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Identification of Flavopiridol Analogues that Selectively Inhibit Positive Transcription Elongation Factor (P-TEFb) and Block HIV-1 Replication

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Abstract

The positive transcription elongation factor (P-TEFb) (CDK9/cyclin T1) regulates RNA Polymerase II dependent transcription of cellular and integrated viral genes. It is an essential cofactor for HIV-1 Tat-transactivation and selective inhibition of P-TEFb blocks HIV-1 replication without affecting cellular transcription, indicating that P-TEFb could be a potential target for developing anti-HIV therapeutics. Flavopiridol, a small molecule CDK inhibitor, blocks HIV-1 Tat-transactivation and viral replication by inhibiting P-TEFb kinase activity, but it is highly cytotoxic. In search for selective and less cytotoxic P-TEFb inhibitors, we prepared a series of flavopiridol analogues and evaluated their kinase inhibitory activity against P-TEFb and CDK2/cyclin A, cellular antiviral potency, and cytotoxicity. We identified several analogues that selectively inhibit P-TEFb kinase activity *in vitro* and show antiviral potency comparable to that of flavopiridol, but with significantly reduced cytotoxicity. These compounds are valuable molecular probes for understanding P-TEFb-regulated cellular and HIV-1 gene transcription and provide potential anti-HIV-1 therapeutics.

Keywords

Enzymes; cyclin-dependent kinases; P-TEFb; inhibitors; antiviral agents

Introduction

The human positive transcription elongation factor (P-TEFb), composed of cyclin dependent kinase 9 (CDK9) and cyclin T1, regulates elongation phase of RNA polymerase II (RNA Pol II) dependent transcription.[1,2] P-TEFb activates elongation of mRNA transcripts by hyperphosphorylating the C-terminal domain (CTD) of RNA Pol II.[2,3] This phosphorylation event increases the processivity of Pol II and allows for complete mRNA transcription.[4,5] P-TEFb exists in cells in two distinct forms, the kinase active form and a large inactive complex comprising HEXIM1 protein and a small nuclear RNA 7SK.[6–8] The dynamic balance between these active and inactive forms is tightly regulated to control P-TEFb levels.[9]

In addition to its role in cellular transcription, P-TEFb specifically activates transcription from the human immunodeficiency virus type 1 (HIV-1) long-terminal repeat (LTR) promoter. [10] After transcription initiation at the LTR, the newly transcribed transactivation responsive (TAR) RNA hairpin recruits the HIV-1 Tat protein, which binds to the cyclin T1 subunit of P-TEFb and recruits the kinase complex to phosphorylate Pol II.[10,11] HIV-1 Tat has recently been suggested to manipulate P-TEFb functional equilibrium by recruiting it from the large inactive complex thus increasing the active pool for efficient HIV-1 transcription.[12,13] The P-TEFb kinase activity is both essential and limiting for viral replication, and inhibition of P-TEFb by small molecules such as DRB **1** (Figure 1) abrogates both viral transcription and replication.[14] The most potent P-TEFb inhibitor flavopiridol **2** effectively blocks HIV-1 Tat-transactivation and viral replication by inhibiting P-TEFb kinase activity at non-cytotoxic concentrations without affecting cellular transcription.[15,16] RNAi-mediated gene silencing of P-TEFb inhibits Tat-transactivation and HIV-1 replication in host cells with no effects on cell viability.[17] Furthermore, direct inhibition of CDK9 using a dominant negative form has been shown to potently inhibit HIV-1 replication without affecting RNA Pol II transcription and cell viability.[14,18] These and other studies indicate that host and viral transcription may be differentially sensitive to P-TEFb inhibition, providing a strong rationale for targeting P-TEFb as a potential strategy for developing anti-HIV therapeutics.[19,20]

HIV-1 currently infects an estimated 33 million people worldwide, and the numbers of infected people and deaths due to AIDS continue to rise despite the availability of antiviral drugs.[21] Since current anti-HIV-1 drugs mainly target viral protease and reverse transcriptase, selective drug pressure coupled with the high rate of HIV-1 infection and high mutation rate during each infection cycle quickly confer resistance to these drugs.[22] Thus, identification of new anti-HIV-1 therapeutics targeting additional viral enzymes and cellular cofactors essential for viral replication remains a necessity. Antiviral drugs that target cellular cofactors without causing cellular toxicity may prove highly effective against multidrug-resistant viruses,[23] and in combination with protease and reverse transcriptase inhibitors could prevent the progression of HIV-1 infections and the AIDS epidemic.

Since P-TEFb is an essential cofactor for HIV-1 Tat-transactivation and viral replication it represents an attractive drug target for antiviral therapy, but lack of selective P-TEFb inhibitors has so far hindered further clinical evaluation.[20] Among small molecule CDK inhibitors that have been evaluated as potential antiviral agents, DRB, flavopiridol, and seliciclib (CYC202) **3** have been extensively studied.[14,15,24,25] The antiviral effects of seliciclib **3** may be due to the inhibition of CDK2/cyclin E and/or P-TEFb,[24,25] DRB and flavopiridol are reported to block HIV-1 viral replication by inhibiting P-TEF-b kinase activity.[14,15,25] Consistent with these observations, DRB and flavopiridol show significant selectivity towards P-TEFb in kinase assays.[19,20] Flavopiridol is the most potent P-TEFb inhibitor currently identified and exhibits about 10-fold selectivity towards P-TEFb compared to other CDKs.[20,26]

Flavopiridol is reported to inhibit P-TEF-b kinase activity with an IC_{50} of 3 nM and block HIV-1 replication with an EC_{50} below 10 nM, which is less than that required to inhibit other CDKs.[15] However, flavopiridol also inhibits several other CDKs with IC_{50} values of 30–300 nM, and exhibits significant cytotoxicity ($CC_{50} = \sim 150$ nM), which is not surprising since it was originally identified as a potent antitumor agent.[27,28] In a recent study, while flavopiridol potently inhibited HIV-1 replication in HeLa cells with limited cytotoxicity, in longer term replication assays it showed reduced antiviral efficacy and greater cytotoxicity in physiologically relevant primary cells.[25] Thus, selective and less cytotoxic P-TEFb inhibitors are needed that could serve as potential anti-HIV-1 therapeutic agents and as molecular probes to decipher the role of P-TEFb in cellular and HIV-1 gene transcription.

As flavopiridol is a highly potent, and to some extent selective, inhibitor of P-TEFb, it is an obvious starting point to identify more selective and less cytotoxic P-TEFb inhibitors by evaluating related analogues. Recently, Baumli *et al.* reported the first crystal structures of P-TEFb and P-TEFb in complex with flavopiridol,[29] which would be very useful to rationally design highly selective P-TEFb inhibitors. Previously, medicinal chemistry efforts have identified flavopiridol mimics with selective activity against CDKs 1 and 4, though with reduced potency. Flavopiridol D-ring olefin analogue **4** was identified as CDK4-selective inhibitor, [30] thio- and oxaflavopiridol **5** and **6**, respectively, were reported as CDK1-selective inhibitors,[31] structure-based design efforts led to the discovery of 2-benzylidene-benzofuranone **7** as potent and selective inhibitor of CDK1,[32] and recently, P-276-008 whose exact structure has not been disclosed, was identified as a novel pan-selective CDK1, 4 and 9 inhibitor[33] (Figure 2).

However, to date no effort has been directed at identifying flavopiridol analogues that may have increased selectivity towards P-TEFb. To identify such analogues, we prepared a series of chiral flavopiridol C-ring and D-ring olefin analogues and evaluated their kinase inhibitory activity against P-TEFb and CDK2/cyclin A, cellular antiviral potency, and cytotoxicity. We report here the identification of flavopiridol analogues that potently and selectively inhibit P-TEFb kinase activity *in vitro* with enhanced antiviral potency and reduced cytotoxicity. *In vitro* kinase profiling indicates that the 2-fluorophenyl analogue, named fluoroflavopiridol, is about 40-fold more selective towards P-TEFb compared to other CDKs. In addition, we show that fluoroflavopiridol selectively inhibits P-TEFb function *in vivo* without affecting CDK2 function, indicating that its antiviral effects are most likely due to the inhibition of P-TEFb.

Results and Discussion

Crystal structures of flavopiridol, deschloroflavopiridol, and thioflavopiridol in complex with CDK2 show that the inhibitor molecules bind in the ATP binding pocket of the enzyme with benzopyran and piperidinyl rings essentially taking the same position as ATP but the C-ring groups point outside the ATP binding pocket.[31,34] In all three structures the inhibitor makes similar contacts with the enzyme except those outside the ATP binding region where the C-ring group resides, indicating that binding differences in the C-ring region are most likely responsible for the observed selectivity of flavopiridol and its analogues towards various CDKs.[35] Therefore, our medicinal chemistry efforts were focused on the synthesis and evaluation of flavopiridol analogues with modifications in the C-ring region.

We prepared a series of chiral flavopiridol analogues with variations in the C-ring following the reaction sequence illustrated in Scheme 1. The key intermediate chiral acetophenone **9** was prepared following reported methods with minor modifications (see Supporting Information for details).[31,36] Treatment of acetophenone **9** with NaH and condensation of the resulting enolate with various aryl- and hetero-aryl esters in dry DMF followed by cyclization of resulting diketones **10a–p** using dry HCl gas provided the dimethoxy chromones **11a–p**. Demethylation using either BBr₃ in 1,2-dichloroethane or pyridine-hydrochloride/quinoline gave the chromone alkaloids, which were converted to the corresponding hydrochloride salts **12a–p** and lyophilized. The D-ring olefin analogues of flavopiridol **16a–c** were prepared from olefin acetophenone **13** using an analogous route as outlined in Scheme 2 (see Supporting Information for details).[30]

In Vitro Kinase Inhibitory Activity

We determined the P-TEFb (CDK9/cyclin T1) and CDK2/cyclin A kinase inhibitory potencies of flavopiridol analogues in enzymatic assays using GST-CTD and histone H1, respectively, as substrates (Table 1). In our assay, flavopiridol inhibits P-TEFb with an IC₅₀ of 2.5 nM, which is highly comparable to the reported IC₅₀ of 3 nM.[15] The unsubstituted analogue,

deschloroflavopiridol **12a**, is slightly less potent than flavopiridol with an IC_{50} of 9.0 nM. The halogen-substituted C-ring analogues show similar inhibitory potency against P-TEFb except the 4-chlorophenyl analogue **12c**, which is about 5-fold less potent than flavopiridol. The 2- and 4-fluorophenyl compounds, **12d** and **12e**, are highly potent inhibitors of P-TEFb with IC_{50} values of 2.8 nM and 2.1 nM, respectively. Introduction of bulky *t*-butyl substituent at 4-position of the phenyl ring (**12g**) results in significant loss of activity against P-TEFb, and similar results were observed with 4-trifluoromethyl-substituted compound **12h**. The P-TEFb inhibitory activity of C-ring pyridine analogues is sensitive to the position of the ring nitrogen; the 2-pyridyl compound **12j** is less potent compared to 3- and 4-pyridyl analogues, **12k** and **12l**.

The D-ring olefin analogues **4** and **16a–c** are also very potent inhibitors of P-TEFb kinase activity with IC_{50} values only slightly higher than those of the corresponding chiral analogues. These results suggest that the absence of the D-ring hydroxyl group in olefin analogues does not significantly affect their P-TEFb kinase inhibitory activity. Similar results were observed in previous SAR studies of D-ring olefin analogues against CDK4.[30]

Deschloroflavopiridol **12a** was reported to be significantly less potent than flavopiridol against CDK2/cyclin A,[28] however, in our assays, it inhibits CDK2/cyclin A kinase activity with an IC_{50} of 196 nM, which is only slightly higher than that of flavopiridol (IC_{50} = 161 nM). The 3-chlorophenyl analogue **12b** is equipotent to flavopiridol, and compound **12c** with 4-chlorophenyl ring showed slight loss of activity against CDK2/cyclin A. The 2-fluorophenyl compound **12d** is also less active against CDK2/cyclin A than flavopiridol, but it has higher selectivity for P-TEFb. In fact, compound **12d** is the most selective inhibitor of P-TEFb kinase activity, showing 127-fold selectivity for P-TEFb compared to 64-fold selectivity exhibited by flavopiridol.

The D-ring olefin analogue **4**, with the same 2-chlorophenyl substituent as flavopiridol, is about 3-fold less potent against CDK2/cyclin A. Similar to the corresponding chiral analogue **12e**, the 4-fluorophenyl compound **16a** is a more potent inhibitor of CDK2/cyclin A than the 2-chlorophenyl analogue; it is almost equipotent to flavopiridol. Interestingly, our SAR study of flavopiridol C-ring analogues against CDK2/cyclin A closely resembles the SAR of olefin analogues against CDK4/cyclin D1.[30]

During the course of current studies Baumli *et al.* reported the first crystal structure of P-TEFb in complex with flavopiridol, revealing that the molecule binds to CDK9 in the ATP binding pocket. Interestingly, flavopiridol interactions with CDK9 are similar to those observed in CDK2-flavopiridol structure except around the C-ring chlorophenyl group, which has different interactions and orientation in each structure.[29] The differences in C-ring chlorophenyl group interactions with CDK2 and CDK9 are most likely responsible for the significant selectivity of flavopiridol towards CDK9 compared to CDK2.

To further examine the selectivity of 2-fluorophenyl analogue **12d** (named fluoroflavopiridol), it was tested against a panel of CDKs and several unrelated kinases (Table 2). Compound **12d** selectively inhibits P-TEFb (IC_{50} = 2 nM) compared to other CDKs; it is significantly less active against CDK1/cyclin B (IC_{50} = 123 nM), CDK3/cyclin E (IC_{50} = 1423 nM), CDK5/p35 (IC_{50} = 86 nM), and has no activity against CDK7/cyclin H/MAT1. Compound **12d** has very weak or no activity against 12 non-CDK kinases. This data suggests that the 2-fluorophenyl analogue **12d** is a more selective inhibitor of P-TEFb *in vitro* than flavopiridol, exhibiting about 40-fold selectivity towards P-TEFb compared to other CDKs.

Antiviral Activity and Cytotoxicity

We used single-round infectivity assays to determine the antiviral potency of flavopiridol analogues, and separately examined their cytotoxicity in MTT-based cell viability assays (Table 1). Consistent with the previously reported antiviral activity,[15] flavopiridol inhibited HIV-1 viral replication with an EC₅₀ of 9 nM but, as expected, it is highly cytotoxic (CC₅₀ = 120 nM). The deschloroflavopiridol **12a** is equipotent to flavopiridol in inhibiting HIV-1 viral replication (EC₅₀ = 7.4 nM), but is less cytotoxic in our cell viability assay. Among the halogen-substituted C-ring analogues, 2- and 4-fluorophenyl compounds, **12d** and **12e**, also show similar antiviral potency as flavopiridol. The 2-fluorophenyl analogue **12d**, which is the most selective inhibitor of P-TEFb kinase activity *in vitro*, potently inhibits HIV-1 replication with an EC₅₀ of less than 10 nM and is less cytotoxic than flavopiridol.

The 4-hydroxyphenyl compound **12i** and pyridinyl analogues **12j–12l**, which are significantly less cytotoxic than flavopiridol, show no antiviral activity. Although these compounds potentially inhibit P-TEFb kinase activity *in vitro*, they probably lose specificity in cellular assays. The 5-methylisoxazole analogue **12n** is the most potent antiviral compound with an EC₅₀ of 3.5 nM, and is considerably less cytotoxicity than flavopiridol and other halogen-substituted C-ring analogues. The D-ring olefin analogues with 2-chloro- and 4-fluoro-phenyl groups, **4** and **16a**, also exhibit potent antiviral activities with EC₅₀ values slightly higher than the corresponding chiral analogues, but these compounds are also highly cytotoxic.

In general, the *in vitro* P-TEFb kinase inhibitory activities of flavopiridol analogues did not directly correlate to their cellular antiviral potencies, and perhaps not surprisingly, the *in vitro* CDK2/cyclin A or P-TEFb kinase activities did not correlate with the analogues' cytotoxicities. The cellular potency of compounds is known to be affected, not only by *in vivo* specificity, but also by factors such as cell membrane permeability, solubility, protein binding, and stability under physiological conditions.[37] It is, therefore, encouraging that several chiral analogues have similar or better antiviral potency than flavopiridol and are significantly less cytotoxic. Specifically, the 5-methylisoxazole analogue **12n** shows highly potent antiviral activity and significantly better cytotoxicity profile than other analogues. Interestingly, the *in vitro* P-TEFb kinase inhibitory activity of **12n** is relatively lower than that of flavopiridol and **12d** yet it shows high antiviral potency, suggesting that its antiviral effects may not be entirely due to the inhibition of P-TEFb.

In Vivo Specificity Profile

Although the antiviral efficacy of flavopiridol analogues was determined in cell-based infectivity assays, this antiviral activity may not necessarily be due to the inhibition of P-TEFb *in vivo*. To determine the *in vivo* specificity profile, we selected two analogues, **12d**, and **12i**, with similar *in vitro* P-TEFb inhibitory activity but different antiviral potency, and studied their effects on the transcription of three genes controlled by P-TEFb and two genes controlled by CDK2. P-TEFb-regulated gene expression was examined by treating HeLa cells overnight with 10 nM flavopiridol, **12d**, or **12i** and measuring the relative levels of c-Fos, Hsp70, and Mcl-1 mRNAs by RT-qPCR. Selectivity of each inhibitor for P-TEFb was also tested by studying the expression of Cdc2 and cyclin A, two transcripts that are upregulated when CDK2 is active. RNA interference (RNAi) against CDK9 and CDK2 was used as a control.

The RNAi-mediated gene silencing of CDK9 inhibited the expression of P-TEFb and P-TEFb-controlled genes, *c-fos*, *hsp70*, and *mcl-1*, but had no effect on the CDK2-controlled genes, Cdc2 and cyclin A. Similarly, CDK2 knockdown by siRNA inhibited Cdc2 and cyclin A expression with no effect on P-TEFb-controlled genes (Figure 3A). Flavopiridol and **12d** significantly down-regulated P-TEFb-controlled genes without affecting the expression of

Cdc2 and cyclin A, indicating that low concentrations of these compounds specifically inhibit P-TEFb (Figure 3B).

To compare the endogenous CDK2 inhibition by flavopiridol, **12d**, or **12i**, at high concentrations, HeLa cells were treated with 200 nM of each compound, and Cdc2 and cyclin A expressions were monitored. Flavopiridol significantly reduced expression of both Cdc2 and cyclin A, whereas analogues **12d** and **12i** had no effect, suggesting that at this high concentration flavopiridol loses selectivity for P-TEFb *in vivo* and inhibits CDK2 (Figure 3C).

Although compound **12i** inhibits P-TEFb *in vitro*, it has no effect on P-TEFb or CDK2 function *in vivo*. The analogue **12d**, which is a more selective P-TEFb inhibitor than flavopiridol *in vitro*, specifically inhibits P-TEFb function *in vivo* and has no effect on CDK2 function. This *in vivo* selectivity indicates that the antiviral effects of **12d** can be attributed to the inhibition of P-TEFb.

Conclusions

Selective inhibition of P-TEFb kinase activity blocks HIV-1 Tat-transactivation and viral replication without affecting cellular transcription and cell viability, providing a strong rationale for targeting P-TEFb and other cellular cofactors as a potential strategy for the development of anti-HIV-1 drugs. We have evaluated structure-activity profile of flavopiridol analogues using *in vitro* kinase, cellular antiviral, and cytotoxicity assays, and have identified a number of analogues that potently and selectively inhibit P-TEFb kinase activity *in vitro* with high antiviral potency in cellular assays. However, our overall results indicate that the *in vitro* P-TEFb kinase inhibitory activities of flavopiridol analogues do not directly correlate to their cellular antiviral potencies, and the *in vitro* CDK2/cyclin A or P-TEFb kinase activities do not correlate with the analogues' cytotoxicities.

The deschloroflavopiridol **12a**, 2-fluorophenyl analogue **12d**, and 5-methylisoxazole analogue **12n** all potently inhibit HIV-1 viral replication in cellular assays (EC₅₀ values < 10 nM), with respective selectivity indexes 2-, 3-, and 14-fold higher than that of flavopiridol. The 2-fluorophenyl analogue **12d** is a more selective inhibitor of P-TEFb than flavopiridol *in vitro*, showing about 40-fold selectivity towards P-TEFb compared to other CDKs. We show that compound **12d** selectively inhibits P-TEFb function *in vivo* with no effect on CDK2 function, whereas flavopiridol at the same concentration also inhibits CDK2. This result indicates that **12d** is a more selective inhibitor of P-TEFb function *in vivo*, and its antiviral effects are most likely due to the inhibition of P-TEFb. This study provides further evidence that P-TEFb is a potential target for anti-HIV-1 drug development, and that selective P-TEFb inhibitors such as **12d** might serve as anti-HIV-1 therapeutics.

The reported structure-activity profile of flavopiridol analogues together with the recently reported crystal structure of P-TEFb in complex with flavopiridol may spur further interest to rationally design selective P-TEFb inhibitors.

Experimental Section

P-TEFb Expression in Baculovirus

Recombinant baculovirus was generated using BaculoGold™ DNA (BD Biosciences) and the plasmid pBAC-HuCDK9-T1 (kindly provided by Dr. David Price).[38] The Sf9 cells (1.2×10^8 total) were infected with recombinant baculovirus, incubated for 3 days, and harvested by centrifugation. Cell pellets were resuspended in 6 mL insect cell lysis buffer (BD Biosciences) containing protease inhibitor cocktail (BD Biosciences) and incubated on ice for 45 min. Cell lysates were centrifuged at $10,000 \times g$ (4 °C, 45 min), and supernatant were removed. Pre-

washed Ni-NTA beads (Qiagen) were added to the supernatants and incubated at 4 °C with constant mixing for 3 h. After incubation, the beads were centrifuged at 500 × g (4 °C, 5 min) and applied to a small screening column for subsequent affinity chromatography. Briefly, beads were washed with 6 mL 6X His wash buffer (BD Biosciences) containing 50 mM imidazole. The P-TEFb was eluted off the beads by the addition of 6X His Elution Buffer (BD Biosciences) containing various concentrations of imidazole (100 mM–1 M). P-TEFb from the 100, 200, and 400 mM eluted fractions were pooled and dialyzed overnight in dialysis buffer (10 mM Tris-HCl, 0.1 mM NaCl, 15% glycerol, 50 µg/mL PMSF, pH 8.0).

GST-CTD Expression

Recombinant GST-CTD protein was generated by growing GST-CTD *Escherichia coli* (kindly provided by Dr. Danny Reinberg)[39] to an OD₇₅₀ of 0.6 and inducing protein expression with 0.5 mM IPTG for 8 h. The cells were centrifuged at 1500 × g (4 °C, 15 min), resuspended in 10 mL resuspension solution (1 × PBS, 50 µg/mL PMSF, 1 µg/mL pepstatin, 2.0 mM EDTA, 1 mM DTT), and disrupted by sonication (10-second treatments, 4–6 times). Cell lysates were centrifuged at 8,000 × g (4 °C, 20 min) and supernatant collected. The supernatant was added to pre-washed glutathione sepharose 4B beads (Amersham Biosciences) and incubated with constant rocking for 1 h at 4 °C. The beads were centrifuged at 900 × g (4 °C, 5 min) and transferred to a small screening column for subsequent affinity chromatography at 4 °C. Beads were washed 3 times with 3 mL wash buffer A (1 × PBS, 1% triton, 1 mM EDTA, 50 µg/mL PMSF, 1 mM DTT) and 3 times with 3 mL TZ Buffer (100 mM KCl, 0.5 mM EDTA, 50 mM Tris-HCl, 12.5 mM MgCl₂, 1 mM DTT, pH 8.0). Proteins were eluted from beads by adding 0.5 mL TZ buffer with various concentrations of reduced glutathione (5–20 mM), resolved by 10% SDS PAGE, and identified on silver-stained gel. Large elution fractions were pooled, and the GST-CTD dialyzed as described for P-TEFb.

P-TEFb (CDK9/Cyclin T1) Kinase Assay

Flavopiridol and analogues (10 mM solutions in DMSO) were serially diluted in kinase buffer 1 (2 mM Tris-HCl, 0.5 mM MgCl₂, 6 mM NaCl, pH 8.0), with final DMSO concentrations in the kinase assay being less than 1%. *In vitro* kinase assays were performed by adding 40 nM P-TEFb, 10 nM GST-CTD, and flavopiridol or analogues in 20 µL sterile distilled water containing kinase buffer 1. Reactions were initiated by adding a hot/cold cocktail of ATP (0.3 µL [γ -³²P] (7000 Ci/mmol)/3.5 µM), and incubated at 30 °C for 10 min. All reactions were simultaneously terminated by snap freezing in liquid nitrogen, immediately followed by adding stop solution (4X SDS gel loading buffer: 200 mM Tris HCl, 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, and 40% glycerol, pH 6.8). For load controls, all samples were spiked with an equal amount of an end-labeled 72-mer oligonucleotide. All samples were heated to 100 °C for 3 minutes and fractionated by electrophoresis on a 4–20% SDS PAGE gel. Protein phosphorylation was quantitated using the FLA-5000 phosphor imager software and GST-CTD band intensities normalized against the load controls. The total kinase activity for each sample was normalized to DMSO control (100% total kinase activity), and data were curve fit using Grafit software.

CDK2/Cyclin A Kinase Assay

The CDK2/cyclin A *in vitro* kinase assay using histone H1 as substrate was based on the protocol recommended by Upstate Cell Signaling Solutions with minor modification (samples were not spotted on paper but fractionated by electrophoresis post reaction). Flavopiridol and analogues were serially diluted in kinase buffer 2 (8.2 mM MOPS, 0.25 mM EDTA, 0.05 mM EGTA, 0.25 mM B-glycerophosphate, 0.0625 mM Na₃VO₄, and 0.75 mM MgCl₂, pH 7.0), with final DMSO concentrations in the kinase assay being less than 1%. *In vitro* kinase assays were performed by adding 2.9 nM CDK2/1.3 nM cyclin A (Upstate Cell Signaling Solutions), 100

nM Histone H1 substrate (Upstate Cell Signaling Solutions), and flavopiridol or analogues in 25 μ L sterile distilled water containing kinase buffer 2. Reactions were initiated by adding a 10 μ L hot/cold cocktail of ATP (1 μ Ci [γ - 32 P]/5 μ M) and incubated at 30 °C for 10 min. All reactions were simultaneously terminated by snap freezing in liquid nitrogen, immediately followed by adding stop solution (4 $\times$ SDS gel loading buffer: 200 mM Tris HCl, 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, 40% glycerol, pH 6.8). For load controls, all samples were spiked with an equal amount of end-labeled 72-mer oligonucleotide. All samples were heated to 100 °C for 3 min and fractionated by 4–20% SDS PAGE. Protein phosphorylation was quantitated using the FLA-5000 phosphor imager software and the histone H1 band intensities were normalized against the load controls. The total kinase activity for each sample was normalized to DMSO control (100% total kinase activity), and data were curve fit using Grafit software.

Kinase Selectivity Assays

All kinase assays were carried out by Upstate's KinaseProfiler™ service with ATP concentration at K_M for each enzyme; assays were performed according to the protocols detailed at <http://www.millipore.com/drugdiscovery/dd3/brochure>.

Cell Viability Assays

The cytotoxicity of flavopiridol and analogues was assessed using an MTT-based cell viability assay. Briefly, HeLa cells were harvested in the log phase of growth and seeded at 1×10^3 cells per well (180 μ L) in a 96-well plate using DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cell growth conditions were defined at 37 °C, 5% CO₂ for the duration of the assay. At 24 h post seeding, 20 μ L of 10 $\times$ stock solutions (flavopiridol or analogues in DMEM) were added to the appropriate wells. Specifically, each column (8 wells total) of the 96-well plate received its own stock solution to give final concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μ M, 3 μ M, 10 μ M, 30 μ M, and 100 μ M. Each test plate included two negative controls: 1) 20 μ L DMEM, and 2) 20 μ L media with 10% DMSO (final concentration of DMSO being 1%, the highest added to the cells). At 72 h post drug addition, 50 μ L of 2 mg/mL MTT (Boehringer Mannheim) solution prepared in PBS (Invitrogen) was carefully dispensed into each well. After 4 h incubation at 37 °C, 5% CO₂, medium was aspirated from all wells and 100 μ L DMSO (Sigma Aldrich, St. Louis, MO) was added and mixed using a multi-channel pipette. Absorbance was read at 550 nm using a Safire plate reader (TECAN) and % viability calculated for each specific concentration. The results were expressed as RA (drug concentrations inhibiting relative MTT absorbance, RA, by 50%), using the equation: $RA (\%) = (A_{\text{treated group}}/A_{\text{control group}}) \times 100\%$. The CC₅₀ for each compound was calculated using Grafit software.

Infectivity Assays

Hela-CD4-LTR/gal indicator cells (kindly provided by Dr. Michael Emerman)[40] were plated in a 96-well microtiter plate (2×10^4 cells per well) in DMEM with 10% calf serum. After 24 h, cells at 50% confluence were pre-treated overnight with various concentrations of flavopiridol or analogues and infected with X4-tropic HIV-1 variant (HIV-1_{LAI}). At 48 h post-infection, the medium was removed and cell monolayers treated with 125 μ L lysis buffer (Promega). All preparations were freeze-thawed once to ensure complete lysis. The β -galactosidase reporter gene activity was assayed by combining total cell lysate in lysis buffer (50 μ L) with 2X β -Gal assay buffer (Promega) and measuring the V_{max} of the enzyme assay at 420 nm over two h in a Spectramax kinetic plate reader (Molecular Devices).

siRNA Transfections in HeLa

As controls for P-TEFb and CDK2 inhibition, we used RNAi to knock down the expression of CDK9 and CDK2, respectively. CDK9 was targeted by a custom designed siRNA (Dharmacon) (sense sequence 5'UGACGUCCAUGUUCGAGUA 3'), whereas CDK2 was targeted using a previously designed and characterized siRNA (5' AGUUGUACCUCCCCUGGAU 3').^[41] siRNA (50–100 nM) was transfected into HeLa cells using Lipofectamine (Invitrogen), and RNA was collected 24–48 h later.

Quantitative PCR (qPCR)

To measure P-TEFb and CDK2 activity in the presence of flavopiridol and analogues, we used quantitative PCR (qPCR) to detect mRNA levels of both P-TEFb and S-phase (CDK2)-activated genes. To monitor P-TEFb activity, we examined c-Fos, hsp70, and mcl-1 transcription in HeLa cells after treating overnight with 10 nM flavopiridol, **12d**, or **12i**. To monitor CDK2 activity, we examined Cdc2 and cyclin A transcription in HeLa cells treated overnight with 200 nM flavopiridol, **12d**, or **12i**. For controls, we also measured these genes after HeLa cells were treated with CDK9 siRNA and CDK2 siRNA. HeLa RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's protocol, and each RNA sample (1 µg) was then reverse transcribed using Superscript II (Invitrogen) with random hexamer primers. The resulting cDNA samples were diluted (1:5) with nuclease-free water and Sybr Green (Abgene) qPCR reactions were performed using an MJ Research PTC-200 thermocycler. Expression levels of mRNAs of interest were calculated by normalizing to GAPDH using the $\Delta\Delta CT$ method.

Typical Procedure for the Synthesis of Chiral C-Ring Flavopiridol Analogues (3*S*,4*R*)-5,7-Dimethoxy-8-(3-hydroxy-1-methyl-4-piperidiny)-2-(5-methylisoxazole)-4*H*-1-benzopyran-4-one (**11n**)

A slurry of anhydrous NaH (0.15 g, 6.25 mmol) in dry DMF (12 mL) was quickly added to the chiral acetophenone **9** (0.375 g, 1.21 mmol) under the flush of N₂ while shaking and swirling the flask. After stirring the mixture for 15 min at room temperature, a solution of methyl 5-methylisoxazole-3-carboxylate (0.52 g, 3.68 mmol) in dry DMF (12 mL) was slowly added in 5 min. The resulting mixture was stirred at room temperature overnight; it was poured on to ice (~30 g) and the pH of the solution was carefully adjusted to 8.5–9.5 with 1N HCl. The reaction mixture was extracted with CHCl₃ (50 mL, 5 ×), and combined CHCl₃ extract was concentrated under reduced pressure. The residue was dissolved in CHCl₃ (15 mL) and dry HCl gas was bubbled through the solution for 10 min followed by stirring at room temperature for 1 h. Reaction mixture was cooled to 0 °C and 5% aqueous Na₂CO₃ was carefully added until the pH of the solution was 9–9.5. The mixture was extracted with CHCl₃ (50 mL, 3 ×), combined organic extract was dried (Na₂SO₄), filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography, eluting with CHCl₃ MeOH (95:05 + 0.1% NH₄OH) mixture to afford the pure dimethoxyflavone **11n** as a light yellow foamy solid (0.44 g, 91%): ¹H NMR (CDCl₃) δ 6.58 (s, 1H), 6.40 (s, 1H), 6.32 (s, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.87 (br. s, 1H), 3.48–3.43 (m, 1H), 3.22–3.10 (m, 1H), 3.03 (m, 2H), 2.49 (s, 3H), 2.35 (s, 3H), 2.32 (m, 1H), 2.12 (m, 1H), 1.57 (m, 1H); ¹³C NMR (CDCl₃) δ 177.56, 171.49, 162.71, 159.93, 157.40, 152.45, 111.92, 111.09, 109.80, 100.60, 92.60, 77.51, 70.0, 63.08, 57.24, 56.59, 56.25, 46.59, 38.51, 24.49, 12.61; MS (ESI): *m/z* 423.61 [M + Na]⁺, 401.60 [M + H]⁺.

(3*S*,4*R*)-5,7-Dihydroxy-8-(3-hydroxy-1-methyl-4-piperidiny)-2-(5-methylisoxazole)-4*H*-1-benzopyran-4-one hydrochloride (**12n**)

A mixture of the dimethoxyflavone **11n** (0.42 g, 1.05 mmol), pyridine hydrochloride (1.7 g), and quinoline (0.6 mL) was heated at 180 °C for 2 h. The reaction mixture was cooled to

ambient temperature, saturated aqueous NaHCO₃ solution was slowly added, and the pH of the solution was adjusted to pH 9–9.5. The reaction mixture was repeatedly extracted with 5% MeOH–CHCl₃ mixture (50 mL, 6 ×); combined organic extract was dried (Na₂SO₄) and concentrated. The residue was subjected to column chromatography on silica gel, eluting with 20% MeOH in CHCl₃ + 0.2% NH₄OH mixture to provide the desired flavone as a yellow solid (0.3 g, 76%). The yellow solid was stirred in MeOH (10 mL) containing 1 N HCl (1 mL) at room temperature for 30 min and then concentrated under reduced pressure. The residue was stirred in MeOH (2 mL) at 80 °C for 10 min; it was cooled to room temperature and diethyl ether (15 mL) was added to the mixture. The precipitated solid was filtered, washed with diethyl ether (3 ×), and dried to afford a pale yellow solid. This solid was dissolved in water (30 mL), filtered through a syringe filter (cellulose acetate membrane), and was lyophilized to provide the title compound **12n** (0.28 g) as a pale yellow solid: ¹H NMR (DMSO-*d*₆) δ 7.29 (d, *J* = 0.8 Hz, 1H), 6.85 (s, 1H), 6.55 (s, 1H), 5.72 (br. s, 1H), 4.01 (s, 1H), 3.50–3.37 (m, 3H, overlapping signal), 3.32–3.10 (m, 3H), 3.78 (s, 3H), 2.57 (s, 3H), 1.76 (m, 1H); ¹³C NMR (DMSO-*d*₆) δ 181.76, 172.38, 163.20, 159.80, 156.86, 156.13, 155.05, 107.26, 106.35, 104.72, 101.47, 99.39, 65.87, 59.36, 54.56, 43.46, 36.22, 21.69, 12.01; MS (ESI): *m/z* 373.85 [*M* + *H*]⁺. Anal. (C₁₉H₂₀N₂O₆·HCl·1.5H₂O) C, H, N.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Price DH. Mol Cell Biol 2000;20:2629–2634. [PubMed: 10733565]
- Peterlin BM, Price DH. Mol Cell 2006;23:297–305. [PubMed: 16885020]
- Zhou Q, Yik JH. Microbiol Mol Biol Rev 2006;70:646–659. [PubMed: 16959964]
- Marshall NF, Price DH. J Biol Chem 1995;270:12335–12338. [PubMed: 7759473]
- Kim YK, Bourgeois CF, Isel C, Churcher MJ, Karn J. Mol Cell Biol 2002;22:4622–4637. [PubMed: 12052871]
- Yang Z, Zhu Q, Luo K, Zhou Q. Nature 2001;414:317–322. [PubMed: 11713532]
- Nguyen VT, Kiss T, Michels AA, Bensaude O. Nature 2001;414:322–325. [PubMed: 11713533]
- Yik JHN, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q. Mol Cell 2003;12:971–982. [PubMed: 14580347]
- He N, Pezda AC, Zhou Q. Mol Cell Biol 2006;26:7068–7076. [PubMed: 16980611]
- Bieniasz PD, Grdina TA, Bogerd HP, Cullen BR. Proc Natl Acad Sci U S A 1999;96:7791–7796. [PubMed: 10393900]
- Zhang J, Tamilarasu N, Hwang S, Garber ME, Huq I, Jones KA, Rana TM. J Biol Chem 2000;275:34314–34319. [PubMed: 10944537]
- Barboric M, Yik JHN, Czudnochowski N, Yang Z, Chen R, Contreras X, Geyer M, Matija Peterlin B, Zhou Q. Nucl Acids Res 2007;35:2003–2012. [PubMed: 17341462]
- Sedore SC, Byers SA, Biglione S, Price JP, Maury WJ, Price DH. Nucl Acids Res 2007;35:4347–4358. [PubMed: 17576689]
- Flores O, Lee G, Kessler J, Miller M, Schlieff W, Tomassini J, Hazuda D. Proc Natl Acad Sci U S A 1999;96:7208–7213. [PubMed: 10377393]

15. Chao SH, Fujinaga K, Marion JE, Taube R, Sausville EA, Senderowicz AM, Peterlin BM, Price DH. *J Biol Chem* 2000;275:28345–28348. [PubMed: 10906320]
16. Chao SH, Price DH. *J Biol Chem* 2001;276:31793–31799. [PubMed: 11431468]
17. Chiu YL, Cao H, Jacque JM, Stevenson M, Rana TM. *J Virol* 2004;78:2517–2529. [PubMed: 14963154]
18. Salerno D, Hasham MG, Marshall R, Garriga J, Tsygankov AY, Grana X. *Gene* 2007;405:65–78. [PubMed: 17949927]
19. Klebl BM, Choidas A. *Future Virology* 2006;1:317–330.
20. Wang S, Fischer PM. *Trends Pharmacol Sci* 2008;29:302–313. [PubMed: 18423896]
21. Joint United Nations Program on HIV/AIDS (UNAIDS). 2008
22. Clavel F, Hance AJ. *N Engl J of Med* 2004;350:1023–1035. [PubMed: 14999114]
23. Schang LM, Bantly A, Knockaert M, Shaheen F, Meijer L, Malim MH, Gray NS, Schaffer PA. *J Virol* 2002;76:7874–7882. [PubMed: 12097601]
24. Agbottah E, de La Fuente C, Nekhai S, Barnett A, Gianella-Borradori A, Pumfery A, Kashanchi F. *J Biol Chem* 2005;280:3029–3042. [PubMed: 15531588]
25. Biglione S, Byers S, Price J, Nguyen VT, Bensaude O, Price D, Maury W. *Retrovirology* 2007;4:47–58. [PubMed: 17625008]
26. Pumfery A, de la Fuente C, Berro R, Nekhai S, Kashanchi F, Sheng-Hao C. *Curr Pharm Des* 2006;12:1949–1961. [PubMed: 16787240]
27. Czech J, Hoffmann D, Naik R, Sedlacek HH. *Int J Oncol* 1995;6:31–36.
28. Sedlacek HH, Czech J, Naik R, Kaur G, Worland P, Losiewicz M, Parker B, Carlson B, Smith A, Senderowicz A, Sausville E. *Int J Oncol* 1996;9:1143–1168.
29. Baumli S, Lolli G, Lowe ED, Troiani S, Rusconi L, Bullock AN, Debreczeni JE, Knapp S, Johnson LN. *EMBO J* 2008;27:1907–1918. [PubMed: 18566585]
30. Murthi KK, Dubay M, McClure C, Brizuela L, Boisclair MD, Worland PJ, Mansuri MM, Pal K. *Bioorg Med Chem Lett* 2000;10:1037–1041. [PubMed: 10843211]
31. Kim KS, Sack JS, Tokarski JS, Qian L, Chao ST, Leith L, Kelly YF, Misra RN, Hunt JT, Kimball SD, Humphreys WG, Wautlet BS, Mulheron JG, Webster KR. *J Med Chem* 2000;43:4126–4134. [PubMed: 11063609]
32. Schoepfer J, Fretz H, Chaudhuri B, Muller L, Seeber E, Meijer L, Lozach O, Vangrevelinghe E, Furet P. *J Med Chem* 2002;45:1741–1747. [PubMed: 11960485]
33. Joshi KS, Rathos MJ, Joshi RD, Sivakumar M, Mascarenhas M, Kamble S, Lal B, Sharma S. *Mol Cancer Ther* 2007;6:918–925. [PubMed: 17363486]
34. De Azevedo WF, Mueller-Dieckmann HJ, Schulze-Gahmen U, Worland PJ, Sausville E, Kim SH. *Proc Natl Acad Sci U S A* 1996;93:2735–2740. [PubMed: 8610110]
35. Huwe A, Mazitschek R, Giannis A. *Angew Chem Int Ed* 2003;42:2122–2138.
36. Naik RG, Kattige SL, Bhat SV, Alreja B, de Souza NJ, Rupp RH. *Tetrahedron* 1988;44:2081–2086.
37. Wang S, Meades C, Wood G, Osnowski A, Anderson S, Yuill R, Thomas M, Mezna M, Jackson W, Midgley C, Griffiths G, Fleming I, Green S, McNae I, Wu SY, McInnes C, Zheleva D, Walkinshaw MD, Fischer PM. *J Med Chem* 2004;47:1662–1675. [PubMed: 15027857]
38. Peng J, Zhu Y, Milton JT, Price DH. *Genes Dev* 1998;12:755–762. [PubMed: 9499409]
39. Akoulitchev S, Makela TP, Weinberg RA, Reinberg D. *Nature* 1995;377:557–560. [PubMed: 7566158]
40. Kimpton J, Emerman M. *J Virol* 1992;66:2232–2239. [PubMed: 1548759]
41. Du J, Widlund HR, Horstmann MA, Ramaswamy S, Ross K, Huber WE, Nishimura EK, Golub TR, Fisher DE. *Cancer Cell* 2004;6:565–576. [PubMed: 15607961]

DRB 1 **Flavopiridol 2** **Seliciclib (CYC202) 3**

Figure 1.
Structures of CDK inhibitors DRB 1, flavopiridol 2, and seliciclib (CYC202) 3.

Figure 2. Structures of flavopiridol mimics: olefin analogue **4**, thio- and oxathioflavopiridol **5** and **6**, 2-benzylidene-benzofuranone **7**, and P276-00 **8** (exact structure not disclosed).

Figure 3.

Flavopiridol analogue **12d** specifically and potently inhibits P-TEFb-regulated genes. P-TEFb activity was examined by using qPCR to measure relative levels of *c-Fos*, *Hsp70*, and *Mcl-1* mRNA, whereas CDK2 activity was assessed by measuring relative levels of *Cdc2* and cyclin A mRNA. HeLa cells were treated overnight with 10 nM flavopiridol, **12d**, and **12i**, or transfected with CDK9 or CDK2 siRNAs. RNAs were harvested and reverse-transcribed, and *c-Fos*, *Hsp70*, *Mcl-1*, *Cdc2*, and cyclin A transcripts were measured by qPCR; expression levels are calculated by normalizing to GAPDH. **A.** CDK9 siRNA specifically inhibits *c-fos*, *hsp70*, and *mcl-1* genes with no effect on expression of *CDK2*, *Cdc2* and cyclin A. **B.** Flavopiridol and analogue **12d** (10 nM) specifically inhibit the P-TEFb-regulated genes *c-*

fos, *hsp70*, and *mcl-1*, but have no effect on Cdc2 and cyclin A expression. Compound **12i** (10 nM) does not inhibit CDK9 or CDK2 *in vivo*. **C.** Flavopiridol at high concentration (200 nM) significantly reduces expression of Cdc2 and cyclin A, but analogues **12d** and **12i** have no effect.

Scheme 1.

Synthesis of chiral flavopiridol C-ring analogues from chiral acetophenone **9**. (a) NaH, DMF, RCO₂Me, 0 °C to RT, overnight; (b) dry HCl (gas), CHCl₃, RT, 1 h, 60–90%; (c) BBr₃, 1,2-dichloroethane, 90 °C, 14 h; (d) 2N HCl, MeOH, 60–70%.

Scheme 2.

Synthesis of flavopiridol D-ring olefin analogues from olefin acetophenone **13**. (a) NaH, DMF, RCO₂Me, 0 °C to RT, overnight; (b) dry HCl (gas), CHCl₃, RT, 1 h, 60–90%; (c) Pyridine-HCl, quinoline, 180 °C, 2 h; (d) 2N HCl, MeOH, 60–70%.

Table 1

In Vitro P-TEFb and CDK2/Cyclin A Kinase Inhibitory Activity, Antiviral Potency, and Cytotoxicity of Flavopiridol Analogues

Compd	R	<i>In Vitro</i> IC ₅₀ (nM) ^a		Antiviral Activity EC ₅₀ (nM) ^b	Cytotoxicity CC ₅₀ (nM) ^b	Selectivity Index (SI) ^c
		P-TEFb (CDK9/Cyc T1)	CDK2/Cyc A			
2	2-chlorophenyl	2.5	161.4	9.1	120	13
12a	phenyl	9.0	196.0	7.4	190	26
12b	3-chlorophenyl	4.4	163.7	30.8	170	5
12c	4-chlorophenyl	12.3	286.7	16.7	200	12
12d	2-fluorophenyl	2.8	356.5	7.5	274	36
12e	4-fluorophenyl	2.1	129.1	11.7	200	17
12f	4-bromophenyl	5.5	223.5	44.9	280	6
12g	4- <i>t</i> -butylphenyl	19.3	567.1	151.6	660	4
12h	4-trifluoromethylphenyl	18.8	301.9	> 1000	1200	< 1.2
12i	4-hydroxyphenyl	9.6	196.0	> 3000	20920	< 7
12j	2-pyridyl	11.3	886.0	> 1000	1490	< 1.5
12k	3-pyridyl	5.5	247.0	> 1000	3300	< 3.3
12l	4-pyridyl	6.5	208.2	> 1000	3350	< 3.3
12m	4-chloro-3-pyridyl	13.5	314.4	> 1000	177	< 0.2

Compd	R	<i>In Vitro</i> IC ₅₀ (nM) ^a		Antiviral Activity EC ₅₀ (nM) ^b	Cytotoxicity CC ₅₀ (nM) ^b	Selectivity Index (SI) ^c
		P-TEFb (CDK9/Cyc T1)	CDK2/Cyc A			
12n	5-methylisoxazole	19.9	238.3	3.5	645	184
12o	3-vinylphenyl	9.9	129.6	32.1	195	6
12p	4-vinylphenyl	9.5	206.2	NT ^d	219	-
4	2-chlorophenyl	8.7	468.3	20.2	194	10
16a	4-fluorophenyl	6.5	208.2	17.8	266	15
16b	2-bromophenyl	5.4	638.7	48.3	331	7
16c	3-pyridyl	12.4	1023.0	83.6	1440	17

^aP-TEFb and CDK2/cyclin A kinase inhibitory activity were determined using GST-CTD and histone H1, respectively, as substrates; IC₅₀ values are the average of at least two independent experiments.

^bAntiviral activity and cytotoxicity were determined in HeLa cells; EC₅₀ and CC₅₀ values are the average of at least two independent experiments.

^cSelectivity Index = CC₅₀/EC₅₀.

^dNT = not tested.

Table 2
In Vitro Kinase Selectivity Profile of Compound **12d**^a

Kinase	IC ₅₀ (μM)
CDK1/cyclin B	0.123
CDK2/cyclin A	0.350
CDK3/cyclin E	1.423
CDK5/p35	0.086
CDK7/cyclin H/MAT1	> 10
CDK9/cyclin T1	0.002
Aurora A	> 1
CK1	> 1
CK2	> 10
cSRC	> 10
EGFR	> 10
GSK3α	> 1
GSK3β	> 1
MAPK2	> 10
PKA	> 10
PKCα	> 10
PKCβII	> 10
SGK	> 10

^a *In vitro* kinase assays were conducted using Upstate's KinaseProfiler™ service with ATP concentration at KM for each enzyme.