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Evaluation of a UCMK/dCK Fusion Enzyme for Gemcitabine-Mediated Cytotoxicity

Adam J. Johnson¹, Melissa N. Brown¹, and Margaret E. Black^{1,#}

¹School of Molecular Biosciences, College of Veterinary Medicine, P.O. Box 647520, Washington State University, Pullman, WA 99164-7520

Abstract

While gemcitabine (2'-2'-difluoro-2'-deoxycytidine, dFdC) displays wide-ranging antineoplastic activity as a single agent, variable response rates and poor intracellular metabolism often limit its clinical efficacy. In an effort to enhance dFdC cytotoxicity and help normalize response rates, we created a bifunctional fusion enzyme that combines the enzymatic activities of deoxycytidine kinase (dCK) and uridine/cytidine monophosphate kinase (UCMK) in a single polypeptide. Our goal was to evaluate whether the created fusion could induce beneficial, functional changes toward dFdC, expedite dFdC conversion to its active antimetabolites and consequently amplify cell dFdC sensitivity. While kinetic analyses revealed the UCMK/dCK fusion enzyme to possess both native activities, the fusion rendered cells sensitive to the cytotoxic effects of dFdC at the same level as dCK expression alone. These results suggest that increased wild-type UCMK expression does not provide a significant enhancement in dFdC-mediated cytotoxicity and may warrant the implementation of studies aimed at engineering UCMK variants with improved activity toward gemcitabine monophosphate.

Keywords

gemcitabine; deoxycytidine kinase; uridine/cytidine monophosphate kinase; pathway engineering; fusion protein; gene therapy

INTRODUCTION

The deoxycytidine analog gemcitabine (2', 2'-difluoro-2'-deoxycytidine, dFdC) is a potent chemotherapeutic agent with wide-ranging activity against many solid tumors [1; 2] and is a first-line standard of care for patients with advanced pancreatic cancer [3; 4]. In the cell dFdC is activated to its monophosphate form (dFdCMP) by deoxycytidine kinase (dCK; EC 2.7.1.74) and subsequently converted to its active di- and triphosphate metabolites, dFdCDP and dFdCTP, by other nucleotide kinases. Both of these active metabolites retain complex self-potentiating activity against multiple cellular targets that reinforces their cytotoxic activity. Specifically, as a potent inhibitor of ribonucleotide reductase, dFdCDP serves to

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[#]To whom correspondence should be addressed. School of Molecular Biosciences, College of Veterinary Medicine, P.O. Box 647520, Washington State University, Pullman, WA 99164-7520. Phone: (509) 335-6265. Fax: (509) 335-4159. blackm@vetmed.wsu.edu.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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deplete available deoxycytidine triphosphate (dCTP) pools necessary for DNA synthesis. This depletion allows for decreased dCK feedback inhibition by dCTP, increased dFdC activation and enhanced dFdCTP incorporation into DNA. Once incorporated, dFdCTP causes DNA synthesis inhibition via masked chain termination that consequently leads to cell apoptosis [5]. The accumulation of dFdCTP pools also augments dFdC potency since dFdCTP retention effectively limits the catabolism of dFdC metabolites by deoxycytidine deaminase and deoxycytidine monophosphate deaminase via feedback inhibition [6; 7]. This self-potentiating activity reduces dFdC and dFdCMP degradation and increases the availability of dFdC and dFdCMP pools for activation by dCK and uridine/cytidine monophosphate kinase (UCMK; EC 2.7.4.14), respectively.

Despite the success of dFdC as an anticancer agent, variable response rates and non-specific toxicity restrict its wide-spread clinical efficacy. Due to these limitations, multiple schedule and dose optimization studies have focused on developing more consistently effective, less toxic treatment regimens than current standard treatment protocols provide [8; 9]. Yet, defective dFdC activation due to lost or reduced expression of drug metabolizing enzymes, such as dCK, remains an important determinant of clinical success [7]. To address this concern researchers have rendered dFdC treatment more effective by introducing dCK through gene therapy approaches [10; 11].

UCMK activity has similarly been suggested to play an important role in the activation of multiple pharmacologically important nucleoside analogs including dFdC and ara-C (1- β -D-arabinofuranosylcytosine) monophosphates to their corresponding diphosphate forms [12; 13]. As a means to facilitate the conversion of the dCK product, dFdCMP, to the active antimetabolite dFdCDP, and reinforce the self-potentiating action of dFdCTP pools, researchers previously engineered a chimeric cDNA that allowed for the co-expression of both dCK and UCMK as separate enzymes. Results demonstrated that dCK and UCMK co-expression reduced IC₅₀ levels and inhibited tumor growth relative to controls [14; 15]. Prompted by prior reports where bifunctional fusion enzymes were shown to possess enhanced prodrug activation capabilities relative to enzyme co-expression [16; 17], we sought to investigate whether the creation of a bifunctional UCMK/dCK fusion enzyme could induce a similar beneficial, functional enhancement of dFdC-mediated cytotoxicity.

In this report we describe the construction and characterization of a novel fusion enzyme that combines the two enzymatic activities of dCK and UCMK into a single polypeptide. Following initial dFdC sensitivity studies in *E. coli*, kinetic analyses were performed to assess the catalytic properties of the UCMK/dCK fusion. The fusion was further characterized *in vitro* to evaluate its ability to render Chinese Hamster Ovary dCK deficient cells (CHO dCK⁻) more sensitive to dFdC relative to dCK alone. Although a prior report demonstrated that the introduction of dCK and UCMK activities into cells can enhance dFdC cell sensitivity relative to dCK expression alone in certain pancreatic cancer cell lines [15], we report that the dual activities of the UCMK/dCK fusion rendered CHO dCK⁻ cells equally sensitive to dFdC treatment relative to dCK expression alone.

MATERIALS AND METHODS

Materials

Oligonucleotides used to amplify and/or sequence the poly-glycine encoding linker, UCMK, dCK, and UCMK/dCK were obtained from Integrated DNA Technologies (Coralville, IA). T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The Wizard PCR prep kit (Promega, Madison, WI), HiSpeed Plasmid Midi Kit (Qiagen, Valencia, CA) or StrataPrep EF Plasmid Midiprep Kit (Stratagene, La Jolla, CA) were used for DNA purification. Alamar Blue was purchased from Serotec Limited

(Oxford, UK). Cell culture reagents were purchased from Gibco (Carlsbad, CA), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Bacterial strains and growth media

Escherichia coli strain NM522 [F^+ *proA*⁺*B*⁺ *lacI*^q Δ (*lacZ*)M15/ Δ (*lac-proAB*) *glnV thi-1* Δ (*hsdS-mcrB*)5] was used for cloning and mutagenesis procedures. *E. coli* strain BL21 (DE3) *tdk*⁻ [F^- *ompT hsdSB(r_{pmB}) gal dcm tdk* λ (DE3)] was used for protein expression and purification. Minimal plates with or without dFdC (0.1 μ M) were prepared from 0.36g yeast synthetic dropout without leucine, 50mL 10X M9 salts (15g KH₂PO₄, 33.9g anhydrous NaHPO₄, 2.5g NaCl, 5g NH₄Cl), 1mM MgSO₄, 2.5mL 20% glucose, 0.1mM CaCl₂, 7.5g agar and 50 μ g/mL carbenicillin per 500mL. 2xYT medium was prepared from 16g tryptone, 10g yeast extract, 5g NaCl, and 50 μ g/mL carbenicillin per 1L.

Construction of expression vectors

UCMK cDNA was initially PCR amplified from pGEXRB:UCMK (a kind gift from Dr. A. Lavie, University of Illinois at Chicago) [18] and subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) to create pCR2.1-TOPO:UCMK. UCMK was then isolated as an *NdeI*(blunt-ended)/*Bam*HI fragment and subcloned in-frame to the His-tag of *NcoI*(blunt-ended)/*Bam*HI digested pETHT, a bacterial expression vector [19]. dCK cDNA was isolated from pET15b:dCK (a kind gift from Dr. B. Stoddard, Fred Hutchinson Cancer Research Center) as an *NcoI*/*Bam*HI fragment and subcloned into *NcoI*/*Bam*HI digested pETHT. Plasmids were verified by sequencing and designated pETHT:UCMK and pETHT:dCK, respectively. DNA sequence analysis confirmed the constructs. Primers MB516 (5'-GACTACGCGTGGTGGAGGGGGAGGCGGTGGGGGAGGTGGTTCCATGGCCACCG GATCC-3') and MB517 (5'-GGTGCTCGAGTCGGATCCGGTGGCCATGGA-3') were used for PCR amplification of the poly-glycine encoding linker. Primers MB421 (5'-GACAAGGAAACGCGTGGATCCCCGGG-3') and MB422 (5'-CCCGGGGATCCACGCGTTTCCTTGTC-3') were used to replace the stop codon of UCMK with an *MluI* site, to create pETHT:UCMK-*MluI*, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Details of the UCMK/dCK fusion gene construction can be found in Figure 1. To subclone the UCMK, dCK, and UCMK/dCK genes into a mammalian expression vector, each gene was initially PCR amplified from pETHT:UCMK, pETHT:dCK, or pETHT:UCMK/dCK using sense primers MB665 (5'-GCCGGGTACCATGAAGCCGCTGG-3') (UCMK and UCMK/dCK) or MB666 (5'-GCCGGGTACCATGGCCACCCCGC-3') (dCK) and the antisense T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3'). Amplified fragments were then gel extracted (Qiagen), digested with *KpnI*/*XhoI*, and ligated into *KpnI*/*XhoI* digested pUB6/V5-HisB (pUB, Invitrogen). DNA sequencing analyses confirmed the constructs and the resulting plasmids were designated pUB:UCMK, pUB:dCK and pUB:UCMK/dCK, respectively.

Protein expression and purification

For protein expression, *E. coli* BL21 (DE3) *tdk*⁻ harboring pETHT:dCK, pETHT:UCMK or pETHT:UCMK/dCK were grown in 2xYT media at 37°C to an OD₆₀₀ of 0.6 and then induced with 1mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4hr. Cells were subsequently harvested, resuspended in lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH 8.0), sonicated, subjected to three freeze thaw cycles and centrifuged at 35krpm at 4°C for 45min. The resultant cleared lysate was then assayed or purified to near (>95%) homogeneity using Ni-NTA chromatography (Qiagen) as described by the supplier. Purified enzyme was then dialyzed overnight at 4°C against 50mM NaCl, 50mM Tris HCl, pH 7.5. Protein concentrations of cleared lysates or purified enzyme were determined by Bradford assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Enzyme assays and kinetics

Purified proteins were used for kinetic analysis with [5-³H(N)] deoxycytidine (specific activity, 23Ci/mmol) and [5-³H] gemcitabine (specific activity, 10.6Ci/mmol) (Moravek Biochemicals, Brea, CA) as substrates for dCK or 5'-deoxycytidine monophosphate (dCMP) (Sigma) as substrate for UCMK. Radiolabeled substrate phosphorylation was detected by a filter binding assay modified from that described by Hruby *et al.* [20]. Briefly, assays were performed in 50mM Tris pH 7.6, 10mM MgCl₂, 2mM dithiothreitol, 0.5mg/ml BSA, and 5mM ATP using a broad range of substrate concentrations (0.5–20μM ³H-dC or 6.25–200μM ³H-dFdC) with 0.1μg dCK or UCMK/dCK. Data were plotted as the double reciprocal of velocity (min/μmol × 10⁴) vs. substrate concentration (μM⁻¹) and intercept values were used to determine K_m and V_{max} values. *k*_{cat} values were determined from the double reciprocal plot data as per active site (one active site per monomer). UCMK activity was determined spectrophotometrically using a pyruvate kinase/lactate dehydrogenase coupling system in 50mM Tris, pH 7.4, 50mM KCl, 5mM MgCl₂, 1mM ATP, 10mM DTT, 1mM phosphoenolpyruvate, 0.2mM NADH, 4U pyruvate kinase, 4U lactate dehydrogenase and 4U NDP kinase with 1–15mM dCMP and 0.05μg UCMK or UCMK/dCK [21]. Data analyses to determine K_m, *k*_{cat}, and *k*_{cat}/K_m values for UCMK and UCMK/dCK were similar to methods used for dCK. Initial dCK and UCMK enzyme assays were performed as described above for dCK with the UCMK assay having [5-³H(N)] dCMP (specific activity, 24.7Ci/mmol) (Moravek) as substrate and modified wash steps [22].

Cell culture

Chinese Hamster Ovary dCK deficient cells (CHO dCK⁻) (a kind gift from Dr. M. Meuth, University of Sheffield) [23] were grown in Dulbecco's Modified Essential Medium containing 5% fetal bovine serum, 1mM sodium pyruvate, 10mM HEPES, 100μM nonessential amino acids, 100U/mL penicillin G, 10μg/mL streptomycin sulfate, 292μg/mL L-glutamine, 100μM sodium citrate and 0.0014% NaCl and maintained in a humidified incubator at 37°C in 5% CO₂.

In vitro cytotoxicity assay

Stable transfectants were created by the addition of 1μg purified endotoxin-free DNA (Stratagene, La Jolla, CA) (pUB (empty vector), pUB:UCMK, pUB:dCK, or pUB:UCMK/dCK) to 1×10⁵ CHO dCK⁻ cells by lipofection using FuGENE 6 transfection reagent (Roche Diagnostic, Penzberg, Germany) at a 3:1 ratio as described by the manufacturer. Stable transfectants were selected by culture in media supplemented with 4μg/mL blasticidin. Immunoblot analyses using rabbit polyclonal antisera to UCMK or dCK followed by goat-anti-rabbit-AP conjugated secondary antibody (Bio-Rad, Hercules, CA) were performed as previously described [24]. Antiserum to purified UCMK was a kind gift from Dr. Y. Cheng (Yale University) [25] and antiserum to dCK was generated by Harlan Laboratories (Indianapolis, IN) using purified full length dCK. An AP Conjugate Substrate Kit (Bio-Rad) was used for colorimetric detection. Stable transfectant pools confirmed by immunoblot were used for *in vitro* cytotoxicity assays. Pools of transfectants were plated at an initial density of 500 cells/well in 96-well microtiter plates. Following cell adherence overnight, dFdC (0–10μM) was added in sets of eight wells for each concentration tested. After a 7 day incubation period, cell viability was determined as previously described [26]. Data were plotted with the standard error of the mean and analyzed for statistical significance using the Student's *t*-test.

RESULTS AND DISCUSSION

Variable factors including insufficient intracellular activation, non-specific toxicity, altered enzyme expression and drug catabolism limit the wide-spread clinical efficacy of current

dFdC treatment regimens for cancer. Thus, there is an unmet medical need for the development of new clinical strategies able to overcome these limitations. While dCK gene therapy has been effective at enhancing dFdC sensitivity to many cancer cell lines [10; 11; 27], this approach is limited in scope due to endogenous dCK levels in healthy tissues. In an attempt to lower necessary prodrug doses and avoid prodrug toxicity and activation in normal tissues, we and others previously performed mutagenesis studies on dCK aimed at enhancing its catalytic activity. While studies by Sabini *et al.* created a mutant with a 4-fold increase in catalytic efficiency (k_{cat}/K_m) for dFdC, this mutant displayed an overall unfavorable relative specificity for dFdC comparative to deoxycytidine [28]. Similarly, via regio-specific random mutagenesis we created dCK mutants with up to a 3-fold increase in catalytic efficiency toward dFdC. Yet, when tested *in vitro* the mutants provided no significant enhancement in dFdC-mediated cytotoxicity relative to wild-type dCK (Willmon and Black, unpublished data) suggesting that a modest increase in dCK activity towards dFdC may not lead to increased dFdC sensitivity.

Pathway engineering strategies using co-expressed or fused, chimeric proteins have been successful at increasing cancer cell chemosensitivity to a number of clinically relevant prodrugs [14; 16; 17; 29]. Reports directly comparing the use of a bifunctional fusion enzyme or enzyme co-expression during therapy has revealed that some fusions contain enhanced prodrug activation capabilities and a more potent ability to sensitize tumors to low prodrug doses. For instance, a mouse guanylate kinase/Herpes Simplex Virus thymidine kinase (MGMK/HSVTK) fusion protein demonstrated a 9-fold enhancement in catalytic efficiency (k_{cat}/K_m) toward the prodrug ganciclovir (GCV) relative to HSVTK alone. While *in vitro* experiments co-expressing separate HSVTK and MGMK proteins demonstrated minimal alterations in GCV sensitivity (IC_{50}) comparative to HSVTK expression, cells expressing the MGMK/HSVTK fusion displayed an approximately 175-fold lower IC_{50} value than HSVTK expressing cells [16; 30]. Likewise, studies characterizing a bifunctional yeast cytosine deaminase (CD)/uracil phosphoribosyltransferase (UPRT) fusion protein revealed the fusion to display 100-fold higher CD activity than CD alone. This enhanced activity significantly increased cancer cell sensitivity to the prodrug 5-fluorocytosine relative to cancer cells transduced with CD alone or co-transduced with adenoviral vectors carrying the CD or UPRT gene [17]. These prior reports led us to investigate whether the creation of a bifunctional UCMK/dCK fusion enzyme could similarly enhance dFdC sensitivity.

The strategy used to create the UCMK/dCK fusion is outlined in Figure 1. Initial dFdC sensitivity studies in *E. coli* revealed that cells harboring pETHT:UCMK/dCK were more sensitive to dFdC than cells harboring pETHT:dCK and enzyme assays demonstrated the fusion to have dual activities (Materials and Methods, data not shown). These results prompted us to perform kinetic analyses on purified proteins to ascertain the catalytic properties of the UCMK/dCK fusion relative to UCMK or dCK alone (Table 1). Overall, the relative catalytic efficiencies (k_{cat}/K_m) of purified UCMK/dCK and dCK towards deoxycytidine (3.0×10^{-2} vs. $2.0 \times 10^{-2} \mu\text{M}^{-1} \text{s}^{-1}$) and dFdC (15.0×10^{-3} vs. $8.3 \times 10^{-3} \mu\text{M}^{-1} \text{s}^{-1}$) are appreciably similar and within standard error. The UCMK activity of the fusion also displayed wild-type UCMK kinetic properties ($k_{\text{cat}}/K_m = 8.2 \times 10^{-2}$ vs. $11.0 \times 10^{-2} \text{mM}^{-1} \text{s}^{-1}$) toward its natural substrate dCMP. The kinetic parameters of the fusion and wild-type UCMK are comparable to previous reports by other investigators regarding UCMK kinetic parameters toward dCMP [12; 25; 31]. These data indicate that the UCMK/dCK fusion maintains both native activities.

We next examined whether the UCMK/dCK fusion could significantly enhance cell sensitivity to dFdC relative to wild-type dCK. To do this the genes for dCK, UCMK or the UCMK/dCK fusion were first cloned into the mammalian expression vector pUB6/V5-HisB

and the resulting vectors were used to stably transfect a CHO dCK⁻ cell line [23]. This cell line was chosen to eliminate background dCK activity during *in vitro* sensitivity assays and to act as a model for acquired drug resistance. Protein expression of stable transfectants was assessed by immunoblot analysis and Figure 2 shows the presence of single UCMK, dCK and UCMK/dCK bands in lanes corresponding to lysates of cells transfected with pUB:UCMK, pUB:dCK or pUB:UCMK/dCK, respectively. Pools of stable transfectants were then assayed for dFdC sensitivity over a wide drug range (0–10 μ M) with representative *in vitro* sensitivity assay results shown as percent survival in Figure 3. No cytotoxicity was observed when control (pUB) or pUB:UCMK transfected cells were subjected up to 10 μ M doses of dFdC (Figure 3), while an inherent cytotoxicity was observed at concentrations above 10 μ M dFdC (data not shown). In contrast, cells transfected with pUB:dCK or pUB:UCMK/dCK were equally sensitive to the dFdC concentrations tested. The dual activities of the UCMK/dCK fusion therefore provided no significant enhancement in ability to render cells sensitive to dFdC relative to dCK expression alone. Instead, a plateau in dFdC-mediated cell killing appeared to emerge above 10nM dFdC suggesting dFdC activation may be a saturable process in this cell culture system, a phenomenon in keeping with data from pre-clinical and clinical studies [9; 32]. The fact that both uridine and cytidine monophosphates exist at high intracellular concentrations [33], and are similarly or more efficiently converted by UCMK than dFdCMP [13; 25], may account in part for the inability of UCMK overexpression in context of the UCMK/dCK fusion to allow for improved dFdC cytotoxicity.

Due to the complex and multifactorial nature of dFdC intracellular activation it is difficult to pinpoint why the dual activities of the UCMK/dCK fusion were unable to improve dFdC cell sensitivity relative to dCK. Prior reports that either co-expressed dCK and UCMK [15], or over-expressed UCMK alone [34], in various cell types did however reveal that UCMK activity plays a modest role in enhancing dFdC-mediated cytotoxicity (IC₅₀) relative to dCK gene therapy (~3 fold) or dFdC treatment alone (~1–2 fold), respectively. The altered metabolic profile of the CHO dCK⁻ cell line and potential variations in the activity levels of enzymes involved in dFdC metabolism, including endogenous UCMK activity, may reflect differences in sensitivity observed between cell types [7; 23]. Taken together, these prior and current results support the notion that UCMK activity may limit the conversion of dFdC to its pharmacologically active antimetabolites and that mere overexpression of wild-type UCMK may not always be sufficient enough to provide a robust enhancement in dFdC activation. Therefore, this bottleneck of dFdC activation may warrant the implementation of studies aimed at engineering UCMK variants with improved activity toward dFdCMP. Indeed, the use of bifunctional fusion enzymes, such as HSVTK/MGMK and CD/UPRT, overlaid with previously described enzyme variants has recently been validated as a viable way to increase prodrug mediated cytotoxicity at low prodrug doses [26; 35]. Furthermore, prior mutagenesis studies on various nucleoside monophosphate kinase family members have created variants with altered substrate specificity [36] or a significantly enhanced ability to activate clinically relevant prodrugs [37; 38]. The use of UCMK variants in cooperation with the dCK activity of our UCMK/dCK fusion may likewise allow for more efficient dFdC conversion at less toxic treatment levels. Such a strategy could improve tumor ablation, limit side effects seen with high dFdC doses, stabilize response rates to dFdC treatment and provide significant survival benefit for patients.

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Highlights

- Goal was to enhance dFdC cytotoxicity by the creation of a UCMK/dCK fusion enzyme
- The UCMK/dCK fusion enzyme possesses both native activities
- The fusion renders cells equally sensitive to dFdC relative to dCK expression alone
- Dual activities of fusion not sufficient to augment cell dFdC sensitivity *in vitro*
- Data may warrant the implementation of UCMK mutagenesis studies

Figure 1.

Schematic of the pETHT:UCMK/dCK construction. To construct the UCMK/dCK fusion gene, a poly-glycine encoding linker was initially PCR amplified using primers MB516 and MB517, digested by *MluI/XhoI*, and ligated in-frame into *MluI/XhoI* restricted pETHT:UCMK-*MluI*. The resulting vector, designated pETHT:UCMK-linker, was then *BamHI(b)/XhoI* digested and the *NcoI(b)/XhoI* digested dCK gene isolated from pETHT:dCK was ligated in-frame 3' to the linker sequence to create pETHT:UCMK/dCK. DNA sequence analysis confirmed the construct. (b) = blunt-ended.

Figure 2.

Immunoblots of stably transfected CHO dCK⁻ cell lysates. Equivalent amounts of cell lysates (~2 million cells) were collected from pools of cells stably transfected with pUB, pUB:UCMK, pUB:dCK or pUB:UCMK/dCK, loaded onto a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and then probed with antisera to UCMK and dCK (**a**) or dCK alone (**b**). Each protein migrated in accord with its predicted molecular mass, with the band for UCMK/dCK corresponding to the combined mass of dCK [28] and UCMK [18] together. A combination of UCMK and dCK antiserum was used to visualize the expression of all enzyme constructs (**a**). Relatively equivalent dCK and UCMK/dCK protein expression levels are observed when probed with dCK antiserum alone (**b**). Endogenous UCMK was unrecognized by the UCMK antiserum. MWM = molecular weight marker.

Figure 3.

Sensitivity of pUB:UCMK, pUB:dCK or pUB:UCMK/dCK transfected CHO dCK⁻ cells to dFdC. Pools of cells stably transfected with pUB (X), pUB:UCMK (▲), pUB:dCK (▼), or pUB:UCMK/dCK (◇) were evaluated for dFdC sensitivity. Cell survival was measured by Alamar Blue treatment and untreated (control) samples (0mM dFdC) were set to 100% cell survival. In this representative plot, each data point (mean ± SEM; performed with 16 replicates) is expressed as a percentage of the value for control samples. Experiments were repeated at least three times using different pools of transfectants and similar results were obtained. Data points of pUB:dCK vs. pUB:UCMK/dCK between 10 and 10⁴ nM dFdC are not statistically significant (*P* 0.05).

Table 1

Kinetic parameters of purified dCK, UCMK and UCMK/dCK enzymes.

	<i>Deoxycytidine</i>				<i>dFdC</i>				<i>dCMP</i>			
	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
UCMK	ND	ND	ND	ND	ND	ND	1.22 (0.32)	9.2e-2 (0.064)	8.2e-2 (0.07)	1.03 (0.21)	11.4e-2 (0.054)	11.0e-2 (0.045)
UCMK/dCK	1.9 (0.71) *	5.3e-2 (0.035)	3.0e-2 (0.016)	24.3 (6.06)	0.335 (0.078)	15.0e-3 (0.007)	1.03 (0.21)	11.4e-2 (0.054)	11.0e-2 (0.045)	1.03 (0.21)	11.4e-2 (0.054)	11.0e-2 (0.045)
dCK	1.5 (0.41)	3.0e-2 (0.012)	2.0e-2 (0.005)	18.4 (2.33)	0.152 (0.057)	8.3e-3 (0.004)	ND	ND	ND	ND	ND	ND

ND, not determined.

* Standard error.

k_{cat} values determined by equation $V_{max} = k_{cat}[E]$ where $[E]$ = total enzyme concentration and is based on one active site per monomer.