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Development of New Deoxycytidine Kinase Inhibitors and Noninvasive in Vivo Evaluation Using Positron Emission Tomography


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Supporting Information

ABSTRACT: Combined inhibition of ribonucleotide reductase and deoxycytidine kinase (dCK) in multiple cancer cell lines depletes deoxycytidine triphosphate pools leading to DNA replication stress, cell cycle arrest, and apoptosis. Evidence implicating dCK in cancer cell proliferation and survival stimulated our interest in developing small molecule dCK inhibitors. Following a high throughput screen of a diverse chemical library, a structure–activity relationship study was carried out. Positron Emission Tomography (PET) using 18F-L-1-(2′-deoxy-2′-FluoroArabinofuranosyl) Cytosine (18F-L-FAC), a dCK-specific substrate, was used to rapidly rank lead compounds based on their ability to inhibit dCK activity in vivo. Evaluation of a subset of the most potent compounds in cell culture (IC50 = 1–12 nM) using the 18F-L-FAC PET pharmacodynamic assay identified compounds demonstrating superior in vivo efficacy.

INTRODUCTION

Mammalian cells rely on two major pathways for the production and maintenance of deoxyribonucleotide triphosphates (dNTPs) for DNA replication and repair: the de novo pathway and the nucleoside salvage pathway. The de novo pathway produces dNTPs from glucose and amino acids. The nucleoside salvage pathway produces dNTPs from preformed nucleosides and phosphorylated purines and pyrimidines by the corresponding kinases. We have shown that dCK and TK1 play important roles in hematopoiesis by regulating dNTP biosynthesis in lymphoid and erythroid progenitors. In addition to its physiological role in nucleotide metabolism, dCK phosphorylates several clinically important antiviral and anticancer nucleoside analog prodrugs (e.g., ganciclovir, acyclovir, valacyclovir, foscamet). Phosphorylation by dCK is critically required for the activation of these prodrugs. Recently, dCK was implicated in the regulation of the G2/M checkpoint in cancer cells in response to DNA damage. The role of dCK in hematopoiesis and cancer has led to our interest in developing a small molecule inhibitor of this kinase. Such dCK inhibitors could represent new therapeutic agents for malignancies and immune disorders. To our knowledge, few dCK inhibitors have been reported, and only one has been demonstrated to inhibit dCK activity in vivo.

Positron emission tomography (PET) is a noninvasive in vivo imaging technique widely used for diagnosing, staging, restaging, and therapy monitoring of various diseases. While PET using the radiotracer 2-18F-fluoro-2-deoxy-D-glucose (18F-FDG) has become an important diagnostic and treatment monitoring tool in cancer, another emerging application of PET concerns its use in drug discovery and development. Thus, by facilitating faster and more effective...
Decision-making early in the drug discovery/development process, PET could accelerate the advancement of promising candidates and reduce failures.\textsuperscript{22–24} For instance, PET can be used to demonstrate the need to modify lead candidates early in the drug discovery process by enabling noninvasive evaluations of drug pharmacodynamic (PD) and/or pharmacokinetic (PK) properties. In the specific context of our drug discovery and development program centered on dCK, PET could play a particularly important role given the availability of validated PET biomarkers to assess dCK activity in vivo. These

\begin{figure}[h]
\centering
\includegraphics{figure1.png}
\caption{Structures and IC\textsubscript{50} values determined using the \textsuperscript{3}H-dC uptake assay in L1210 cells for the initial HTS hits (1 and 2) and for commercially available compounds containing similar structural scaffolds (3–7).}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{compd} & \textbf{R\textsubscript{1}} & \textbf{R\textsubscript{2}} & \textbf{R\textsubscript{3}} & \textbf{R\textsubscript{4}} & \textbf{IC\textsubscript{50} (µM) L1210 cells} & \textbf{IC\textsubscript{50} (µM) CEM cells} \\
\hline
\hline
8 & H & OCH\textsubscript{2}CH\textsubscript{2}F & OCH\textsubscript{3} & H & 0.808 (±0.040) & 1.612 (±0.543) & 0.421 (±0.075) & 0.534 (±0.012) \\
9 & H & OCH\textsubscript{3} & OCH\textsubscript{2}CH\textsubscript{2}F & H & 0.538 (±0.014) & 0.528 (±0.015) & 0.230 (±0.042) & 0.506 (±0.138) \\
10 & H & OCH\textsubscript{2}CH\textsubscript{3} & OCH\textsubscript{2}CH\textsubscript{2}F & H & 0.513 (±0.100) & 1.226 (±0.450) & 0.251 (±0.020) & 0.512 (±0.409) \\
11 & H & CH\textsubscript{3} & OCH\textsubscript{2}CH\textsubscript{2}F & H & 2.381 (±0.042) & 3.201 (±0.566) & 1.960 (±1.001) & 1.922 (±0.573) \\
12 & H & OCH\textsubscript{3} & OCH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}F & H & 0.330 (±0.160) & 0.603 (±0.140) & 0.197 (±0.109) & 0.297 (±0.020) \\
13 & OCH\textsubscript{3} & H & OCH\textsubscript{2}CH\textsubscript{2}F & H & 1.445 (±0.060) & 2.649 (±0.902) & 1.041 (±0.084) & 0.849 (±0.183) \\
14 & H & OCH\textsubscript{2}CH\textsubscript{2}F & H & OCH\textsubscript{3} & 5.469 (±1.336) & ND\textsuperscript{b} & 2.367 (±0.238) & ND\textsuperscript{b} \\
\hline
\end{tabular}
\caption{In Vitro Biological Data in L1210 and CEM Cells for Compounds 8–14\textsuperscript{a}}
\end{table}

\textsuperscript{a}Inhibitory activity measured by \textsuperscript{3}H-deoxycytidine (\textsuperscript{3}H-dC) uptake in murine L1210 cells and in CCRF-CEM human cells. Values reported are the mean ± SD of at least \textit{n} = 2 independent experiments. \textsuperscript{b}ND = not determined (compound was not synthesized).
PET PD biomarkers of dCK activity include a series of 18F-fluoro-arabinofuranosylcytosine analogs substrates of dCK developed by our group which include 18F-1-(2′-deoxy-2′-fluoro-arabinofuranosyl) Cytosine (18F-FAC) and 18F-L-1-(2′-deoxy-2′-fluoro-arabinofuranosyl) Cytosine (18F-L-FAC).

Herein we describe the development of potent dCK inhibitors and demonstrate their in vivo efficacy using 18F-L-FAC PET as a noninvasive and clinically applicable PD biomarker.

**RESULTS AND DISCUSSION**

Identification of Lead Compound 15c. To identify new small molecule inhibitors of dCK, we performed a high throughput screen (HTS) of a set of selected chemical libraries totaling ~90,000 small molecules. We screened the library for dCK inhibitory function using a Firefly luciferase-coupled assay with recombinant human dCK enzyme. In this assay, inhibition of dCK prevents ATP depletion by dCK, thus resulting in higher luminescent signals in positive wells. The screen yielded two hit compounds, 1 and 2, which were validated to inhibit the uptake of tritiated deoxycytidine (3H-dC) with micromolar potency in the L1210 murine leukemia cell line (Figure 1).

Based on these results, five commercially available compounds containing similar structural scaffolds were tested; their IC50 values against L1210 cells were determined by measuring inhibition of 3H-dC uptake (Figure 1). Strikingly, compounds 6 and 7 were inactive, suggesting that the bis-amino functionality on the pyrimidine ring is crucial for dCK inhibition. Based on these results, we initiated a structure–activity relationship (SAR) study to develop a lead structure, which could be further optimized to compounds with potent in vivo activity.

We initially studied two main structural classes of compounds, pyrimidines and 1,3,5-triazines (Table 1). Two cell lines were used to determine the IC50 values: the L1210 murine leukemia cells and the CCRF-CEM human acute T-lymphoblastic leukemia cells. In nearly all cases, substitution of the pyrimidine ring with the 1,3,5-triazine motif reduced dCK inhibitory activity; in some instances an approximate 2-fold reduction in potency was observed. Consequently, the pyrimidine motif was utilized as the preferred scaffold to advance. At this stage of the SAR, the presence of a fluoroethoxy side-chain on the phenyl ring was considered for eventual 18F-radiolabeling purposes. Substitutions around the phenyl ring with respect to the position of the fluoroethoxy side-chain were also examined. Moving the fluoroethoxy side-chain from the para position in 8a to the meta position in 9a increased the inhibitory activity approximately 2-fold. It was also apparent that alkoxy substituents in the para position were better than alkyl moieties, since compound 11a had substantially lower activity than either the methoxy 9a or ethoxy 10a analogs. Compound 12a, which contains a side-chain that was extended by one carbon to give a fluoroarylpropanoyl group at the meta position, gave slightly greater inhibitory activity, albeit not a significant increase from compounds 9a and 10a. Substitution at the ortho position of the phenyl ring, e.g. in compounds 13a and 14a, resulted in substantially lower dCK inhibitory activity, an approximate 10-fold decrease in potency was observed for compound 14a when compared to 9a. A general synthetic scheme for compounds in Table 1 can be found in the Supporting Information (Scheme S2).

While the presence of fluorine in the small molecule may eventually enable the synthesis of an 18F-isotopolog of the dCK inhibitor, fluorine introduction also affects nearly all the physical and ADME (adsorption, distribution, metabolism, and excretion) properties of a compound. The capacity of fluorine to enhance metabolic stability has become increasingly apparent in recent years. Thus, a series of compounds were synthesized which contained fluorine attached directly on the aromatic ring of the inhibitors rather than linked by an ethoxy side-chain (compounds 16–18, Table 2). For each compound in this series, a set of three derivatives (a–c) were synthesized; in each case the group on the 5-position of the thiazole was either a methyl, ethyl, or propyl substituent. For compounds 15a–c the fluoroethoxy side-chain was retained at the meta position of the phenyl ring, as was a methoxy group at the para position due to the favorable inhibitory results from the initial SAR in Table 1.

Increasing nonpolar functionality at the 5-position of the thiazole resulted in increased inhibitory activity (Table 2). The IC50 values in CCRF-CEM cells illustrate the same trend in potency as observed in L1210 cells with one exception; set 16 shows little difference between the methyl, ethyl, or propyl

**Table 2. In Vitro Biological Data in L1210 and CEM Cells for Compounds 15–18**

<table>
<thead>
<tr>
<th>compd</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>IC50 (μM) L1210 cells</th>
<th>IC50 (μM) CEM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a Y = Me</td>
<td>b Y = Et</td>
<td>c Y = Pr</td>
<td>a Y = Me</td>
<td>b Y = Et</td>
</tr>
<tr>
<td>15</td>
<td>H</td>
<td>OCH3</td>
<td>OCH2CH2F</td>
<td>0.035 (±0.015)</td>
<td>0.030 (±0.077)</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>0.595 (±0.163)</td>
<td>0.620 (±0.170)</td>
</tr>
<tr>
<td>17</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>0.395 (±0.134)</td>
<td>0.265 (±0.163)</td>
</tr>
<tr>
<td>18</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>0.255 (±0.021)</td>
<td>0.510 (±0.014)</td>
</tr>
</tbody>
</table>

“Inhibitory activity measured by 3H-deoxycytidine (3H-dC) uptake in murine L1210 and in CCRF-CEM human cells. Values reported are the mean ± SD of at least n = 2 independent experiments.

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substituents. However, for all compounds tested against L1210 cells, the propyl substituent yielded better inhibitory activity than the corresponding methyl derivatives. The best example in L1210 cells was the 12-fold increase in activity when comparing compound 15c to compound 15a. In addition, comparisons between the propyl substituents against their respective methyl derivatives in CCRF-CEM cells also showed an increasing inhibitory trend in activity: 6-fold (compare 17c to 17a) or 3-fold (compare 18c to 18a). The most drastic effect from modifications at the S-position of the thiazole ring was the change exhibited from 9a in Table 1 to 15c in Table 2, where the substitution of a hydrogen for a propyl moiety resulted in a 180-fold increase in potency in L1210 cells. In addition, removal of the fluoroethoxy side-chain (e.g., compound series 16–18) resulted in a significant decrease in potency in both cell lines. Compound 15c, the most potent compound in this series, contains both the fluoroethoxy side-chain at the meta position on the phenyl ring and also a propyl group at the 5-position of the thiazole ring.

**Synthesis.** Compounds 15a–c were synthesized in six steps (Scheme 1). The commercially available 3-hydroxy-4-methoxybenzonitrile 19 was functionalized via alkylation with 1-bromo-2-fluoroethane in DMF with cesium carbonate as the base to obtain the nitrile 20 in 99% yield. Subjection of 20 to an aqueous ammonium sulfide solution under basic conditions afforded the thioamide 21 in excellent yield.31 Cyclization to

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**Scheme 1. Synthesis of Compounds 15a–c**

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form the thiazole core of 15a–c was achieved via condensation of thioamide 21 with the respective ethyl 3-bromo-2-oxoalkanolate32 in refluxing ethanol.33 Reduction of the resulting compounds with disobutylaluminium hydride afforded the respective alcohols 23a–c in 88–99% yield. The alcohols 23a–c were converted to the respective bromides 24a–c under mild conditions34 in 74–80% yield. Finally, nucleophilic displacement of the bromide with 4,6-diamino-2-mercaptopyrimidine35 generated the desired products 15a–c in 71–87% yield.

**X-ray Crystal Structure of Compound 15a Bound to Human dCK.** X-ray crystallographic studies of compound 15a were initiated to obtain information about its binding to dCK. Detailed analysis of the dCK-inhibitor interactions for this series of compounds was performed (Nomme, unpublished results). In short, the crystal structure of the dCK:15a complex was solved at 1.9 Å resolution (Figure 2). Human dCK, a dimer of two identical subunits with a molecular weight of ∼30 kDa per monomer, can bind either ATP or UTP as the phosphoryl donor for catalysis; in addition, dCK can adopt an open or closed conformation.36,37,3 In complex with 15a, the enzyme adopts the open conformation. We observed two 15a molecules in each protomer of the dimeric enzyme (shown in blue (15a-I) and cyan (15a-II), Figure 2A). Note that binding of 15a to dCK does not preclude nucleotide binding (UDP is shown in red, Figure 2A). The parallel orientation between 15a-I and 15a-II allows for optimal π–π stacking interactions between the phenyl and thiazole rings of each molecule.

While two molecules of 15a bind in the active site, it appears that 15a-I forms more key interactions and shorter hydrogen bond distances than 15a-II (Figure 2B). The extensive hydrogen-bond network that exists between the pyrimidine moiety of 15a-I and residues E53, Q97, and D133 in the dCK nucleoside binding site are illustrated in Figure 2B. Figure 2C illustrates the hydrophobic pocket that exists, via V55, L82, and F96, around the methyl group of compound 15a. This figure demonstrates that the pocket will accept larger substituents, explaining the increased trend in potency obtained for compounds 15b and 15c.

**Monte Carlo-Based Computational Modeling.** A Monte Carlo (MC)-based computational modeling approach using the free energy perturbation (FEP) method39,40 was used to further investigate the inhibitory effects of alkyl chain lengthening at the S-position of the thiazole. FEP allows calculation of the difference in binding energy of two molecules. The perturbation of molecule A into molecule B in a complex with a protein [ΔG<sub>protein</sub>(A → B)] and in solution alone [ΔG<sub>water</sub>(A → B)] is part of a complete thermodynamic cycle (Figure 3A). Because the sum of all components in such a cycle must equal zero, the binding energy difference may be calculated as the difference in free energies:

\[
\Delta \Delta G_{\text{binding}} = \Delta G_{\text{binding}}(B) - \Delta G_{\text{binding}}(A) \\
= \Delta G_{\text{protein}}(A \rightarrow B) - \Delta G_{\text{water}}(A \rightarrow B)
\]

Models of structures 15b and 15c (Figure 3B) each in a monomeric complex with dCK and in solution alone were equilibrated using MC. The equilibrated structure of 15c was subsequently perturbed into the structure of 15b (“shrinking” the propyl chain into an ethyl) and vice versa (“growing” the ethyl chain into a propyl) using FEP. These calculations were performed using the MCPRO 2.041 software package. The free energy changes for these perturbations are illustrated in Figure 3C. Averaging the ΔΔG<sub>binding</sub> obtained from the two

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**Figure 3.** (A) The complete thermodynamic cycle relating the binding energies to the perturbation of molecule A into molecule B. ΔG<sub>protein</sub>(A → B) denotes the change in free energy upon perturbation of A into B in the solvated inhibitor–protein complex, while ΔG<sub>water</sub>(A → B) denotes the free energy change when the perturbation takes place in water alone. The difference in free energies of binding, ΔΔG<sub>binding</sub>, is equal to the change in free energy when molecule A binds with the protein [ΔG<sub>binding</sub>(A)] subtracted from the change in free energy when molecule B binds [ΔG<sub>binding</sub>(B)]. Because the sum of all components in a complete thermodynamic cycle must equal zero, ΔΔG<sub>binding</sub> is therefore also equivalent to ΔG<sub>protein</sub>(A → B) − ΔG<sub>water</sub>(A → B). (B) Computational model of compound 15c (orange) in complex with dCK. Binding pocket residues Glu 53, Gln 97, Arg 114, and Asp 133 are shown explicitly, while the remainder of the protein is illustrated as a ribbon structure. (C) Free energy changes (kcal/mol) associated with the perturbation of the alkyl chain at the S-position of the thiazole. ΔG<sub>water</sub> is the free energy change for the inhibitor in water alone. The change in free energy upon binding is denoted as ΔΔG<sub>binding</sub>.
simulations indicates that the propyl chain of 15c confers a 1.210 kcal/mol more favorable free energy of binding in comparison to the ethyl chain of 15b; this favorable effect is due to desolvation. The change in free energy upon extension of the alkyl chain is unfavorable both in the complex with the protein and in water alone (positive \( \Delta G \) for chain lengthening, negative \( \Delta G \) for chain shortening); however, the magnitude of the unfavorable \( \Delta G \) is larger in solvent. The fact that this produces an overall favorable \( \Delta \Delta G \) of binding suggests that the propyl chain is better able to exclude water from the interior cavity of the protein, allowing a greater association between the protein and the inhibitor.

**Lead Optimization and SAR.** Based on the potency trend in Table 2 and the existence of a hydrophobic pocket around the 5-position of the thiazole ring of 15a, all further compounds in the SAR were made with the propyl chain installed at that position, to increase nonpolar interactions between the dCK enzyme pocket and the inhibitors. The fluorine atom terminating the ethoxy side-chain was substituted for a hydroxyl or sulfonamide group, with the goal of improving the molecule’s solubility properties as well as potential hydrogen bonding interactions that might exist in the active site. Moreover, since inhibitory activity in L1210 and CCRF-CEM cells demonstrated the same trend in potency, the SAR for all subsequently synthesized compounds were examined only in CCRF-CEM cells. The results are summarized in Table 3.

Compounds 25−27 showed excellent (1−2 nM) potency against CCRF-CEM cells (Table 3). Substitution of the end-chain hydroxyl for a methyl sulfonamide resulted in a decrease in inhibitory activity of about 3-fold (compare 27 to 29) or 5-fold (compare 25 to 28). The initial SAR in Table 1 indicated that the presence of an alkoxy substituent at the para position led to increased inhibitory activity; therefore, the methoxy group was reinstalled at the para position. As expected, removal of the ethoxy side-chain (e.g., compound 31) resulted in a substantially lower inhibitory activity, reinforcing the data observed for compounds 16−18 (Table 2). The presence of the methoxy moiety at the para position, in addition to the hydroxylethoxy side chain at the meta position, generated compound 33, which has an inhibitory potency of 1 nM. To our surprise, removal of one of the amino groups from the pyrimidine ring led to a mere 2.5-fold decrease in inhibitory activity (compare 33 to 34). Initially, we observed that removal of both amino groups from the pyrimidine ring resulted in complete loss of inhibitory activity (compounds 35−37, Figure 1); however, the presence of one amino group can provide suitable key hydrogen bonding interactions to inhibit the enzyme. Compound 32, which contains a side-chain that has been extended by one carbon to give a hydroxypropoxy group, was also synthesized. However, this modification resulted in slightly decreased inhibitory activity in comparison with the hydroxylethoxy group. While compound 33 was a potent compound in cell culture, the presence of a primary hydroxyl group in the molecule raised concerns of a metabolic liability as a consequence of potential oxidation or glucuronidation.

Thus, compounds 35−37 were synthesized to decrease the possibility of metabolic degradation of 33. Eight of these compounds in Table 3, whose IC\(_{50}\) values were lower than 15a and whose structural properties suggested that they would have the best in vivo efficacy, were selected for further investigation.

**Steady-State Kinetic Analysis of Selected dCK Inhibitors.** In order to confirm that the cell-based values truly reflect the potency of the compounds we also determined the \( K_{i,pp} \) values for select compounds using steady-state kinetic simulations.
assays. The cell-based assays indicated that compound 15a was 6–12-fold (depending on the cell line used for the assay) less potent than compound 15c (Table 2). Correspondingly, the steady-state data showed a 6-fold higher $K_{i,app}$ value for compound 15a (Table 4). Likewise, the low nanomolar IC$_{50}$ observed in CEM cells for compounds 36 and 37 (Table 3) was recapitulated in the steady-state kinetics derived $K_{i,app}$ values for these compounds (Table 4). Hence, we conclude that our cell-based assays are providing us with relatively accurate data as to the strength of the interactions between the compounds and dCK.

**Evaluation of in Vivo Inhibition of dCK Activity via a New PET PD Assay.** The nucleoside analog PET probe $^{18}$F-L-FAC is a high affinity substrate for dCK, which can be used to noninvasively estimate dCK enzymatic activity in vivo. A schematic depicting the mechanism by which $^{18}$F-L-FAC accumulates in cells in a dCK-specific manner is shown in Figure 4A. We reasoned that $^{18}$F-L-FAC PET could be used to rapidly identify the most potent dCK inhibitors based on their effectiveness at inhibiting the accumulation of the $^{18}$F-labeled dCK substrate PET tracer in various tissues. For the in vivo PET PD assay we selected dCK inhibitors that demonstrated 1–12 nM inhibitory activity in the cell culture $^3$H-dC uptake assay (Table 3). Mice were treated with a single dose (50 mg/kg) of a given dCK inhibitor administered by intraperitoneal injection. Control mice received vehicle (4% Captisol in water) injections. Four hours later, treated mice were injected intravenously with $^{18}$F-L-FAC; one hour after probe injection, mice were imaged by mPET/CT. The readout for the PET PD assay was the reduction in the accumulation of $^{18}$F-L-FAC in dCK-positive tissues in dCK inhibitor versus vehicle treated mice. Previously, we showed that $^{18}$F-L-FAC accumulates in a dCK-dependent manner into various tissues such as the thymus, spleen, bone marrow, and liver. Accumulation in the bladder is a result of nonenzymatic renal clearance of the unmetabolized probe. Since the reproducibility in the dCK-inhibitor efficacy of the various dCK inhibitors. Optimal conditions for the PET PD assay were determined by performing a dose escalation and time course study using compound 33 (Supporting Figure S4).

Results from the $^{18}$F-L-FAC mPET/CT scans are summarized in Figure 4. Transverse PET images of the $^{18}$F-L-FAC liver scans for mice treated with either vehicle or compounds 15a, 36, or 37 are shown in Figure 4B. Figure 4C illustrates the uptake of $^{18}$F-L-FAC in the livers of mice treated dCK inhibitors. The more efficacious compounds induced a greater reduction in the $^{18}$F-L-FAC uptake relative to vehicle treatment, as a result of their greater inhibition of dCK-mediated phosphorylation of its $^{18}$F-labeled substrate. Note the approximate 30% decrease in $^{18}$F-L-FAC signal compared to vehicle control induced by compounds 28, 29, 36, and 37, indicating their superior in vivo efficacy relative to the other dCK inhibitor candidates. In addition, compounds 30 and 32 show about a 20% decrease in probe uptake. Compound 33, one of the most potent dCK inhibitors in the cell culture assay (Table 3), showed poor in vivo efficacy in the $^{18}$F-L-FAC PET assay, presumably due to its poor PK properties. As hypothesized, substitution of the hydroxyl group at the end of the ethoxy chain (e.g., compound 33) for the metabolically stable methylsulfonamide (compounds 28, 29, and 37) or hindering the hydroxyl group (compound 36) proved advantageous for in vivo efficacy. Compounds 36 and 37 have the

<table>
<thead>
<tr>
<th>compd</th>
<th>$K_{i,app}$ (nM)</th>
<th>error (nM)</th>
<th>$R^2$</th>
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</thead>
<tbody>
<tr>
<td>15a</td>
<td>9.5</td>
<td>2.3</td>
<td>0.974</td>
</tr>
<tr>
<td>15c</td>
<td>1.5</td>
<td>0.3</td>
<td>0.998</td>
</tr>
<tr>
<td>36</td>
<td>0.8</td>
<td>0.7</td>
<td>0.982</td>
</tr>
<tr>
<td>37</td>
<td>0.5</td>
<td>0.5</td>
<td>0.988</td>
</tr>
</tbody>
</table>

“Data from Nomme et al.

Figure 4. In vivo evaluation of dCK inhibitors via PET analysis. (A) Schematic of the mechanism by which $^{18}$F-L-FAC accumulates in dCK expressing cells. (B) Representative transverse images of $^{18}$F-L-FAC PET/CT liver scans of C57Bl/6 mice treated with compounds 15a, 36, and 37. (C) Quantification of $^{18}$F-L-FAC uptake in the liver for a sample of inhibitors with low nanomolar in vitro potency. Data are mean values ± SEM for at least $n = 3$ mice/group. * $P < 0.03$. (D) Representative images and quantification of $^{18}$F-L-FAC PET/CT scans of CCRF-CEM tumor bearing NSG mice that were treated with vehicle or compound 36. Data are displayed as box and whisker plots for at least $n = 4$ mice/group. * $P < 0.0012.$
lowest IC₅₀ values among all the efficacious compounds and were chosen for further study. Here we focus on compound 36, while compound 37 will be described in a subsequent publication (Nathanson, unpublished results).

Next we determined the efficacy of compound 36 at inhibiting dCK activity in tumor tissues in vivo. Mice bearing CCRF-CEM tumor xenografts were treated with compound 36 four hours prior to injection of ¹⁸F-L-FAC (Figure 4D). One hour after the ¹⁸F-L-FAC injection, mice were imaged by mPET/CT. The retention of ¹⁸F-L-FAC in tumor xenografts from mice treated with compound 36 was reduced by about 30% compared to the retention of ¹⁸F-L-FAC in tumors from vehicle treated mice (Figure 4D). To complement the PET assay, the pharmacokinetics of compound 36 was determined using standard analytical techniques, and the approximated values are reported in Figure 5.

X-ray Crystal Structure of Compound 36 Bound to Human dCK. X-ray crystallographic studies of compound 36 were initiated to obtain information about its binding to dCK. The crystal structure of the dCK:36 complex was solved at 1.94 Å resolution (Figure 6 and Table 5). Similar to our observations for compound 15a (Figure 2), in the case of 36, the enzyme also adopts the open conformation. We observed one 36 molecule (green) in each protomer of the dimeric enzyme (Figure 6A). This is in contrast to the observation of two molecules bound per active site when the substituent at the 5-position is smaller than the propyl present in 36 (Figure 2, determinants of single versus double molecule binding to the dCK active site was analyzed by Nomme, unpublished results). Note that binding of 36 to dCK does not preclude nucleotide binding (UDP is shown in red, Figure 6A). The specific dCK:36 interactions are shown in Figure 6B. These include an extensive hydrogen-bond network between the pyrimidine moiety of 36 and residues E53, Q97, and D133 in the dCK nucleoside binding site, as well as several hydrophobic interactions.

‖ CONCLUSION ‖

The identification of potent small molecule human dCK inhibitors that demonstrate in vivo target inhibition is reported. Optimization of inhibitory activity was achieved by extending an alkyl chain from the 5-position of the thiazole ring. In vivo efficacy was improved by manipulation of the ethoxy side-chain present at the meta position of the phenyl ring. The utility of PET as a powerful tool for noninvasive measure of target inhibition and, consequently, as a measure of lack of target inhibition (most likely due to substrate metabolism in vivo) is also presented. Although the major clinical applications of PET are primarily for central nervous system (CNS) and oncology-based diagnostics/therapeutics, PET is playing an increasingly important role in drug development, given the capability of this molecular imaging platform to address key challenges that include evaluation of biodistribution, absorption, target affinity, plasma binding, metabolism, and dosing. Here we used the
**EXPERIMENTAL SECTION**

**High-Throughput Screen.** Recombinant human dCK at a concentration of 1 μM was incubated with 10 μM of drug, 10 μM of dC, and 0.5 μM ATP with 50 mM Tris (pH 7.6), 5 mM MgCl2, and 2 mM DTT. The reaction was incubated at 37 °C for 4 h before adding CellTiter-Glo (Promega): Briefly, 40 μL of dCK enzyme was dispensed into 384 well plates (Greiner, Bahlingen, Germany) using a multichannel 384 (Thermo, Turku, Finland) at concentration of 12.5 μg/mL; compounds were added using a Beckman-Coulter Biomek FX (Beckman Coulter, Brea, CA) equipped with a 500 μL custom pin tool (V&F Scientific, San Diego, CA). Columns 1, 2, 23, and 24 received only DMSO instead of any drugs. In addition, no dCK was added to columns 23 and 24 as these columns served as additional controls (see below). After 30 min incubation at 37 °C, dC and ATP were added to a final concentration of 10 μM and 0.5 μM, respectively, for columns 1–22 using the multichannel in a volume of 10 μL. For columns 23 and 24 the following controls were used: 10 μL of a 2.5 μM ATP solution containing the following additional controls was added: for wells A-D23 1 μM dCTP, for wells E-H23 10 μM dCTP, for wells I-L23 10 μM L-FAC, for wells F-P23 10 μM FAC, for wells A-D24 0.5 μM ATP standard, for wells E-H24 0.1 μM ATP standard, for wells I-L24 1 μM DCK only, and for wells F-P24 10 μM UTP was added, respectively. These controls were included on each plate to exclude equipment failure. This was followed by a 4 h incubation at 37 °C and addition of 25 μL of Cell titer glo reagent (Promega, Fitchburg, WI) by multichannel followed by reading on an Acquast plate reader (Molecular Devices, Sunnyvale, CA). The libraries used were custom sets of compounds from the compound manufacturers Asinex (Winston-Salem, NC) and Enamine (Mornmouth Jct, NJ). These sets consisted of compounds selected extensively for drug-like using the Lipinski rule of five, rotatable bonds, and maximal diversity using custom clustering algorithms.

**Chemistry. General Procedures.** Unless otherwise noted, reactions were carried out in oven-dried glassware under an atmosphere of nitrogen using commercially available anhydrous solvents. Solvents used for extractions and chromatography were not anhydrous. 4,6-Diamino-2-mercaptoypyrimidine was obtained from drying the hydrate over dynamic vacuum at 110 °C for 20 h. All other reagents obtained from commercial suppliers were reagent grade and used without further purification unless specified. Reactions and chromatography fractions were analyzed by thin-layer chromatography (TLC) using Merck precoated silica gel 60 F_{254} glass plates (250 μm). Visualization was carried out with ultraviolet light, vanillin stain, permanganate stain, or p-anisaldehyde stain. Flash column chromatography was performed using E. Merck silica gel 60 (230–400 mesh) with compressed air. 1H and 13C NMR spectra were recorded on ARX500 (500 MHz) or Avance500 (500 MHz) spectrometers. Chemical shifts are reported in parts per million (ppm, δ) using the residual solvent peak as the reference. DMSO-d6 (δ 2.50 ppm for 1H; δ 39.5 ppm for 13C) was used as the solvent and reference standards unless otherwise noted. The coupling constants, J, are reported in Hertz (Hz), and the resonance patterns are reported with notations as the following: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Electrospray mass spectrometry data were collected with a Waters LCT Premier XE time-of-flight instrument controlled by MassLynx 4.1 software. Samples were dissolved in methanol and infused using direct loop injection from a Waters Acquity UPLC into the Multi-Mode Ionization source. The purity of all final compounds was determined to be >95%. Analytical HPLC analysis was performed on a Knauer Smartline HPLC system with a Phenomenex reverse-phase Luna column (5 μm, 4.6 × 250 mm) with inline Knauer UV (254 nm) detector. Mobile phase: A: 0.1% TFA in H2O, B: 0.1% TFA in MeCN. Eluent gradient is specified.
for each described compound in the Supporting Information. All chromatograms were collected by a GinaStar (raytest USA; Inc; Wilmington, NC, USA) analog to digital converter and GinaStar software (raytest USA, Inc.).

**General Procedure for the Synthesis of Compounds 15a–c.** 3-(2-Fluoroethoxy)-4-methoxybenzonitrile (20). To a solution of 3-hydroxy-4-methoxybenzotriazole 19 (3.0 g, 20.1 mmol) in DMF (100 mL) was added CaCO₃ (10.5 g, 32.2 mmol) and 1-bromo-2-fluorobenzene (5.1 g, 40.2 mmol). The mixture was stirred for 18 h at 50 °C. After concentration to remove residual solvent, the resulting residue was washed with brine and extracted with ethyl acetate. The organic layer was washed with water three times, dried over anhydrous MgSO₄ and concentrated in vacuo to yield crude 20 (3.91 g, 20.03 mmol, 99%) as a cream-colored solid. 1H NMR (500 MHz, CDCl₃) δ: 7.28 (dd, J = 8.5, 2.0 Hz, 1H), 7.10 (d, J = 2.0 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 4.83–4.81 (m, 1H), 4.73–4.71 (m, 1H), 4.28–4.26 (m, 1H), 4.23–4.21 (m, 1H), 3.89 (s, 3H); 13C NMR (125 MHz, CDCl₃) δ: 153.6, 148.1, 127.3, 119.1, 110.9, 108.3, 82.3 (d, JCF = 30.0, 3.5 Hz, 2H), 3.79 (s, 3H), 2.41 (s, 3H); 13C NMR (125 MHz, CDCl₃) δ: 164.1, 151.2, 148.1, 148.0, 137.1, 126.4, 120.4, 111.6, 111.5, 82.4 (d, JCF = 169.9 Hz), 68.4 (d, JCF = 20.5 Hz), 55.9, 25.8, 11.4.

![Image of text content](6705 dx.doi.org/10.1021/jm4004557/J. Med. Chem. 2013, 56, 6966–6708)
dCK inhibitor (resuspended in 40% Captisol) 4 h prior to intravenous were intraperitoneally (i.p.) injected with the indicated amounts of 3H-dC (Moravek Biochemicals) were added to the cells were systematically varied from the original to the steps of double-wide sampling. During FEP, the system underwent 5 allowed and of 10^6 con of 100 L/well. After 1 h at 37 °C, cells were washed four times with ice cold phosphate-buffered saline (PBS) using the Millipore Vacuum Manifold. The amount of incorporated probe was measured by scintillation counting with the PerkinElmer Microbeta.

Protein Expression and Purification. Details on C4S S74E dCK variant expression and purification are detailed in Nomme et al.

Crystalization, X-ray Data Collection, and Refinement. Crystallization, data collection, and structure determination of dCK in complex with 15a and 36 were performed following the general procedure as detailed in Nomme et al. Specifically for compound 36, crystals of dCK in complex with UDP, MgCl2 and a 2.5-fold excess of the 36 inhibitor were grown using the hanging drop vapor diffusion method at 12 °C. The reservoir solution contained 0.9–1.5 M trisodium citrate dehydrate and 25 mM HEPES (pH 7.5). Diffraction data were collected at the Advanced Photon Source, Argonne National Laboratory on Life Sciences-Collaborative Access Team (LS-CAT) beamlines 21-ID-G.

Kinetic Assay. Steady-state kinetic assay and data fitting were performed as described in Nomme et al.

Computational Modeling. All simulations were performed using the MCPRO 2.0 package. Initial coordinates were obtained from the X-ray structure of dCK in complex with compound 15c. The protein was solvated in a 30 Å water cap, represented by the TIP4P44 classical water model. Solute atoms represented by the OPLS-AA force field were used. Equilibrations were performed using Metropolis Monte Carlo (MC) in the NPT ensemble at 25 °C and 1 atm. The backbone of the protein and all bond lengths within the protein were fixed; angles and torsions within 1 Å from the center of the bound molecule were sampled. All degrees of freedom of the inhibitor compound were sampled during equilibration simulations. Equilibration consisted of 5 × 10^8 configurations of sampling in which only solvent moves were allowed and of 10 × 10^8 subsequent configurations for the protein-inhibitor complex and for the lone inhibitor in solution. The equilibrated systems were then subject to free energy perturbation (FEP)/MC simulations. These simulations consisted of 14 perturbing steps of double-wide sampling. During FEP, the system underwent 5 × 10^8 configurations of solvent equilibration, followed by 10 × 10^8 configurations of full equilibration, and 25 × 10^8 configurations of data collection. All degrees of freedom of the inhibitor were sampled except those bonds undergoing perturbation. The perturbed bond lengths were systematically varied from the original to the final length.

In Vivo MicroPET/CT Imaging Studies. Animal studies were approved by the UCLA Animal Research Committee and were carried out according to the guidelines of the Department of Laboratory Animal Medicine at UCLA. For the PET liver assay, C57BL/6 mice were intraperitoneally (i.p.) injected with the indicated amounts of dCK inhibitor (resuspended in 40% Captisol) 4 h prior to intravenous injection of 70 μCi of 35F-L-FAC. For the tumor xenograft assay, NOD scid IL-2 receptor gamma chain knockout (NSG) bearing subcutaneous CCRF-CEM tumor xenografts were injected with 50 mg/kg of compound 36 or vehicle. Four hours post-treatment mice were injected intravenously with 70 μCi of 35F-L-FAC. For all mPET/CT studies, a 1 h interval was allowed between probe administration and mPET/CT scanning (Inveon, Siemens Medical Solutions USA Inc.; microCAT, Imtek Inc.). Static mPET images were acquired for 600 s. Images were analyzed using OsirIX Imaging Software Version 3.8.

Pharmacokinetic Studies. C57BL/6 female mice, 8 weeks of age, were injected with a single dose of indicated compounds (50 mg/kg, i.p.). Blood samples (approximately 70 μL) were collected through retro-orbital bleeding into heparinized tubes at 5 min, 15 min, 30 min, 35 min, 40 min, 45 min, 1 h, 2 h, 4 h, and 6 h. The blood samples were centrifuged at 20,000g for 5 min to isolate plasma. One mL of acetonitrile was added to 30 μL of plasma. The supernatant was transferred to new tubes and was evaporated using a SpeedVac. Samples were then resuspended in 50 μL of neat DMSO, and supernatant was transferred to LC/MS sample vials. Samples were then run on an Agilent 6460 Triple Quad LC/MS.

Statistical Analyses. All statistics presented as means of biological replicates with standard error of the mean (±SEM), standard deviation (±SD), or box plots with max and min whiskers. P-value significances were calculated using one sample Student’s t test function in GraphPad Prism 5 (GraphPad Software).

ASSOCIATED CONTENT

Supporting Information

Experimental details and spectroscopic data for compounds 8–14, 16–18, and 25–36; synthetic schemes for 8a, 8b, and 37; dose escalation/time course for PET assay. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Figures 2 and 3: dCK + 15a + UDP Code: 4JLK; Figure 6: dCK + 36 + UDP Code: 4LSB

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Notes

The authors declare the following competing financial interest(s): C.G.R., J.C. and M.E.P. are co-founders of Soﬁe Biosciences, a molecular diagnostic company. They hold equity in Soﬁe Biosciences. The University of California also holds equity in Soﬁe Biosciences. Intellectual property that C.G.R. and J.C. invented and which was patented by the University of California has been licensed to Soﬁe Biosciences. The University of California has patented additional intellectual property invented by C.G.R., J.C., H.L., D.N., J.M.M., A.L.A., M.E.J., and A.L.

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ABREVIATIONS USED

ADA, adenosine deaminase; dCK, deoxycytidine kinase; dNTP, deoxyribonucleotide triphosphate; I11F-FAC, 11F-1-(2′-deoxy-2′-Fluoroarabinofuranosyl) Cytosine; TK1, thymidine kinase 1; HTS, high throughput screen; dC, deoxycytidine; dG, deoxyguanosine; dA, deoxyadenosine; I18F-FDG, 2′-18F-fluoro-2′-deoxy-glucose; I18F-I-1-(2′-deoxy-2′-Fluoroarabinofuranosyl) Cytosine; H3C, H3-deoxycytidine; ATP, adenosine triphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; ES3, glutamic acid 53; D133, aspartic acid 133; Q97, glutamine 97; V55, valine 55; L82, leucine 82; Fluorodeoxyglucose; 18F-L-FAC, 18F-L-1-(2′-Fluoro-2′-deoxy-arabino-furanosyl) Cytosine; 11C, 11C-deoxycytidine; 18F, 18F-fluorodeoxyglucose; 11C-fluoro-2-deoxy-D-glucose, 11C-fluorodeoxyglucose.

REFERENCES


