ABSTRACT

Gemcitabine (dFdC, 2',2'-difluorodeoxycytidine) is metabolized by cytidine deaminase (CDA) and deoxycytidine kinase (DCK), but the contribution of genetic variation in these enzymes to the variability in systemic exposure and response observed in cancer patients is unclear. Wild-type enzymes and variants of CDA (Lys27Gln and Ala70Thr) and DCK (Ile24Val, Ala119Gly, and Pro122Ser) were expressed in and purified from Escherichia coli, and enzyme kinetic parameters were estimated for cytarabine (Ara-C), dFdC, and its metabolite 2',2'-difluorodeoxyuridine (dFdU) as substrates. All three CDA proteins showed similar $K_m$ and $V_{max}$ for Ara-C and dFdC deamination, except for CDA70Thr, which had a 2.5-fold lower $K_m$ and 6-fold lower $V_{max}$ for Ara-C deamination. All four DCK proteins yielded comparable metabolic activity for Ara-C and dFdC monophosphorylation, except for DCK24Val, which demonstrated an approximately 2-fold increase ($P < 0.05$) in the intrinsic clearance of dFdC monophosphorylation due to a 40% decrease in $K_m$ ($P < 0.05$). DCK did not significantly contribute to dFdU monophosphorylation. In conclusion, the Lys27Gln substitution does not significantly modulate CDA activity toward dFdC, and therefore would not contribute to interindividual variability in response to gemcitabine. The higher in vitro catalytic efficiency of DCK24Val toward dFdC monophosphorylation may be relevant to dFdC clinical response. The substrate-dependent alterations in activities of CDA70Thr and DCK24Val in vitro were observed for the first time, and demonstrate that the in vivo consequences of these genetic variations should not be extrapolated from one substrate of these enzymes to another.

INTRODUCTION

The deoxycytidine analog gemcitabine [2',2'-difluorodeoxycytidine (dFdC)] is active toward numerous solid tumor types, but has a narrow-therapeutic index and variable responses ranging from lack of efficacy to severe cytotoxicity, which may be attributed to variability in drug exposure. Genetic variation of cytidine deaminase (CDA) and deoxycytidine kinase (DCK) may help explain the variable response due to their important roles in dFdC metabolism. Appropriate genotyping may provide much-needed biomarkers for optimizing patient care (Ciccolini et al., 2011). More than 90% of an i.v. dose of dFdC is metabolized by CDA to its inactive metabolite 2',2'-difluorodeoxyuridine (dFdU). dFdC is phosphorylated by DCK to its monophosphate (dFdCMP) and, subsequently, to the active triphosphorylated form (dFdC triphosphate), which is incorporated into DNA and leads to apoptosis. Cells lacking DCK activity are resistant to the dFdC cytotoxicity (Mini et al., 2006). Additionally, dFdU was reported to form dFdU monophosphate (dFdUMP) via direct phosphorylation (Veltkamp et al., 2008), but it is unknown whether DCK is involved (Supplemental Fig. 1). Understanding the regulation of dFdUMP is important because dFdUMP may lead to DNA damage by inhibiting thymidylate synthase (Mini et al., 2006).

Numerous single-nucleotide polymorphisms (SNPs) of CDA and DCK have been identified, but their impact on dFdC clinical response is not well understood. These polymorphisms include two non-synonymous SNPs in CDA [79A>C (Lys27Gln) and 208G>A (Ala70Thr)] and three nonsynonymous SNPs in DCK (Ile24Val, Ala119Gly, and Pro122Ser) (Kocabas et al., 2008; Deenen et al., 2011). The haplotypes harboring 79A>C and 208G>A were designated CDA*2 and CDA*3, respectively. The effects of the 208G>A mutation on clinical outcomes to dFdC treatment have been demonstrated (Deenen et al., 2011). However, conflicting data exist on the association of the CDA 79A>C mutation with response. In comparison with the C-allele (Gln27) carriers, the CDA79A allele (Lys27) has been associated with significantly decreased, increased, or comparable deaminase activity, suggested by clinical outcomes or dFdC pharmacokinetics (Deenen et al., 2011). On the other hand, the Pro122Ser variant of DCK was not associated with the dFdC treatment.
outcomes in 107 malignant mesothelioma patients (Erçul et al., 2012), although it was associated with decreased DCK expression and activity in vitro (Kocabas et al., 2008).

In vitro functionality assay data help clarify the functional consequences and elucidate the role of these nonsynonymous SNPs as potential predictive pharmacogenomics biomarkers for dFdC. However, previous in vitro studies addressed the direct effects of some of these variants on dFdC metabolism using crude cell extracts, which may be confounded by other enzymes with overlapping substrate specificities (Gilbert et al., 2006; Kocabas et al., 2008). Additionally, the effects of Ala70Thr substitution on the CDA catalytic activity for dFdC or the role of DCK in dFdU metabolism is unknown. The current study used purified recombinant enzymes of these nonsynonymous SNPs of CDA and DCK to better understand their impact on dFdC using in vitro comparative kinetic studies.

Materials and Methods

Materials. dFdC, dFdU, dFdCMP, 2',3'-deoxy- 2',2'-difluorouridine-5'-monophosphate (dFUDP), di-lithium dFdU, and [3H]dFdU were synthesized at Eli Lilly and Company (Indianapolis, IN). The following reagents were obtained commercially: cytosine β-D-arabinofuranoside-5'-MP (Moravek Biochemicals, Brea, CA), cytarabine-13C,15N2 5'-monophosphate (Santa Cruz Biotechnology, Santa Cruz, CA), Tris acetate buffer (Teknova, Hollister, CA), and tris-HCl buffer (In vitrogen, Carlsbad, CA). The following were obtained from Sigma-Aldrich (St. Louis, MO): cytarabine (ara-C), arabinofuranosyl1 uridine, KCl, MgCl2, ATP-Mg, cytidine 5'-monophosphate, thymidine 3'-monophosphate sodium salt, and bovine serum albumin (BSA).

Expression and Purification of Human Recombinant CDA and DCK Variants. A cDNA clone of wild-type (WT) CDA (NM_0010578.2) was obtained from Open Biosystems (Lafayette, CO) (clone ID: LIFEOSEQ744666, catalog number IHS1380-97652440), and CDA variants were subsequently generated using polymerase chain reaction–mediated mutagenesis. Full-length cDNAs encoding DCK-WT (NM_007788.2) and variants of interest were synthesized (Gene Oracle, Mountain View, CA). All of the CDA clones were inserted into pET21d (Novagen, Darmstadt, Germany) with N-terminal His-SUMO (In vitrogen) fusion, and all DCK clones were inserted into pET21d with an N-terminal His tag (In vitrogen). Bacterial BL21(DE3)pLysS (Novagen) was used as an expression host, and the induction of protein expression was carried out with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 18°C overnight. All proteins were purified using Ni-NTA Agarose (Qiagen, Valencia, CA) according to the standard protocol (Qiagen), followed by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare Biosciences, Piscataway, NJ). The His-tagged DCK proteins were eluted in the storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 10% glycerol) and stored at −80°C. The SUMO-CDA fusion protein was further digested with SUMO protease (In vitrogen) to generate untagged CDA protein, which was subsequently purified using ion exchange chromatography on a Mono Q 10/100GL column (GE Healthcare Biosciences). The purified CDA proteins were stored in buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, 10% glycerol) at −80°C. Protein concentrations were determined by the Bradford assay using BSA as standard. Protein identities of CDA and DCK were confirmed by N-terminal sequencing and matrix-assisted laser desorption/ionization, respectively. Enzyme purity and molecular mass were determined by SDS-PAGE and visualized by Comassie Blue staining (In vitrogen).

Molecular Weight Estimation and Zinc Content Analysis in CDA. The molecular mass of the recombinant CDA was determined by gel filtration using a fast protein liquid chromatography system, and the zinc content of CDA proteins was quantified with inductively coupled plasma optical-emission spectrometry, as described previously (Vincenzetti et al., 1996).

In Vitro Kinetics Studies. For the incubations with CDA proteins, the final reaction mixtures (100 μl) consisted of 50 mM Tris acetate buffer (pH 7.4), substrate (ara-C: 20–2560 μM, or dFdC: 15.63–2000 μM), BSA, and recombinant CDA proteins (Miwa et al., 1998). Substrates were preincubated in Tris buffer for approximately 3 minutes at 37°C, and reactions were then initiated with the addition of expressed CDA protein (0.1 μg) and BSA (0.05 mg), and incubated for 1 minute at 37°C, reflecting linear rate conditions.

For the incubations with DCK proteins, the final reaction mixtures (100 μl) consisted of 50 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, 5 mM MgCl2,1 mM ATP-Mg, substrate, and recombinant DCK proteins (Sabini et al., 2003). When examining the metabolism of dFdC to dFdCMP or Ara-C to cytosine β-D-arabinofuranoside-5’-MP(Ara-CMP), substrate (dFdC or Ara-C: 0.156–320 μM) was preincubated with buffer for approximately 3 minutes at 37°C. Reactions were then initiated with the addition of DCK enzyme (40–100 ng) and incubated for 1 minute (dFdC) or 3 minutes (Ara-C) at 37°C, reflecting linear rate conditions. Incubations designed for the dFdU to dFUMP conversion were carried out under the same conditions, except that reactions were initiated with the addition of 4 ng of DCK-WT and incubated with a range of dFdU concentrations (7.8–500 μM) for 10 minutes at 37°C. All incubations were stopped by adding 100 μl acetonitrile containing the appropriate internal standard, and centrifuged to remove denaturated protein. The supernatant was subjected to liquid chromatography-tandem mass spectrometry analysis using methods described in Supplemental Table 1.

Data Analysis. The estimation of enzyme kinetic parameters was conducted by nonlinear regression (WinNonlin; Pharsight Corp., Mountain View, CA) using the best-fit model, and the intrinsic clearance (CLint) was calculated as Vmax/Km. Comparisons of kinetic parameters between the individual variant and WT were made using one-way analysis of variance followed by Dunnett’s t test. Statistical significance of kinetic parameter differences between enzymes was defined as P < 0.05.

Results and Discussion

Expressed recombinant proteins of CDA (WT, Lys27Gln, and Ala70Thr) and DCK (WT, Ile24Val, Ala19Gly, and Pro122Ser) used in this study were characterized and validated. The purity of all proteins was >90%, as demonstrated by the major band at an expected size of 16 kDa (CDAs) (Vincenzetti et al., 1996) and 30.5 kDa (DCKs) (Sabini et al., 2003) on SDS-PAGE (Supplemental Fig. 2). The patterns of Michaelis-Menten and substrate inhibition kinetics exhibited by all CDA and DCK proteins (Fig. 1; Table 1), respectively, toward the prototypical substrate Ara-C were similar to other studies (White and Capizzi, 1991; Laliberte et al., 1992). The Km values of 138 μM in CDA-WT and 3.04 μM in DCK-WT for Ara-C (Table 1) were consistent with previous reports, which were 140–169 μM (Laliberte et al., 1992; Yue et al., 2003) and 1.3–15.5 μM (White and Capizzi, 1991; Sabini et al., 2003), respectively, for CDA and DCK. The physicochemical properties of these three CDA proteins were shown to be comparable. All three CDA proteins were found to have a major peak at an estimated size of 57 kDa by gel-filtration methods, suggesting comparable levels of tetrameric enzyme components among the three allelic variants (unpublished data). In the zinc content assay, a 1-mol zinc/mol of subunit for each of the three CDA proteins was observed, as expected, indicating an identical level of incorporated zinc within each of the variants. In the current study, only the Km and Vmax values for CDA70Thr were approximately 2.5- and 6-fold lower (P < 0.05), respectively, than those of the CDA-WT, which caused a 2.5-fold decrease in the catalytic efficiency of Ara-C deamination (Fig. 1; Table 1). Therefore, the relative order of catalytic activities among the three CDA variants for Ara-C (WT = CDA27Gln > CDA70Thr) was similar to previous studies (Yue et al., 2003; Vincenzetti et al., 2004).}

The effects of the three CDA variants on the kinetics of deamination of dFdC were investigated. The formation of dFdU from dFdC yielded Michaelis-Menten kinetics for all three CDA variants, consistent with a single enzyme responsible for the conversion of dFdC to dFdU (Fig. 1). Furthermore, in contrast to Ara-C, for which CDA70Thr demonstrated reduced activity, all three CDA variants exhibited similar Km and Vmax for dFdC conversion to dFdU (Table 1), suggesting that they have a similar binding affinity and deamination efficiency for dFdC.
One major finding from this study was that the CDA Lys27Gln substitution alone does not appear to be a significant contributor to alterations in CDA activity toward dFdC; Lys27Gln did not affect CDA kinetics in our study, and an earlier study demonstrated this amino acid change does not impact CDA protein expression as shown by immunoblots (Gilbert et al., 2006). However, our CDA kinetic result differs from that of Gilbert et al. (2006), which reported a modest $K_m$ value increase in CDA27Gln (397 $\pm$ 40 $\mu$M) relative to the WT (289 $\pm$ 20 $\mu$M) for dFdU formation. Purified proteins were used in the present study to exclude the interference of other enzymes with overlapping substrate specificities, as opposed to the crude cell extracts from transiently overexpressed COS-1 cells used by Gilbert et al. (2006). Therefore, these data provide a potential mechanistic explanation for the inconsistent results regarding the relationships of CDA*2 haplotype and dFdC clinical response in many candidate-gene association studies that genotyped 79A>C (Lys27Gln) to represent the *2 haplotype. It may be the imbalanced distribution of additional functional variants among the Lys27 and Gln27 carriers that led to imbalanced responders versus non-responders/adverse responders, and thus complicated the interpretation of this SNP contribution to dFdC response. In fact, additional CDA functional polymorphisms that are in partial linkage disequilibrium with 79A>C have been found to be associated with increased gene expression (Parmar et al., 2011).

Additionally, other factors, such as genetic changes in linkage disequilibrium with 208G>A and/or epigenetic regulation, may also contribute to the lower CDA activity associated with the CDA*3 haplotype in vivo, which was not observed in this in vitro kinetic study (Fig. 1; Table 1). Therefore, identification of predictive genetic biomarkers for dFdC clinical responses requires a better understanding of the genetic and epigenetic regulation of the CDA gene that modulates its phenotypic activity.

In this first study to examine the direct effect of Ala70Thr on the CDA activity of dFdC, a significant finding is that the magnitude of the reduction in catalytic activity with 208G>A and/or epigenetic regulation, may also contribute to the lower CDA activity associated with the CDA*3 haplotype in vivo, which was not observed in this in vitro kinetic study (Fig. 1; Table 1). Therefore, identification of predictive genetic biomarkers for dFdC clinical responses requires a better understanding of the genetic and epigenetic regulation of the CDA gene that modulates its phenotypic activity.

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in whom the frequency of the 70Thr allele variant is 3.7% and 13%, respectively (Fukunaga et al., 2004; Deenen et al., 2011).

This is also the first report on the substrate-dependent alterations in the catalytic activity of DCK24Val. All four DCK proteins (WT, Ile24Val, Ala119Gly, and Pro122Ser) exhibited patterns of substrate inhibition kinetics for dFdC and Ara-C monophosphorylation (Fig. 1; Table 1). The average apparent Km value of the DCK-WT was 2.15 μM for dFdC (Table 1), which was comparable to the previously reported values (Sabini et al., 2003). Relative to the WT, DCK24Val was the only variant that demonstrated an increase (~2-fold) in the intrinsic clearance of dFdC phosphorylation (P = 0.0122) due to a 40% decrease in Km (P < 0.05). DCK119Gly had similar Km and Vmax values as the WT, and DCK122Ser had Km and Vmax values that were ~50% lower than that of WT (P < 0.05) and yielded a Cint that trended lower. Although none of these DCK variants had a Km value significantly different from that of the WT for dFdC in a previous study (Kocabas et al., 2008), a direct comparison of the two studies should be done cautiously due to the use of different expression systems (purified proteins versus crude cell extracts from transiently overexpressed COS-1 cells). In contrast, relative to the WT, all three proteins had comparable Km and Vmax values toward Ara-C, although the Cint value of DCK122Ser for Ara-C monophosphorylation trended lower than that of the WT due to Km and Vmax values that were approximately 2- and 2.7-fold lower (P < 0.05), respectively (Fig. 1; Table 1). The mechanism for the substrate dependency is unclear. Conceivably, the position 24 lies close to a highly conserved P-loop motif that is critical to the interactions of enzyme, substrate, and phosphoryl donor (Sabini et al., 2003), and the substitution from Ile24 to a smaller amino acid Val24 may bring a locally destabilizing conformational change to the interactions. This may result in differential catalytic activity toward Ara-C versus dFdC when the 2'-sugar position changes from a hydroxyl group in Ara-C to fluorines in dFdC. Although this variant is specific to individuals of African descent at an allelic frequency of 2.5% (Kocabas et al., 2008), its impact, as demonstrated in the current kinetic study, warrants further examination of its clinical relevance with regard to treatment with dFdC or other deoxycytidine analogs.

For the first time, direct evidence was provided that DCK is unlikely to have a significant effect on the conversion of dFdU to dFdUMP. The average Cint value for the dFdU monophosphorylation was 0.027 ml/min/mg, which is more than 18,000 times lower than that of dFdCMP formation from dFdC by the DCK-WT (491.9 ml/min/mg). The slope of the initial linear formation rate was used to determine the Cint since there was a linear relationship between dFdCMP formation by DCK and dFdU concentration. The high dFdUMP concentrations previously reported (Veltkamp et al., 2008) either were formed through direct phosphorylation of dFdU by another kinase, or resulted from the biotransformation of dFdCMP to dFdUMP by deoxycytidine monophosphate deaminase (Mini et al., 2006). As a result, no further studies were conducted on the effects of other DCK variants in the conversion from dFdU to dFdUMP.

In conclusion, the CDA Lys27Gln polymorphism does not significantly modulate CDA activity toward dFdC, and therefore would not be expected to contribute to interindividual variability in response to gemcitabine. DCK is unlikely to have a significant effect on the conversion of dFdU to dFdUMP. The higher in vitro catalytic efficiency of DCK24Val toward dFdC monophosphorylation may be relevant to dFdC clinical response, and warrants further examination. This is the first study to show substrate-dependent alterations in catalytic activities of CDA70Thr and DCK24Val, and demonstrates that conclusions regarding pharmacogenomic contributions to response for one substrate should not be extrapolated to another.

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Authorship Contributions
Participated in research design: Baker, Wickremesinhe, Ring, Qian, Wrighton, Dantzig, Hall, Guo.
Conducted experiments: Baker, Oluyedun, Li.
Contributed new reagents or analytic tools: Baker, Oluyedun, Qian, Guo.
Performed data analysis: Baker, Oluyedun, Ring, Li, Wrighton, Dantzig, Hall, Guo.
Wrote or contributed to the writing of the manuscript: Baker, Wickremesinhe, Ring, Qian, Wrighton, Dantzig, Hall, Guo.

References

Address correspondence to: Dr. Yingying Guo, Drug Disposition of Eli Lilly and Company, Drop Code 0714, Indianapolis, IN, 46285, E-mail: guoying@illy.com


Pharmacogenomics of Gemcitabine Metabolism: Functional Analysis of Genetic Variants in Cytidine Deaminase and Deoxycytidine Kinase

Jessica A. Roseberry Baker, Enaksha R. Wickremsinhe, Claire H. Li, Olukayode A. Oluypedun, Anne H. Dantzig, Stephen D. Hall, Yue-wei Qian, Barbara J. Ring, Steven A. Wrighton and Yingying Guo

Supplemental Figures Legends

Supplemental Figure 1. Structure of gemcitabine (dFdC) and its metabolic pathways. The dotted line indicates a proposed but unproven pathway. dFdU, 2’,2’-difluorodeoxyuridine; dFdC-MP, dFdC monophosphate; dFdC-TP, dFdC triphosphate; dFdU-MP, dFdU monophosphate; dFdU-TP, dFdU triphosphate.

Supplemental Figure 2. SDS-PAGE analyses of purified recombinant human cytidine deaminase (a) and deoxycytidine kinase (b) variants.
### Catalytic Activity Table

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<td><strong>Catalytic Activity</strong></td>
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*: columns purchased from Phenomenex (Torrance, CA)
#: columns purchased from Thermo Fisher Scientific (Waltham, MA)

Calibration curves for metabolites were developed in the following ranges: dFdU (3.90-1000 µM), ara-U (3.90-500 µM), dFdC-MP (100 - 25600 nM), ara-CMP (100 - 12800 nM), dFdU-MP (100- 12800 nM). The correlation coefficients were calculated by least-square regression analysis.
Supplemental Figure 1

Gemcitabine (dFdC) → Deoxycytidine kinase (DCK) → dFdC-MP → dFdC-TP

Cytidine deaminase (CDA) → dFdU → dFdU-MP → dFdU-TP

Deoxycytidylate deaminase (DCTD) → DCK?