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5-Fluorocytosine derivatives as inhibitors of deoxycytidine kinase

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ABSTRACT

A series of potent piperidine-linked cytosine derivatives were prepared as inhibitors of deoxycytidine kinase (dCK). Compound **9h** was discovered to be a potent inhibitor of dCK and shows a good combination of cellular potency and pharmacokinetic parameters. Compound **9h** blocks the incorporation of radiolabeled cytosine into mouse T-cells in vitro, as well as in vivo in mice following a T-cell challenge.

Deoxycytidine kinase (dCK) is an enzyme which is responsible for 5′-phosphorylation of deoxynucleosides deoxycytidine (dC), deoxyguanosine (dG) and deoxyadenosine (dA).[1] This phosphorylation is an important part of the nucleoside salvage pathway and is the first and rate-limiting step in the bio-activation of dA and dC. Numerous publications have reviewed the role of the salvage pathways in cancer and inflammation.[2,3] In addition to its natural function, dCK is also responsible for in vivo phosphorylation of several known nucleoside drugs (such as ddC, 3TC, AraC, Cladribine, and Gemcitabine).[4] Knockout mice deficient in dCK show reduced levels of circulating B- and T-lymphocytes.[5] This suggested that dCK would be a good target for immunomodulation. Other connections have been made between the nucleoside salvage pathway and modulation of immune function, such as the genetic deficiency in adenosine deaminase, which leads to severe combined immunodeficiency.[6]

We conducted a high-throughput screen of our library to find inhibitors of dCK[7] and identified hit structures **1** and **2** (Fig. 1). Both compounds showed submicromolar enzyme potency against dCK with IC_{50}s of 510 nM for **1** and 120 nM for **2**, respectively.

Neither compound showed significant potency in the cellular assay (IC_{50} >4 μM). Compound **2** as a dC analog attracted our attention since other dC analogs have been reported to inhibit dCK with IC_{50} values in the micromolar or millimolar potency range.[8] In general, nucleoside derivatives such as **2** demonstrated kinetic behavior consistent with the compounds acting as substrates as well as inhibitors. It also should be noted that dCK displays feedback inhibition through deoxynucleotide triphosphates.[9] An X-ray co-crystal structure of dCK with bound dC has been published.[10]

Several attempts were made to merge substructures from **1** and **2** to enhance potency. We sought to attach the hydrophobic right-

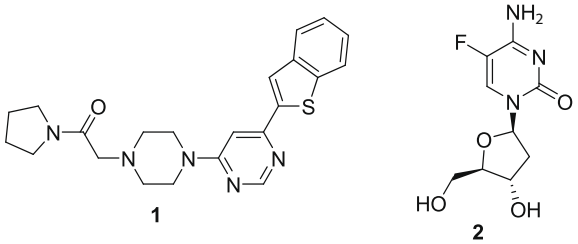

Figure 1. Initial hits from HTS screening against dCK.

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hand side piece of compound **1** to the fluorocytosine core of **2** using a suitable linker.

Our synthetic approach is shown in Scheme 1. Treatment of BOC-protected amino alcohols such as **3** with 5-fluorocytosine under Mitsunobu conditions[11] afforded iminophosphorane-protected cytosine derivatives such as **4**, which were isolable via silica gel chromatography. Subsequent bis-deprotection of **4** under acidic conditions gave access to coupled fluorocytosine salts such as **5** with >10:1 ratios of O- to N-linkage. In most cases, trituration of the solid hydrochloride salt afforded O-linked derivative **5** in high purity and good yield. Substitution regiochemistry at cytosine could be distinguished by the ^{13}C chemical shifts of the corresponding N- or O-coupled carbon atom of the piperidine.[12] In general, the major O-linked derivatives were >5X more potent than their N-linked isomers (see Table 3). Direct-linked scaffold leading to compound **7a** was prepared by direct displacement of 4,6-dichloropyrimidine with 5-fluorocytosine.

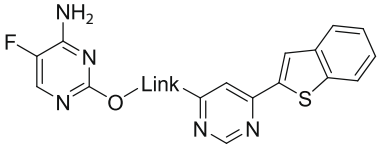
Coupling of linked amines such as **5** to substituted di-halopyrimidines was generally fast and highly selective for mono-addition. Mono-halide intermediates (e.g., **6**) could be purified on silica gel, but were often sufficiently pure after aqueous workup to telescope the final step. Standard Suzuki coupling[14] under microwave conditions afforded biaryl derivatives such as **7a–d**, while a second nucleophilic aromatic substitution with substituted phenols at elevated temperature led to products **8a**–**p** and **9a**–**j**.

In vitro potency (IC_{50}) was obtained using a filter-binding assay to measure inhibition of human dCK.[7] Cell potency (EC_{50}) was determined by measuring the inhibition of Ara-C phosphorylation in CCRF-CEM cells by an Ara-C rescue assay.[7] IC_{50} and EC_{50} values shown are averages of at least 2 determinations.

As shown in Table 1, piperidine and spaced piperidine scaffolds served as good linkers between the cytosine mimic and the hydrophobic region. Piperidine **7b** was chosen for further SAR exploration because it had slightly better solubility than compounds **7a**, **7c**, or **7d**. We sought to further improve both potency and physical properties by incorporating more hetero atoms and flexibility. As shown in Table 2, phenoxy-linked piperidine derivatives **8a**–**8p** showed very promising increases in potency. Unsubstituted phenoxy compound **8a** compared reasonably well with benzothiophene **7b**. Substitution of the phenyl ring allowed significant increases in potency. As can be observed in **8b**–**8d** and **8h**–**8j**, substitution was tolerated at all positions, but the strongest potency improvements (both primary and cellular) were realized by substitution of the 2-position as in **8d**, **8f**, **8g**, **8j**, and **8l**. Even large substitutions at the 2-position such as biphenyl derivative **8g** maintained good primary and cellular potency.

The steric affects of phenyl substitution seemed to have a larger impact on activity compared to the electronic factors; for instance, SAR trends for methoxy-substituted compounds **8b**–**8d** were gen-

Table 1
In vitro and cell potency of **7a–d**



7a-d

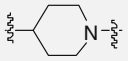
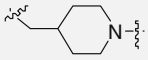
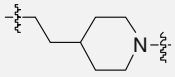
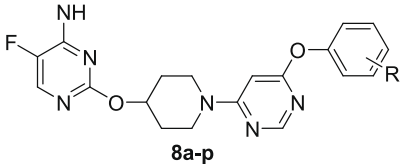
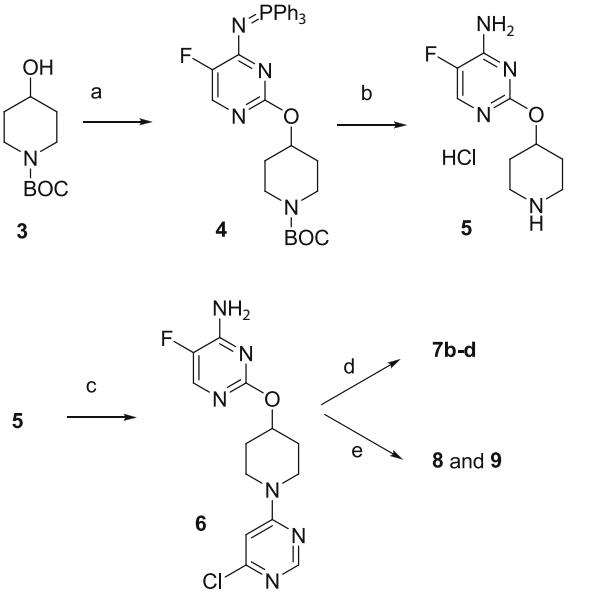
Example	Linker	IC_{50} (μM)	EC_{50} (μM)
7a	Bond	0.72	13
7b		0.038	0.7
7c		0.034	0.79
7d		0.003	0.16

Table 2
In vitro and cell potency of **8a–p**



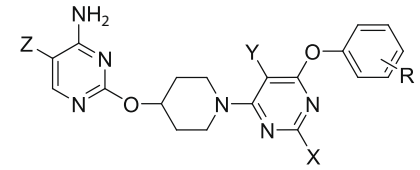
8a-p

Example	R	IC_{50} (μM)	EC_{50} (μM)
8a	H	0.095	0.34
8b	4-OCH_3	0.033	0.89
8c	3-OCH_3	0.023	0.036
8d	2-OCH_3	0.005	0.017
8e	4-CH_3	0.021	0.060
8f	2-CH_3	0.009	0.019
8g	2- Ph	0.003	0.026
8h	4-Cl	0.019	0.057
8i	3-Cl	0.022	0.035
8j	2-Cl	0.003	0.013
8k	2-CH_2CH_3	0.010	0.061
8l	2-CN	0.003	0.014
8m	3-CN	0.013	0.061
8n	2-F	0.006	0.015
8o	2-CH_3, 4-Cl	0.009	0.027
8p	2-CN, 4-OCH_3	0.003	0.012

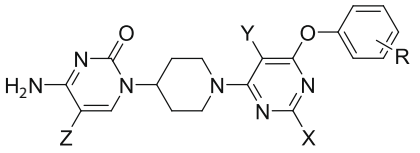


Scheme 1. Reagents and conditions: (a) diethylazodicarboxylate, triphenylphosphine, THF, rt, overnight; (b) HCl/Dioxane rt, overnight 50% (two steps); (c) 4,6-dichloropyrimidine, K_2CO_3, acetonitrile, 4→25 °C, 4–12 h, 90%; (d) $PdCl_2DPPF$, Na_2CO_3, boronic acid, acetonitrile, 140 °C, 10 min, 50–90%; (e) ArOH, K_2CO_3, DMF, 140 °C, 10 min, 60–90%; All compounds were characterized by ^1H NMR, LC/MS, and HPLC.[13]

Table 3
In vitro and cell potency of **9a–j, 10**



9



10

Example	R	X	Y	Z	IC_{50} (μM)	EC_{50} (μM)
8l	2-CN	H	H	F	0.003	0.014
9a	2-CN	H	CH_3	F	0.021	0.054
9b	2-CN	CH_3	H	F	0.010	0.14
9c	2-CN	H	Br	F	0.005	0.080
9d	2-CN	H	F	F	0.001	0.004
9e	4-OCH_3	H	F	F	0.004	0.014
9f	3-CN	H	F	F	0.004	0.013
9g	4-CN	H	F	F	0.005	0.020
9h	2-OCH_3, 5-CN	H	F	F	0.001	0.002
9i	2-CN, 4-OCH_3	H	F	F	0.002	0.005
9j	2-CN	H	F	H	0.0007	0.005
10	2-CN	H	F	H	0.063	0.35

erally very similar to those of chloro-analogs **8h–8j**, both in primary and cellular potency. Within the SAR for mono-substitution, appropriate disubstitution of the ring was also tolerated, as in **8o–8p**. The overall scope of Table 2 shows that the phenoxy substituent is a useful moiety to modulate potency as well as physico-chemical properties required for cell penetration.

Further analogs covering pyrimidine ring substitutions are shown in Table 3. Compared to analog **8l**, methyl substitutions at the 2- or 5-position of the central pyrimidine (compounds **9a–9b**) led to somewhat reduced potency, both primary and cellular. Bromo substitution at the pyrimidine 5-position was tolerated (**9c**), while 5-fluoro substitution led to increased potency (for instance: **9d–8l**). The potency gain with the 5-fluoropyrimidine seemed to hold with other aryl substitutions. In fact, the potency gains from pyrimidine 5-fluorination were even more pronounced with some of the simple mono-substituted aryl analogs. For example, comparison of **9e–8b**, and **9f–8m,** show dramatic potency gains, especially in the cellular assay.

Substitution of the cytosine 5-position is also shown in Table 3. Comparison of compound **9j** with **8l** shows that the cytosine 5-fluoro substituent had little effect on potency. N-Linked piperidine derivative **10** illustrates the common ~10-fold potency difference seen between N-linked and O-linked piperidine derivatives (compound **9j**).

Compounds such as **9h** and **9i** were the most potent dCK inhibitors discovered in this series, showing excellent potency in both primary and cellular assays. Shown in Table 4, the pharmacokinetic behavior of selected compounds was examined in vivo by administering the compound to mice by intravenous injection (iv) or oral gavage (po) at doses of 1 mpk and 10 mpk, respectively. Several compounds of the class showed very favorable pharmacokinetics, characterized by low clearance, moderate volume of distribution, robust oral exposure, and high oral bioavailability. Compounds of this series showed low protein binding. For example, compound **9h** showed 73% protein binding in mouse and 45% in human.

Table 4
Mouse pharmacokinetic parameters of **9f, 9h, 9i**

Example	1 mg/kg iv		10 mg/kg po	
	CL (mL/min/kg)	V_{dss} (L/kg)	AUC (μMol * h)	*%F*
9f	5.7 ± 0.6	1.6 ± 0.1	108 ± 10	~100
9h	5.0 ± 0.3	0.9 ± 0.1	101 ± 19	~100
9i	3.3 ± 0.5	1.1 ± 0.1	236 ± 13	~100

Activity of the compounds was further established in vitro by testing their ability to inhibit the uptake of ^{3}H-dC by stimulated primary murine splenic T cells, see Figure 2. Compounds **9f** and **9h** dramatically inhibited uptake of ^{3}H-dC by splenocytes stimulated with anti-CD3+anti-CD28 antibodies in a standard 48 h proliferation assay.[15,16] The same effect was also observed in human peripheral blood T cells (not shown). Under these conditions, incorporation of ^{3}H-dT is minimally affected (not shown).

Compound **9h** was tested for pharmacologic activity by determining its ability to inhibit ^{3}H-dC uptake in vivo, see Figure 3. Compound was administered to mice by oral gavage beginning at day −1 relative to ip injection of anti-CD3 antibodies to activate T cells, and continuing through day 2 for a total of 3 doses.[17] Compound treatment resulted in a 75% or greater inhibition of ^{3}H-dC uptake by spleen T cells in vivo at a dose of 100 mpk. Subsequent experiments revealed that a single dose of compound given on the same day as anti-CD3 (day 0) was sufficient to block ^{3}H-dC uptake. Similar results were obtained if ^{3}H-dC incorporation into bone marrow was measured without exogenous stimulation (data not shown).

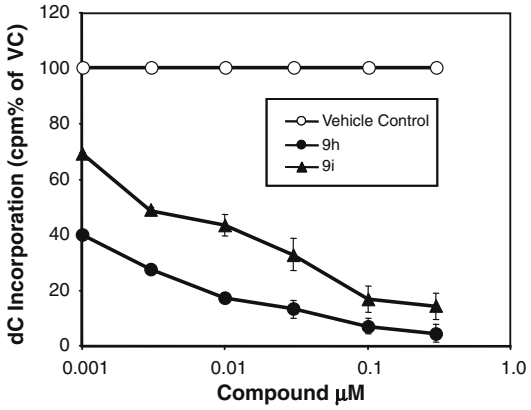


Figure 2. In vitro ^{3}H-dC incorporation into mouse splenocytes blocked by **9h, 9i**.

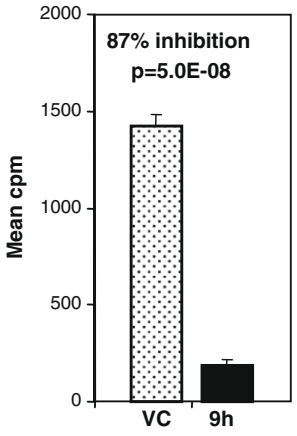


Figure 3. In vivo ^{3}H-dC incorporation into mouse spleen, compound **9h**.

In summary, we have described a novel class of potent dCK inhibitors.[15] Compounds such as **9h** inhibit dCK in enzyme and cellular assays, and inhibit the target in isolated mouse cells and inhibit the incorporation of radiolabeled ^{3}H-dC in vivo.

Acknowledgements

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15. In vitro dC uptake: Single cell suspensions from murine spleens were stimulated in vitro in 96-well plates with immobilized anti-CD3 antibodies and soluble antibodies to CD28 for 48 h to induce DNA synthesis. Compound was diluted in DMSO and added to each culture at the indicated concentration. DMSO was kept constant in all wells at 0.1%. Before (16 h) harvest 0.5 μCi ^{3}H-dCTP was added to each well. Cultures were harvested onto glass fiber filters and counted by liquid scintillation.
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17. In vivo dC uptake: To measure uptake of dC by T cells in vivo, mice were dosed with compound by oral gavage beginning on day −1 relative to ip injection of anti-CD3 antibodies. On day 0, mice received the second dose of compound followed by injection of anti-CD3, and a third dose of compound on day 1, followed by ip injection of 3H-dCTP. Spleens were harvested from the mice on day 2, dissociated into single cell suspensions, and an aliquot of cell suspension harvested onto glass fiber filters and counted by liquid scintillation.