowa (Berlin, Germany).

Transport experiments and cytotoxicity assays in MDCK cells. The sensitivity of MDCK control, MDCK-CNT1, and MDCK-CNT3 cells to CP-4200 and azacytidine, respectively, was assessed with the CellTiter-Blue assay (Promega). MDCK cells were seeded (1 × 10⁶ per well) in 384-well plates and incubated for 24 hours before exposure to graded concentrations of each drug for 24 or 72 hours. S-(4-Nitrobenzyl)-6-thioinosine (NBTI; Biozol) was added at a subtoxic concentration (10 μmol/L) 20 minutes before drug incubation. The IC₅₀ concentration was defined as the drug concentration required to reduce cell survival, as determined by the relative fluorescence of reduced resazurin, to 50%. For transport assays, MDCK cells were seeded in six-well plates at a density of 2 × 10⁶ per well and cultured for 24 hours to confluence. Cells were induced with 10 mmol/L sodium butyrate for additional 24 hours, as described previously (23). Uptake studies examining the Na⁺-dependent uptake of [³H]uridine (0.6 TBq/mmol; Moravek Biochemicals) were done using Na⁺-containing buffer (100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 10 mmol/L HEPES, pH 7.5) and Na⁺-free buffer in which NaCl was replaced by equimolar choline chloride. Cells were first washed three times with Na⁺-free buffer and then incubated with 1 mL of Na⁺-containing buffer containing the labeled nucleoside at the indicated concentration in the absence or presence of unlabeled nucleoside analogues for 5 minutes. After incubation at 37°C, cells were washed three times with ice-cold Na⁺-free buffer. Intracellular radioactivity was determined by liquid scintillation counting, after lysing the cells with 0.2% SDS.

In vivo experiments. Human acute lymphatic leukemia ALL-SCID 6 cells (10⁷; ref. 24) were injected i.v. into 48 nonobese diabetic/severe combined immunodeficient female mice on day 0. Groups of eight mice were randomized to treatment and control groups, respectively. Treatment was initiated on day 3 and repeated every 3 days for five times (q3dx5). The control group received solvent only (10% Tween 80/saline, i.p.). The following treatment modalities were tested: CP-4200 in Phares solubilization—15 mg/kg/inj., i.v., or 20 mg/kg/inj., i.v.; CP-4200 in 10% Tween 80/saline—15 mg/kg/inj., i.v., or 20 mg/kg/inj., i.p.; 5-azacytidine in 10% Tween 80/saline—5 mg/kg/inj., i.v. Mice were sacrificed on day 69 and spleens were resected and weighed.

Results

Azanucleosides are generally characterized by complex modes of action that are based on cytotoxicity and epigenetic modulation of gene expression (2). To determine the effect of CP-4200 on the proliferation of human cancer cell lines, we incubated a panel of three cell lines (HCT116 colon carcinoma, MCF-7 breast cancer, and HL-60 myeloid leukemia) with increasing concentrations of CP-4200 and measured the metabolic activity of the cells after 24, 48, and 72 hours of drug incubation. The results showed well-defined dose-response curves after 48 and 72 hours and established IC₅₀ concentrations of 6, 15, and 2 μmol/L for HCT116, MCF-7, and HL-60 cells, respectively (data not shown). These concentrations were slightly higher but overall similar to the corresponding
IC$_{50}$ concentrations for azacytidine (5, 4, and 1 μmol/L, respectively; data not shown).

To characterize the epigenetic activity of CP-4200, we analyzed the ability of the drug to deplete the DNMT1 protein. This effect is a prominent characteristic of azanucleosides and a consequence of their incorporation into DNA. DNA containing the modified base covalently traps DNMTs and triggers the degradation of DNMT proteins (25). HCT116, MCF-7, and HL-60 cells were incubated with the respective cell line–specific IC$_{50}$ concentrations of CP-4200 or azacytidine to compare the effects between individual cell lines under equitoxic conditions. Protein extracts were prepared after 24 hours and DNMT1 protein was detected by Western blotting. The results showed a rapid CP-4200–dependent depletion of DNMT1 in all cell lines, which was highly similar to the

Figure 2. CP-4200 causes global and gene-specific demethylation in cancer cells. 
A, global DNA methylation levels, as determined by capillary electrophoresis. Bars, SD from three or more measurements. 
B, array-based analysis of gene-specific DNA methylation. Histograms show the numbers of probes (y axis) over the methylation scores (x axis). Methylation scores range from 0 (completely unmethylated) to 1 (completely methylated). 
Blue histograms, results from untreated cells; orange histograms, methylation patterns from treated cells.
DNMT1 depletion observed with azacytidine (Fig. 1B). Differences in the kinetics of DNMT1 depletion between cell lines have been observed before and are probably related to differences in the metabolic activation of the drug (9). These results strongly suggest that intracellular CP-4200 can be metabolized to aza-dCTP and become incorporated into DNA. The observed trapping also predicted that CP-4200 has demethylating activity in human cancer cell lines.

To directly investigate the demethylating activity of the drug, HCT116, MCF-7 and HL-60 cells were incubated with 1 μmol/L CP-4200. Cells were harvested after 24 hours, and after 72 hours, and genomic DNA was isolated. Total cytosine methylation levels were quantified by capillary electrophoresis, which established control methylation levels of 3.1% for HCT116 cells, 2.5% for MCF-7 cells, and 2.8% for HL-60 cells (Fig. 2A). These methylation levels were significantly decreased after 72 hours in all cell lines (Fig. 2A), which confirmed the demethylating activity of CP-4200. A parallel analysis of genomic DNA isolated from the same cell lines treated with 1 μmol/L azacytidine revealed similar effects (Fig. 2A), thus suggesting that the epigenetic activity of CP-4200 is similar to that of the parent drug azacytidine.

To characterize the demethylating activity of CP-4200 in greater detail, we used Illumina HumanMethylation27 DNA Analysis BeadChips to interrogate the methylation status of 27,578 CpG sites across the human genome. The methylation profiles from all three cell lines showed that the majority of sites were unmethylated, with methylation scores ranging from 0 to 0.2 (Fig. 2B). In addition, the methylation profiles also showed a second, smaller peak between 0.8 and 1, which corresponds to highly or completely methylated sites (Fig. 2B). After 72 hours of drug treatment, CP-4200 (1 μmol/L) caused a distinct shift in the hypermethylation peaks toward lower methylation scores (Fig. 2B), which illustrates the demethylation of highly methylated sites. This effect was observed in all three cell lines and was similar to the demethylation effects induced by 1 μmol/L azacytidine (Fig. 2B). Our results thus provide an important confirmation for CP-4200–mediated DNA demethylation and suggest that the drug induces widespread demethylation at numerous hypermethylated loci. These findings also suggest that CP-4200 might be able to revert epigenetic mutations.

Hypermethylation of the TIMP-3 tumor suppressor gene represents a well-described epigenetic mutation in HCT116 cells (26). The TIMP-3 promoter CpG island is represented on the array by four independent probes that were strongly methylated in control HCT116 cells (Fig. 3A). Methylation was distinctly reduced after 72-hour treatment with CP-4200 (Fig. 3A). Similar results could also be observed in DNA obtained from HCT116 cells treated with azacytidine under the same conditions (Fig. 3A). We then used quantitative RT-PCR to determine the amount of TIMP-3 mRNA in HCT116 control and drug-treated cells. The results showed that TIMP-3 is silenced in control HCT116 cells (Fig. 3B). The expression level was not detectably altered after 24 hours of treatment with 1 μmol/L CP-4200, but the gene became strongly upregulated after 72 hours of drug treatment (Fig. 3B). Again, similar effects were observed with the parent drug azacytidine (Fig. 3B), and a tight correlation between TIMP-3 demethylation and activation could be observed in all experiments.

To confirm CP-4200–mediated reversion of epigenetic mutations in an independent model, we analyzed the DAPK-1 epimutation in HL-60 cells (27). The array contains four independent probes that are closely associated with the DAPK1 promoter CpG, and these probes indicated high levels of methylation in control HL-60 cells (Fig. 3C). Methylation was distinctly reduced after 72-hour treatment with CP-4200 (Fig. 3C). Quantitative RT-PCR confirmed that DAPK-1 expression was not detectable in control HL-60 cells (Fig. 3D), but robust
induction was detectable after 72 hours of drug treatment (Fig. 3D). Again, similar effects were observed with the parent drug azacytidine both by DNA methylation analysis and by expression analysis (Fig. 3C and D). Together, these results convincingly illustrate the ability of CP-4200 to revert established epigenetic mutations in human cancer cell lines.

It has been shown recently that azacytidine inhibits DNMT2-mediated tRNA methylation, and it has been suggested that this effect might be an important aspect of the antiproliferative drug effects (28). To investigate whether CP-4200 also inhibits tRNA methylation, we used RNA bisulfite sequencing of tRNAAsp, an established substrate of DNMT2. Sequencing of 300 to 1,000 molecules per RNA sample showed variable CP-4200-dependent demethylation of C38, the primary target residue of DNMT2 in tRNAAsp (Fig. 4). More specifically, CP-4200 treatment induced weak demethylation in HCT116 cells, but readily detectable demethylation in MCF-7 cells after 72 hours of drug incubation and in HL-60 cells after 24 hours of drug incubation (Fig. 4). These observations establish tRNAAsp demethylation as a nontreated cells. Black columns, methylation levels from azacytidine-treated cells; dark gray columns, CP-4200-treated cells; light gray columns, nontreated cells.

It has been shown before that elaidic acid esterification of nucleoside drugs can modulate their cellular uptake mechanisms (15, 17, 29). Transport proteins that have been associated with azacytidine uptake include hCNT1 (13), hCNT3, (30) and hENT1 (31). To elucidate whether CP-4200 enters into the cells by similar pathways, we used MDCK cells, a standard model system for transport studies that is characterized by a basal Ent-dependent uptake of azacytidine (Fig. 5A). After 24 hours of drug incubation, MDCK-CNT1 cells showed a 1.9-fold reduction in the IC50 value for CP-4200 (relative to MDCK control cells) but a 170-fold reduced IC50 value for azacytidine (Fig. 5B). Similarly, increased cytotoxicity was also observed in MDCK-CNT3 cells with 2.7- and 170-fold reductions in the IC50 values for CP-4200 and azacytidine, respectively (data not shown). These results indicate that CP-4200 is not a high-affinity substrate for hCNT1 and hCNT3 and further illustrate the differences between CP-4200 and its parent drug azacytidine in their cellular uptake mechanisms.

To further confirm these findings, we measured the uptake of 1 μM [3H]uridine in MDCK-CNT1 and MDCK-CNT3 cells for 5 minutes in the absence or presence of CP-4200, azacytidine, and decitabine at 100 μM/L concentrations (Fig. 5C). Under these experimental conditions, the nucleoside drugs compete with the radioactively labeled uridine for cellular uptake, if they share the same transport system. The results showed that azacytidine and decitabine were able to inhibit the Na+-dependent uptake of uridine in MDCK-CNT1 and MDCK-CNT3 after 5 minutes (Fig. 5C). In contrast, the
The presence of CP-4200 did not cause inhibition of the hCNT1- and hCNT3-mediated uptake of [3H]uridine. These results again suggest that CP-4200 is not a high-affinity substrate for hCNT1 and hCNT3 and thus further confirm that the cellular uptake mechanisms for CP-4200 and azacytidine are different.

Having shown that the elaidic acid modification of azacytidine modifies the cellular uptake mechanism of the drug without affecting its epigenetic potency, we also analyzed the therapeutic efficacy of CP-4200. To this end, an orthotopic in vivo tumor model was used (24). Forty-eight mice were randomized to six different treatment and control groups, respectively, and treatment was started at day 3 after i.v. injection of acute lymphocytic leukemia cells. In the course of the experiment, it was observed that the combination of Phares solubilization and i.v. administration seemed to be better tolerated (data not shown). After 69 days, mice were sacrificed and spleen weights were measured to determine the effect of the drug on splenomegaly, a prominent characteristic of the tumor model. The results showed that all four treatment modalities induced a significant decrease of the spleen weight with respect to the control group, which was independent of the route of application and dose (Fig. 6B). Notably, the decrease in splenomegaly consistently seemed to be more pronounced for CP-4200 than for an equitoxic dose of azacytidine, and the differences were statistically significant for three of four groups (Fig. 6B). These results suggest an increased therapeutic efficacy of CP-4200 when compared with azacytidine.

Discussion

Over the past few years, azacytidine has been approved for the treatment of myelodysplastic syndrome and has found increasing clinical use for the treatment of myeloid leukemias (33). Attempts to develop azacytidine for the clinical management of other tumor indications have remained largely unsuccessful. The underlying reasons are complex and include historical factors as well as specific biological features of myeloid leukemias (1). However, it is also becoming clear that efficient intracellular accumulation of azacytidine depends on the expression of specific transport proteins. As such, it is reasonable to assume that tissues with
a low level of intracellular drug uptake and/or a high level of drug efflux are less sensitive to the effects of azacytidine.

The esterification of nucleoside drugs with fatty acids represents an established strategy for modifying the cellular uptake of these drugs. CP-4200 is an elaidic acid ester of azacytidine that has been synthesized with the intention of rendering drug uptake less dependent on conventional nucleoside transport systems. Indeed, our results indicate that the cellular uptake mechanisms of azacytidine and CP-4200 seem to be fundamentally different. More specifically, the endogenously expressed Ent proteins of MDCK cells were found to be important for the cellular uptake of azacytidine, but seemed to be largely dispensable for the uptake of CP-4200. Furthermore, our data also show that CP-4200 is not a high-affinity substrate for hCNT1 and hCNT3, whereas these proteins have been shown to efficiently mediate cellular uptake of azacytidine (15, 30). It is possible that CP-4200 enters cells through the membrane, either by a passive, concentration-dependent uptake mechanism or by an active transport pathway (34). However, alternative possibilities, including the extracellular cleavage of CP-4200 by plasma membrane lipases, followed by a secondary uptake of the free azacytidine group through hCNT1 or hCNT3 should also be considered in future studies.

Protection from inactivation by cytidine deaminase and efficient metabolic activation by cytidine kinase are important biochemical processes that modulate the potency of azacytidine (2). It was shown recently that elaidic acid esterification of the cytidine analogue gemcitabine strongly increases resistance to cytidine deaminase–mediated drug inactivation (35). As such, it is very likely that CP-4200 is a similarly poor substrate for cytidine deaminase. In addition, our results show that CP-4200 effectively depletes DNMT1 protein, which suggests that the added metabolic steps required for the activation of CP-4200 do not reduce the concentration of the active metabolite in human cancer cells. Our results also show that elaidic acid esterification does not affect the epigenetic potency of the drug. Genome-wide DNA methylation profiling of three different human cancer cell lines treated with CP-4200 showed widespread DNA demethylation of hypermethylated markers. In agreement with this finding, CP-4200 also caused efficient reactivation of epigenetically silenced tumor suppressor genes. Overall, we noticed that the epigenetic modulatory effects of CP-4200 seemed to be similar to that of azacytidine. The low dependency on nucleoside transporters, combined with the high epigenetic modulatory potency, establishes CP-4200 as an intriguing drug candidate for epigenetic cancer therapy.

Although azacytidine has shown significant clinical benefits in the treatment of myeloid leukemias, only little clinical activity has been shown in solid tumors and in lymphocytic leukemias. A good example is provided by acute lymphocytic leukemia, in which epigenetic lesions have been found to be prevalent (36–39) but in which clinical activity of demethylating agents has only been shown for isolated cases (40). We have tested azacytidine and CP-4200 in an orthotopic ALL mouse tumor model. Our results showed a stronger therapeutic effect for CP-4200 in all treatment groups, with the difference being statistically significant in three of four groups. These results further support the notion that CP-4200 could be effective for the treatment of tumor entities that show little or no effect with azacytidine.

**Disclosure of Potential Conflicts of Interest**

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**References**


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