In vitro assessment of cytochrome P450 inhibition and induction potential of azacitidine

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Abstract

Purpose To assess the potential inhibitory and inductive effects of azacitidine on cytochrome P450 isozymes in vitro.

Methods The inhibitory effects of azacitidine on various CYP isozymes were determined in human liver microsomes. In addition, the ability of azacitidine to induce CYP enzymes in cultured human hepatocytes was evaluated.

Results Azacitidine did not inhibit CYP2B6-, CYP2C8-, CYP2C9-, CYP2C19-, CYP2D6-, and CYP3A4-mediated activities in human liver microsomes up to a concentration of 100 μM, while weak inhibition (<30% inhibition) of CYP1A2 and CYP2E1 activities was observed at 100 μM azacitidine. In vitro azacitidine did not induce CYP1A2, CYP2C19, or CYP3A4/5 activities in cultured human hepatocytes.

Conclusions Azacitidine is not an inhibitor or inducer of the cytochrome P450 isozymes tested; therefore, clinically relevant pharmacokinetic drug–drug interactions are unlikely to occur between azacitidine and co-administered substrates of these CYP isozymes.

Keywords Azacitidine · Cytochrome P450 · CYP inhibition · CYP induction · Drug–drug interactions

Introduction

Azacitidine (5-azacitidine) is an analogue of the naturally occurring pyrimidine nucleoside cytidine possessing pharmacological effects on cell differentiation, gene expression, and DNA synthesis and metabolism [1–4]. Mechanistically, azacitidine is believed to exert its antineoplastic effects by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow [4, 5]. Azacitidine is used for the treatment of all subtypes of myelodysplastic syndrome. It has also demonstrated activity for the treatment of acute myelogenous leukemia [6, 7].

Cytochrome P450 (CYP) is a superfamily of mixed function oxidases that are responsible for the metabolism of many drugs including oral contraceptives, dexamethasone, and many anticancer agents such as taxanes. Some of these enzymes are polymorphically expressed (e.g., CYP2D6, CYP2C19) resulting in wide variability of pharmacokinetics of their substrate drugs. CYP isozymes are subject to inhibition via competitive or other mechanisms by drugs such as ketoconazole, ritonavir, and clarithromycin, leading to clinically relevant increases in the exposure of the affected drug. Further, some of the CYP isozymes are also subject to induction by xenobiotics via activation of nuclear receptors, with a consequent result of decreased exposure of the affected compound (e.g., rifampicin and oral contraceptive steroids) leading to therapeutic failure or toxicological implications due to higher levels of an undesired metabolite [8, 9].

For a new molecular entity, it is important to assess its likely inhibitory or inductive effects on the metabolic reactions catalyzed by CYP isozymes. Anticancer therapeutic regimens often involve polypharmacy comprising of multiple therapeutic agents and/or palliative therapies. Recent clinical studies have explored combinations of azacitidine
with other anticancer agents to enhance the therapeutic activity [10–13]. It is likely that azacitidine will be administered with other therapeutic agents in clinical settings. This study was conducted to evaluate the potential for drug–drug interactions with azacitidine as an inhibitor or inducer of CYP enzymes. The clinical dosing regimen of azacitidine is by subcutaneous injection or intravenous infusion of 75 mg/m² for 7 days every 4 weeks (Vidaza® package insert). The peak plasma concentrations of azacitidine at the therapeutic dose are approximately 3 μM [14, 15]. The concentrations of azacitidine employed in this study span and exceed the therapeutic concentration range.

Materials and methods

Azacitidine was synthesized by Ash Stevens™ (Riverview, MI). Pooled human liver microsomes were obtained from In Vitro Technologies (NJ, USA), Tissue Transformation Technologies (T3) (NJ, USA), BD Biosciences Discovery Labware (Woburn, MA), and CellzDirect™ Invitrogen Corporation (Durham, NC). NADPH was purchased from Sigma–Aldrich Co Ltd (St. Louis, USA). NADP+ (disodium salt) and glucose 6-phosphate (disodium salt) were obtained from Roche Products Ltd (Exeter, UK). S-mephenytoin was purchased from Research Biochemicals International (Natick, MA). Resorufin and 7-ethoxyresorufin were purchased from Molecular Probes (Junction City, OR). All other reagents including rifampin, β-naphthoflavone (BNF), and testosterone were obtained from Sigma–Aldrich Chemical Company, Ultrafine Chemicals Ltd (Manchester, UK), Affiniti Research Products Ltd (Exeter, UK), FSA Laboratory Supplies (Loughborough, UK), BDH Ltd (Poole, UK), Helget Gas (Kansas City, MO), Gibco BRL (Grand Island, NY), Pierce Chemical Co (Rockford, IL), J.T. Baker Inc. (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), Cohesion (Santa Clara, CA), and Toronto Research Chemicals Inc (North York, ON, Canada). All chemicals were of analytical grade or higher.

Inhibitory effect on cytochrome P450s

Stock solution of azacitidine and subsequent serial dilutions were freshly prepared and refrigerated until use. Other stock solutions of substrates and inhibitors were made in either aqueous or organic solvents, and the final percentage of organic solvent in incubation mixture was less than 1%. Incubations were conducted at 37°C either in pH 7.4 potassium phosphate buffer or Tris buffer in the presence of human liver microsomes (concentrations from 0.05 to 2 mg/mL) and azacitidine at concentrations ranging from 0.1 to 100 μM. The incubation mixture was preincubated for 5 min prior to the initiation of metabolic reaction with NADPH cofactor, in either duplicate or triplicate (CYP2B6 and CYP2C8), alongside vehicle control. After incubation, the reactions were terminated by addition of quenching reagents such as acetonitrile, hydrochloric acid, or perchloric acid. Incubation conditions for evaluating CYP inhibition are summarized in Table 1. Experiments with positive control inhibitors were conducted in parallel, and furafylline as an inhibitor for CYP1A2 was preincubated for 15 min prior to initiation with substrate.

Inductive effect on cytochrome P450s

Human hepatocytes were prepared from three non-transplantable human liver tissue samples procured from Blue Color Scientific (Chapel Hill, NC). Fresh hepatocytes were isolated and cultured according to established methods [16–20]. Hepatocytes were seeded (approximately 1 million hepatocytes per mL) on 60-mm Permanox culture dishes (Fisher Scientific, Pittsburgh, PA), coated with collagen (PureCol), and placed in a humidified culture chamber (37 ± 1°C, at 95% relative humidity, 95/5% air/CO2). After an attachment period of 2–3 h, media and dead or unattached cells were removed by aspiration, and the media were replaced with modified Eagle’s medium Dr. Chee’s modification containing ITS+ (6.13 μg/mL insulin, 6.13 μg/mL transferrin, and 6.13 ng/mL selenious acid), linoleic acid (5.25 μg/mL), BSA (1.23 mg/mL), penicillin (49 U/mL), streptomycin (49 μg/mL), dexamethasone (0.098 μM), and Matrigel (250 μg/mL). The viability of hepatocytes was assessed by the trypan blue exclusion method. Cultures were allowed to adapt to the culture environment for 3 days with daily media replacement. Hepatocyte cultures were treated once daily for three consecutive days, with medium containing 0.1% dimethyl sulfoxide (DMSO) (vehicle, negative control), one of the three concentrations of azacitidine (1.0, 10 or 100 μM), or one of the two known human CYP enzyme inducers (positive controls: 33 μM β-naphthoflavone and 20 μM rifampin). At the end of the treatment period, microsomal samples were prepared and stored at −80°C until analysis. Microsomal incubation was carried out with the CYP1A2, CYP2C19, and CYP3A4/5 isozyme-specific probe substrates at the concentrations and incubation conditions indicated in Table 2. The assessment of three CYP1A2, 2C19, and 3A4/5 activities was carried out as nuclear receptors, such as the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR), have been recognized as key mediators of drug-induced changes in the
expression of CYP1A, CYP3A, CYP2B, and the CYP2C enzymes. All incubations were conducted in duplicate wells at 37 ± 1°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 Unit/mL). Reactions

CYP1A2:
Ethoxyresorufin
O-deethylation, 0.4 μM
3; 0.1; 50 mM PPB
Fluorescence
λ<sub>e</sub>x = 530 nm,
λ<sub>e</sub>m = 585 nm
Azacitidine (0.1–100)
Furafylline (10)
19.4% at 100 μM; 53.7%

CYP2C9:
Tolbutamide 4-methyl hydroxylation, 100 μM
30; 2; 50 mM Tris
HPLC–UV/β-Ram
Radiochemical
Azacitidine (0.1–100)
Sulfaphenazole (10)
NI; 80.4%

CYP2C19:
S-mephenytoin 4-hydroxylation, 96.4 μM
30; 2; 100 mM Tris
HPLC–UV/β-Ram
Radiochemical
Azacitidine (0.1–100)
Tranylcypromine (50)
NI; 51.8%

CYP2E1:
Chlorzoxazone 6-hydroxylation, 40 μM
15; 0.8; 50 mM PPB
HPLC–UV (297 nm)
Radiochemical
Azacitidine (0.1–100)
Diethyldithiocarbamate (50)
27.1% at 100 μM; 75.0%

CYP2D6:
Bufuralol 1'-hydroxylation, 10 μM
30; 0.05; 100 mM PPB
HPLC–Fluorescence,
λ<sub>e</sub>x = 252 nm,
λ<sub>e</sub>m = 302 nm
Azacitidine (0.1–100)
Quinidine (1)
NI; 80.8%

CYP2B6:
Bupropion Hydroxylation, 50 μM
20; 0.25; 100 mM PPB
HPLC–MS/MS
Azacitidine (0.1–100)
DI: Orphenadrine
NI; 195 μM

CYP2C8:
Paclitaxel 6α-hydroxylation, 10 μM
10; 0.5; 100 mM PPB
HPLC–MS/MS
Azacitidine (0.1–100)
DI: Montelukast
NI; 0.46 μM

CYP3A4:
Testosterone 6β-hydroxylation, 65 μM
10; 0.4; 50 mM PPB
HPLC–UV/β-Ram
Radiochemical
Azacitidine (0.1–100)
Ketoconazole (1)
NI; 70.8%

NI No inhibition, DI Direct inhibition, HPLC High performance liquid chromatography, MS Mass spectrometry, PPB potassium phosphate buffer

* Azacitidine concentration range for each CYP isozyme was 0.1–100 μM

Table 2 Experimental conditions for CYP induction study

| CYP enzymes: | CYP1A2 | CYP2C9 | CYP3A4/5 |
|--------------|--------|--------|----------|
| CYP-mediated reactions: | 7-Ethoxyresorufin O-dealkylation | S-mephenytoin 4'-hydroxylation | Testosterone 6β-hydroxylation |
| Microsome content; incubation time | 0.1 mg; 30 min | 0.04 mg; 240 min | 0.05 mg; 20 min |
| Organic solvent (% in incubation mixture) | DMSO (0.4%) | Methanol (1.2%) | Methanol (2%) |
| Substrate; concentration | 7-ethoxyresorufin; 10 μM | S-mephenytoin; 400 μM | Testosterone; 250 μM |
| Reaction stop reagents | Acetone | Methanol | Methanol |
| Metabolites monitored | Resorufin | 4'-hydroxy-mephenytoin | 6β-hydroxy-testosterone |
| Analytical method | Fluorimetric λ<sub>e</sub>x = 535 nm, λ<sub>e</sub>m = 585 nm | HPLC–UV (204 nm) | HPLC–UV (254 nm) |

* HPLC–UV high performance liquid chromatography–ultraviolet detection

Ref [26, 27] for additional details

expression of CYP1A, CYP3A, CYP2B, and the CYP2C enzymes. All incubations were conducted in duplicate wells at 37 ± 1°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 Unit/mL). Reactions
were initiated by addition of the NADPH-generating system and were terminated after the appropriate incubation time by addition of acetonitrile. Precipitated protein was removed by centrifugation (920 g for 10 min at 10°C), and supernatants were analyzed for the respective metabolites (Table 2). The catalytic activities in cultures treated with azacitidine and positive control inducers were compared to DMSO-treated cultures. A one-way analysis of variance (ANOVA) for repeated measures was carried out to determine whether there were significant differences between the group means. The ANOVA was followed by a Dunnett’s post hoc test to identify the group means that were significantly different from the controls (P < 0.05 or 5% level of significance). This statistical test is designed for multiple comparisons with a mean, such as comparing multiple treatment groups with a control group. Statistical analyses were performed using SigmaStat Statistical Analysis System (version 2.03, SPSS Inc, Chicago, IL).

Results

Inhibitory effects on cytochrome P450s

The effect of azacitidine on isozyme-specific CYP activities in human liver microsomes is summarized in Table 1. As expected, all positive control inhibitors produced inhibitory effects on the respective catalytic activities. Azacitidine did not inhibit CYP2B6-, CYP2C8-, CYP2C9-, CYP2C19-, CYP2D6- and CYP3A4-mediated activities up to 100 μM concentration under the in vitro conditions tested. Although limited inhibition of CYP1A2-dependent ethoxyresorufin O-dealkylation (19%) and CYP2E1-dependent chlorzoxazone 6-hydroxylation (27%) was observed at the highest concentration of azacitidine (100 μM) tested, no inhibition of these two CYP enzymes was observed at 70 μM or lower concentrations of azacitidine tested.

Inductive effects on cytochrome P450s

At the time of isolation, the viability of each hepatocyte preparation was between 73 and 89%. During the adaptation followed by a 72-h incubation period, human cultured hepatocytes were judged to be morphologically normal and adequately confluent for treatment with test and control articles. In general, the cells were characteristically cuboidal and contained intact cell membranes and granular cytoplasm with one or two centrally located nuclei. Enzymatic activities (pmol/mg protein/min) and fold-over DMSO (negative control) data are listed in Table 3. As expected, the positive control inducers produced marked elevations in most of the isozyme-specific catalytic activities tested. For all three CYP isozymes tested, (CYP1A2, CYP2C19, and CYP 3A4/5) treatment of azacitidine at 1 and 10 μM resulted in catalytic activities that were 0.86–1.20-fold of the catalytic activities of the vehicle-treated cultures, demonstrating no notable inductive effect of azacitidine. At 100 μM azacitidine, the CYP enzyme activities showed a 29% decrease of CYP1A2-mediated 7-ethoxyresorufin O-dealkylation, 56% decrease of CYP2C19-catalyzed S-mephenytoin 4′-hydroxylation, and a 78% decrease of CYP3A4/5-catalyzed testosterone 6β-hydroxylation. However, only the CYP3A4/5 activity decrease was statistically significant. Though morphologically azacitidine-treated hepatocytes were observed to be similar to those of vehicle-treated hepatocytes, it is possible that azacitidine, being a cytotoxic compound, may have adverse effects on primary hepatocytes at high concentrations. Overall, the results of this study demonstrate that treatment of cultured human hepatocytes with clinically relevant concentrations of

Table 3  Effect of azacitidine on isozyme-specific cytochrome P450 enzymatic activities in cultured human hepatocytes

| Treatment          | Concentration | Enzymatic activity (pmol/mg protein/min)§ (Mean fold over control) |
|--------------------|---------------|-------------------------------------------------|
|                    |               | 7-Ethoxyresorufin O-dealkylation (CYP1A2) | S-mephenytoin 4′-hydroxylation (CYP2C19) | Testosterone 6β-hydroxylation (CYP3A4/5) |
| DMSO               | 0.1% (v/v)   | 4.09 ± 0.86                                    | 7.80 ± 0.78                                 | 4190 ± 1350 |
| Azacitidine        | 1 μM          | 4.11 ± 0.94 (1.0)                              | 9.47 ± 4.76 (1.2)                           | 4570 ± 1960 (1.1) |
| Azacitidine        | 10 μM         | 3.78 ± 0.72 (0.93)                             | 6.82 ± 2.89 (0.86)                          | 3650 ± 1320 (0.86) |
| Azacitidine        | 100 μM        | 2.91 ± 0.77 (0.71)                             | 3.51 ± 1.16 (0.44)                          | 936 ± 443* (0.22) |
| β-Naphthoflavone  | 33 μM         | 36.3 ± 1.5* (9.2)                              | 33.6 ± 13.6* (4.4)                          | 1880 ± 1150 (0.42) |
| Rifampin           | 20 μM         | 6.75 ± 1.29* (1.7)                             | 59.0 ± 10.2* (7.5)                          | 13100 ± 200* (3.3) |

§/v: volume/volume, DMSO: dimethyl sulfoxide

* Values are the mean ± standard deviation of three human hepatocyte preparations

* Significantly different from control (0.1% DMSO) according to Dunnett’s test (P < 0.05)
Azacitidine, once daily for three consecutive days, did not induce CYP1A2, CYP2C19, and CYP3A4/5 activities.

Discussion

Azacitidine is shown to undergo non-enzymatic degradation to N-formylamidinoribosyl guanylurea and is susceptible for further decomposition both in vitro and in vivo. In addition, cytidine deaminase and uridine-cytidine kinase play a role in the metabolism of azacitidine via deamination and phosphorylation, respectively [21–24]. In humans, azacitidine and/or its metabolites were eliminated renally, with 50–98% of the radioactive dose recovered in urine [24], and CYP enzymes do not play a role in metabolic clearance. Therefore, co-administration of potent CYP inhibitors (e.g., ketoconazole, fluconazole, fluoxamine, and quinidine) or inducers is not likely to result in clinically relevant effects on the exposure of azacitidine.

Based on early promising results in recent in vitro and in vivo studies, azacitidine could potentially be administered with other anticancer drugs in clinical settings [10, 12, 25], some of which are likely to be CYP substrates. It is important to understand any potential drug–drug interactions due to inhibition or induction of CYP enzymes by azacitidine. The results of these studies demonstrate that azacitidine does not inhibit or induce CYP enzymes in vitro. The peak plasma concentrations of azacitidine at therapeutic dose are approximately 3 μM, which are considerably lower than the highest concentration (100 μM) evaluated in this in vitro study. Hence, azacitidine is not anticipated to precipitate clinically relevant pharmacokinetic drug–drug interaction when co-administered with CYP substrates either due to inhibition or induction.

Conclusion

Azacitidine is not an inhibitor of CYP enzymes nor inducer of CYP group of enzymes via activation of AhR, CAR, or PXR nuclear receptors; therefore, clinically relevant pharmacokinetic drug–drug interactions are unlikely to occur between azacitidine and co-administered substrates of these CYP isoenzymes.

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