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Chemical Library Screens Targeting an HIV-1 Accessory Factor/Host Cell Kinase Complex Identify Novel Antiretroviral Compounds

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ABSTRACT Nef is an HIV-1 accessory protein essential for AIDS progression and an attractive target for drug discovery. Lack of a catalytic function makes Nef difficult to assay in chemical library screens. We developed a high-throughput screening assay for inhibitors of Nef function by coupling it to one of its host cell binding partners, the Src-family kinase Hck. Hck activation is dependent upon Nef in this assay, providing a direct readout of Nef activity *in vitro*. Using this screen, a unique diphenylfuropyrimidine was identified as a strong inhibitor of Nef-dependent Hck activation. This compound also exhibited remarkable antiretroviral effects, blocking Nef-dependent HIV replication in cell culture. Structurally related analogs were synthesized and shown to exhibit similar Nef-dependent antiviral activity, identifying the diphenylfuropyrimidine substructure as a new lead for antiretroviral drug development. This study demonstrates that coupling noncatalytic HIV accessory factors with host cell target proteins addressable by high-throughput assays may afford new avenues for the discovery of anti-HIV agents.

Nef is one of several accessory proteins encoded by HIV-1, HIV-2, and SIV with essential functions in viral pathogenicity (*1, 2*). Deletions within the SIV *nef* gene reduce viral replication *in vivo* and delay the onset of AIDS-like disease in macaques (*3*). Similarly, HIV isolates from some infected individuals that fail to develop AIDS exhibit defective *nef* alleles (*4–6*), supporting a role for Nef in disease progression. Nef has no known catalytic function and targets signaling pathways in infected cells through direct protein–protein interactions (*7*). Nef binding influences several classes of signaling molecules, including immune receptors, trafficking proteins, guanine nucleotide exchange factors, and protein kinases (*7–9*). These Nef-mediated interactions enhance viral replication in some cell types and contribute to immune evasion as well as survival of infected cells (*10–12*).

Members of the Src family of nonreceptor protein-tyrosine kinases (SFKs) represent an important class of Nef target proteins. Nef binds to the Src homology 3 (SH3) domains of the Src family members Fyn, Hck, Lck, Lyn and c-Src, all of which are expressed in HIV-1 target cells (*13–16*). Nef induces constitutive activation of Hck through a mechanism that involves displacement of the SH3 domain from a negative regulatory interaction with the catalytic domain (*17, 18*). Nef activates c-Src and Lyn through a similar mechanism, suggesting that Nef-mediated SFK activation is a common feature of HIV-infected cells (*19*).

A growing body of evidence suggests that Nef–SFK interaction is important for HIV replication and AIDS pro-

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gression. Komuro et al. demonstrated a strong positive correlation of macrophage-tropic HIV-1 replication with Hck expression in primary cultures of human macrophages; HIV replication was blocked following suppression of Hck protein levels with antisense oligonucleotides (*20*). In transgenic mice, targeted expression of Nef to T-cells and macrophages induced an AIDS-like syndrome characterized by CD4+ T cell depletion, diarrhea, wasting, and 100% mortality (*21*). Strikingly, mice expressing a Nef mutant lacking the highly conserved PxxPxR motif essential for SH3 binding and Hck activation showed no evidence of the AIDS-like phenotype (*22*). When transgenic mice expressing wild-type Nef were crossed into a *hck*-null background, appearance of the AIDS-like phenotype was delayed and mortality was reduced (*22*). Taken together, these observations suggest a critical role for Nef–SFK interactions in AIDS and identify complexes of Nef with these host cell protein kinases as attractive targets for anti-HIV drug discovery.

Lack of a catalytic function makes it difficult to design direct high-throughput screening (HTS) approaches to identify small molecule inhibitors of Nef function. As an alternative, we developed an HTS assay that couples Nef to the activation of Hck (*15, 18, 19, 23*). Using this strategy, we identified three compounds that inhibited Nef-mediated Hck activity and also blocked Nef-dependent HIV-1 replication *in vitro*. These compounds represent valuable chemical probes for future analyses of Nef biological functions. Moreover, these findings demonstrate that chemical library screens directed against an HIV-1 virulence factor in complex with a host cell signaling partner may provide a new avenue to antiretroviral drug discovery.

RESULTS AND DISCUSSION

Development of an *in vitro* Kinase Assay for Nef-Induced Hck Activation Amenable to HTS. Hck and other SFKs adopt an inactive conformation *in vivo* as a result of phosphorylation of a conserved tyrosine residue in their C-terminal tails (*24*). This regulatory phosphorylation event requires an independent kinase known as Csk. To recapitulate this key aspect of SFK regulation in our *in vitro* kinase assay with Nef, we expressed and purified a form of Hck with a modified C-terminal tail (Hck-YEEI) that drives Hck downregulation independently of Csk (*25*). Previous studies from our group have shown that Nef activates both native Hck and Hck-YEEI to the same extent in cell-based assays (*23*), suggesting that Hck-YEEI would provide a useful surrogate for Csk-phosphorylated Hck in our HTS assay. Hck-YEEI was expressed in Sf9 insect cells and purified to homogeneity. Mass spectrometry revealed the presence of a single phosphotyrosine residue in the C-terminal tail, indicating that the kinase was predominantly in the downregulated conformation (data not shown).

The assay platform used for the screen was the Z′-Lyte method (Invitrogen), an intramolecular FRET-based assay that takes advantage of the differential sensitivity of phosphorylated and nonphosphorylated peptide substrates to proteolytic cleavage. In the first step, the Nef–Hck kinase complex phosphorylates a single tyrosine residue in a synthetic FRET-peptide substrate that is tagged on the N-terminus with coumarin and on the C-terminus with fluorescein. The kinase reactions are then developed with a site-specific protease that selectively cleaves the nonphosphorylated FRET-peptide. Cleavage interrupts FRET between the donor and acceptor fluorophores on the N- and C-termini of the FRET-peptide, whereas the uncleaved phosphopeptide maintains the FRET signal. Activity is expressed as an "Emission Ratio" of donor to acceptor emission after excitation of the donor fluorophore at 400 nm:

$$\text{Emission Ratio} = \frac{\text{Coumarin Emission (445 nm)}}{\text{Fluorescein Emission (520 nm)}}$$

The Emission Ratio (ER) remains low if the FRET-peptide is phosphorylated (i.e., no kinase inhibition) and is high if the FRET-peptide is not phosphorylated (i.e., kinase inhibition). By following the reaction progress with this ratio, well-to-well variations in FRET-peptide concentration and signal intensities can be readily controlled.

We first established assay conditions under which Hck-YEEI activation was dependent upon the presence of Nef. As shown in Figure 1, panel a, FRET-peptide substrate phosphorylation increased as a function of the amount of Hck-YEEI added to the assay. Under these conditions, kinase activation is likely due to random intermolecular collisions of Hck-YEEI that result in tyrosine phosphorylation of the activation loop. These events increase in frequency as the concentration of Hck-YEEI rises. This experiment was then repeated in the presence of a 10-fold molar excess of HIV-1 Nef at each Hck-YEEI concentration. The presence of Nef markedly shifted the Hck-YEEI activation curve to the left, indicative of its ability to bind to Hck and relieve the inhibitory

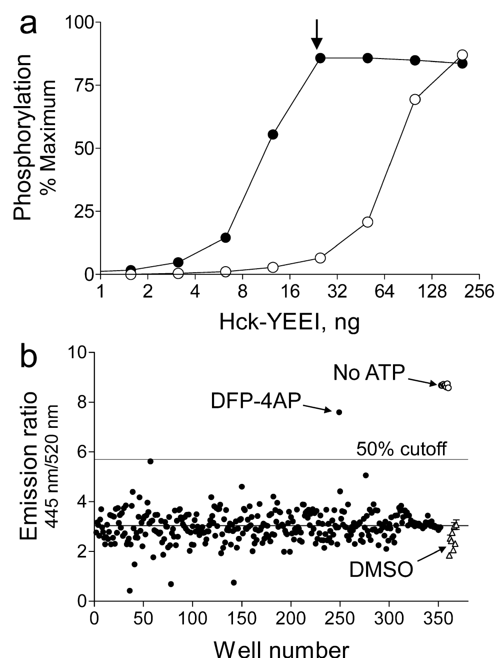

Figure 1. Screening for Nef–Hck inhibitors using the Z′-Lyte assay. (a) Nef stimulates Hck protein-tyrosine kinase activity *in vitro*. Recombinant Hck was purified from Sf9 insect cells in its downregulated form (Hck-YEEI; see text) and assayed with a FRET-peptide substrate and ATP as described under Materials and Methods. Reactions were run in the presence of increasing amounts of Hck-YEEI either alone (○) or in the presence of a 10-fold molar excess of purified recombinant HIV-1 Nef (●). Each condition was repeated in quadruplicate, and the extent of phosphorylation is expressed as mean percent of phosphorylation relative to a control phosphopeptide ± SD. Chemical library screens were performed under conditions where Hck-YEEI activation is dependent upon Nef (*arrow*). (b) Chemical libraries (10 000 compounds total) were screened for inhibitors of Nef-induced Hck kinase activity. Shown are representative emission ratios for 350 compounds from one plate which includes a hit (DFP-4AP; see text for details). The average emission ratio and 50% inhibition cutoff are indicated by the horizontal lines. Clusters of control points include wells with no ATP (○) and DMSO vehicle control (△).

effect of the SH3 domain on kinase activity as reported previously (*17*). Importantly, Nef-induced activation of Hck *via* SH3 domain binding occurs without tail dephosphorylation or release from the SH2 domain (*23*), suggesting that Nef may induce a novel active conformation of Hck and other SFKs in HIV-infected cells. By including Nef in the assay, we hoped to retain this important aspect of Nef signaling. Subsequent chemical library screens were therefore conducted under conditions where Hck activation was dependent on Nef (Figure 1, panel a; arrow).

Identification of Nef–Hck Inhibitors by HTS. The Nef–Hck-YEEI assay was then used to screen chemical libraries consisting of approximately 10 000 discrete compounds for inhibitory activity. The libraries were populated with structures biased toward kinase and phosphatase inhibitors as well as more diverse structures. All of the compounds were initially screened in duplicate at 10 μM, with a positive inhibitory compound being defined as one that caused 50% inhibition relative to untreated controls. The assay routinely produced Z′-factors, a measure of HTS statistical robustness (*26*), in the 0.7–0.8 range for each 384-well plate. Results for a representative plate are shown in Figure 1, panel b. The primary screen yielded four candidate inhibitors, three of which were confirmed in subsequent concentration–response assays. All three confirmed inhibitors were obtained from the kinase inhibitor-biased library, and their structures are shown in Supplementary Figure S1, panel a. They include a methoxyphenyl purine derivative **[1]**, an indeno-isoquinolinedione **[2]** reminiscent of the tyrosine kinase inhibitor staurosporine (*27*), and a 4-amino substituted diphenylfuropyrimidine **[3]**.

Inhibitors of Nef–Hck Activity Block HIV Replication. Inhibitors of Nef-mediated Hck activity identified in the library screen were then evaluated for anti-HIV activity. For these experiments we used U87MG astroglioma cells engineered to express the HIV-1 receptors CD4 and CXCR4 (*28, 29*). Importantly, HIV-1 replication is dependent upon Nef in these cells as demonstrated in Figure 2A. In this experiment, U87MG cells were infected with HIV-1 (NL4–3 strain) as well an isogenic variant that fails to express Nef (ΔNef) over a wide range of viral titers. Wild-type HIV replicated about 60-fold more efficiently than the ΔNef mutant in these cells, providing a unique system to evaluate the impact of the compounds on Nef-dependent HIV replication. In addition, Nef expression stimulates endogenous SFK autophosphorylation in this cell line as assessed by immunoblotting with phosphospecific antibodies (Supplementary Figure S1, panel b).

U87MG cells were infected with wild-type HIV-1 in the presence of each compound at 5 μM and viral replication was assessed as HIV p24 antigen release 4 and

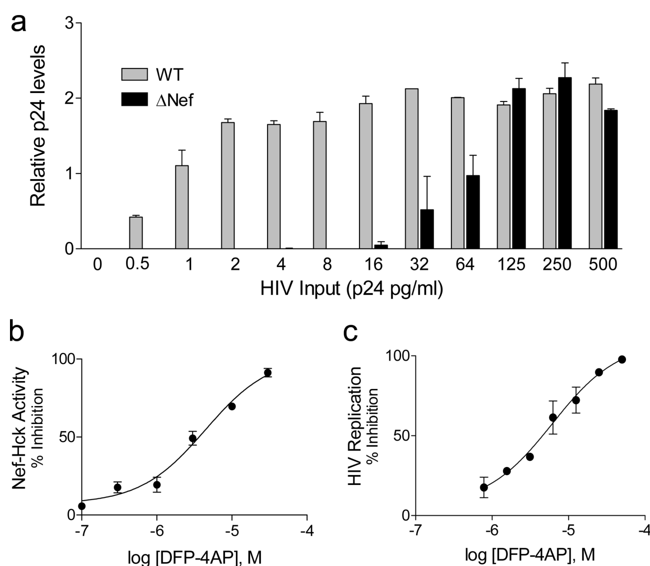

Figure 2. Inhibition of Nef-induced Hck activation and HIV-1 replication by DFP-4-aminopropanol (DFP-4AP). (a) Nef-dependence of HIV-1 replication in U87MG cells. Cells were infected with wild-type HIV strain NL4−3 (WT) or a mutant that fails to express Nef (ΔNef) over the range of viral titers shown. Relative HIV p24 levels were determined by ELISA 5 days later. (b) Inhibition of Nef-induced Hck activation by DFP-4AP. The Nef–Hck complex was assayed *in vitro* with a peptide substrate in the presence of DFP-4AP over the range of concentrations shown. Each concentration was assayed in triplicate, and data are expressed as percent inhibition relative to control reactions run in the presence of solvent only. The data were best-fit by nonlinear regression analysis (GraphPad Prism Software), yielding an IC_{50} value of 4 μM. (c) Inhibition of HIV-1 replication by DFP. U87MG cells were infected with HIV strain NL4−3 in the presence of DFP-4AP over the range of concentrations shown. Release of viral p24 was determined by ELISA 5 days later. The data were best-fit by nonlinear regression analysis, yielding an IC_{50} value of 6 μM.

5 days later. Supplementary Figure S1, panel c, shows that each of these compounds displayed anti-HIV activity; compound **[3]** was most remarkable, suppressing HIV replication to undetectable levels in this experiment. This compound, 3-(5,6-diphenylfuro[2,3-*d*]pyrimidin-4-ylamino)-propan-1-ol (referred to hereafter as DFP-4AP; Figure 3), is structurally related to a class of recently described protein-tyrosine kinase inhibitors built around a 5,6-biarylfuro[2,3-*d*]pyrimidine pharmacophore (*30*). We resynthesized DFP-4AP and confirmed its structure by NMR and mass spectrometry. We then performed a detailed concentration–response study using the Nef–Hck-YEEI assay and found that DFP-AP blocked Nef-induced kinase activity with an IC_{50} of about 4 μM (Figure 2, panel b). We next titrated the anti-HIV activity of DFP-4AP in the U87MG system, and found that it blocked HIV replication with an IC_{50} value of about 6 μM (Figure 2, panel c), which is consistent with the IC_{50} value for inhibition of Nef-induced Hck activity *in vitro*. The anti-HIV potency of resynthesized DFP-4AP was about 10-fold lower than that observed with the compound originally obtained from the chemical library in terms of the maximum extent of viral inhibition observed. This difference may reflect the presence of contaminants or breakdown products present in the commercial preparation.

Antiretroviral Activity of Diphenylfuropyrimidines Requires Nef. The remarkable anti-HIV efficacy of DFP-4AP led us to investigate whether the presence of Nef affected the inhibitory action of diphenylfuropyrimidines against Hck *in vitro* and whether Nef was required for their antiretroviral activity. For these studies, we included three additional DFP-4AP analogs: DFP-4AB, which has a slightly longer 4-aminobutanol side chain; DFP-4PF, with a bulkier 4-aminopropylfuran substituent; and DFP-4A, which bears an unsubstituted 4-amino group (Figure 3). Each of these compounds along with DFP-4AP were then tested in concentration–response experiments against Nef-activated Hck vs Hck alone using the Z′-Lyte assay. As shown in Figure 4, the lead compound (DFP-4AP) showed a modest but highly reproducible 2- to 3-fold increase in potency in the presence of Nef. This effect was magnified with the

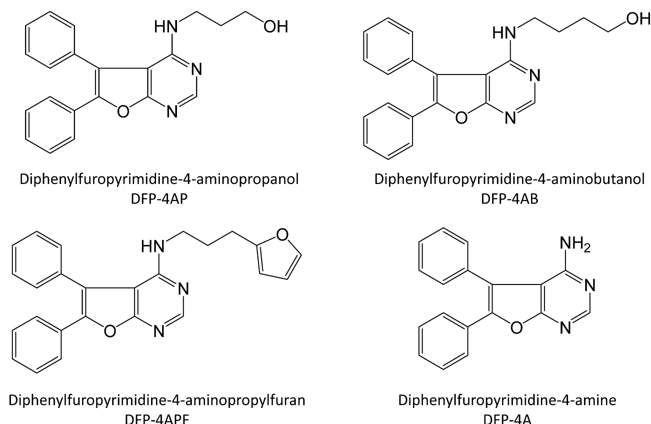

Figure 3. Structures of DFP-4-aminopropanol and related 4-amino derivatives.

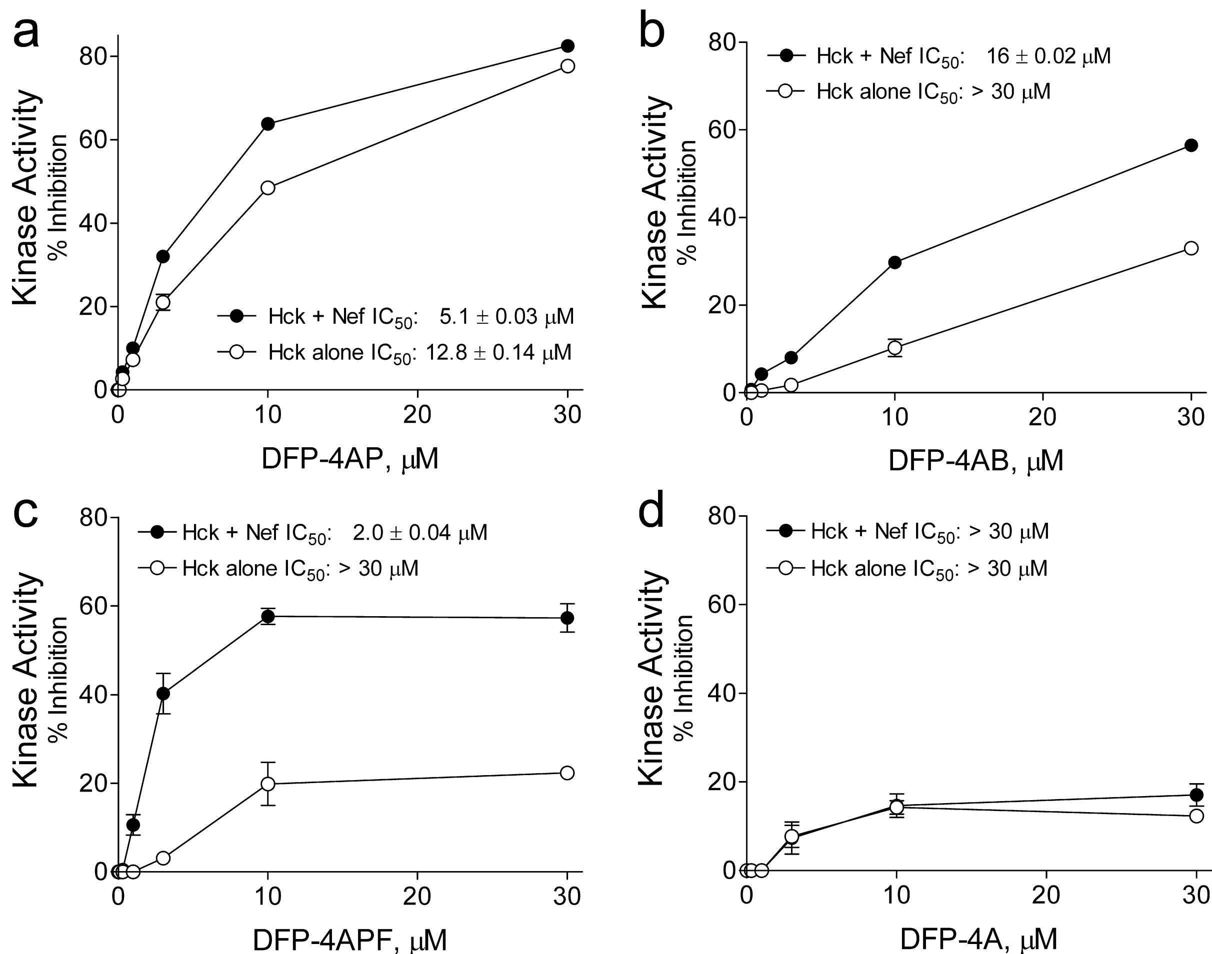

Figure 4. Selective inhibition of Nef-induced Hck activation by diphenylfuropyrimidines. The kinase activities of the Nef–Hck complex and Hck alone were assayed *in vitro* with a peptide substrate in the presence of DFP-4AP and the three analogs shown in Figure 3 over the range of concentrations shown. Each concentration was assayed in triplicate, and data are expressed as percent inhibition relative to control reactions run in the absence of compound. Where possible, nonlinear regression analysis was used to estimate IC_{50} values shown (GraphPad Prism).

4-aminobutanol analog, which also showed less efficacy against Hck alone (Figure 4, panel b). The 4-aminopropylfuranyl derivative showed a remarkable difference in both potency and efficacy for Nef-activated Hck vs Hck alone (Figure 4, panel c). Finally, the unsubstituted analog (DFP-4A) was virtually inactive against Hck in the presence or absence of Nef, indicating that the 4-amino substitutent is a key activity and specificity determinant (Figure 4, panel d). The presence of Nef also sensitized Lyn, another SFK activated by Nef, to inhibition by DFP-4AP, DFP-4AB, and to a lesser extent DFP-4APF (Supplementary Figure S2). As for Hck, very little inhibition of Lyn was observed with DFP-4A in the presence or absence of Nef. Note that a recent X-ray crystal structure of the Lck kinase domain bound to a related diphenylfuropyrimidine-based inhibitor (*30*) suggests that the furopyrimidine moiety of DFP-4AP and the active analogs occupy the Hck and Lyn ATP-binding sites (Supplementary Figure S3). Indeed, substitution of the Hck active site "gatekeeper" residue (Thr338) (*31*), which comes in close contact with the DFP pharmacophore in the Lck crystal structure, with a bulkier methionine dramatically reduced the inhibitory potency of DFP-4AP toward Nef-activated Hck (Supplementary Fig-

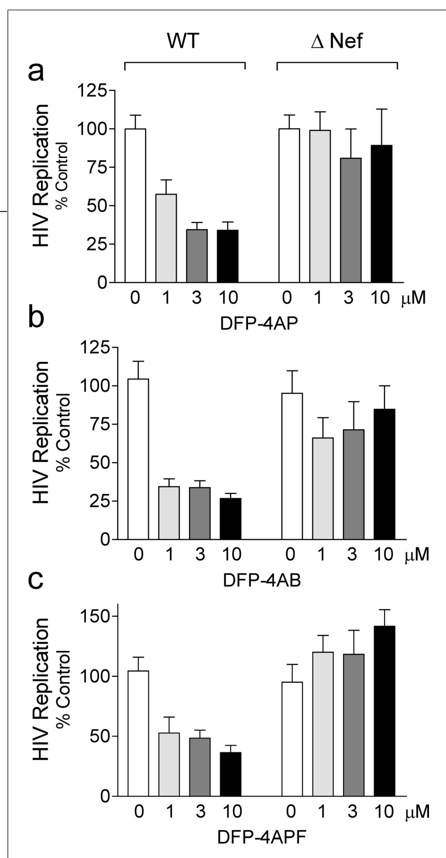

Figure 5. Inhibition of HIV-1 replication by diphenylfuropyrimidines requires Nef. U87MG cells were infected in 96-well plates (200 μL final volume) with equal titers (100 pg/mL p24) of wild-type HIV strain NL4−3 (WT) or a mutant that fails to express Nef (ΔNef) in the presence of the DFP analogs (Figure 3) over the range of concentrations shown. HIV p24 levels were determined by ELISA 5 days later. Data are presented as percent of p24 release observed in the absence of compound ± SD.

ure S3). Furthermore, data presented in Figures 4 and S2 suggest that engagement of SFK SH3 domains by Nef may influence the conformation of the active site to favor compound binding. Indeed, experiments with a mutant of Hck in which the SH3 domain is locked to its internal docking site on the back of the kinase domain shows no difference in inhibitor sensitivity in the presence or absence of Nef (Supplementary Figure S4).

To address whether Nef is required for the antiretroviral effects of these compounds, we next performed HIV replication assays in U87MG cells infected with either wild-type or Nef-defective HIV. As shown in Figure 5, panel a, the lead compound, DFP-4AP, blocked wild-type HIV replication with an IC_{50} value in the low μM range, consistent with the results presented in Figure 2, panel c. In contrast, DFP-4AP had no effect on replication of the HIV-ΔNef mutant, even at 10 μM. Similar experiments were then performed with the three DFP analogs. Both of the analogs that showed selectivity for Nef-activated Hck in the *in vitro* kinase assay also demonstrated potent inhibition of wild-type HIV replication, with IC_{50} values of 1 μM or less. As with DFP-4AP, however, neither of these analogs inhibited the replication of HIV-ΔNef (Figures 5, panels b and c). These data provide strong evidence that 4-amino substituted DFP analogs block HIV replication through a Nef-dependent mechanism. Finally, we examined the activity of DFP-4A, with an unsubstituted 4-amino group. This compound was devoid of antiretroviral activity (not shown), consistent with its poor activity profile *in vitro* (Figures 4 and S2). None of these compounds displayed cytotoxicity toward U87MG cells up to 10 μM as assessed by resazurin reduction assay (Supplementary Figure S5).

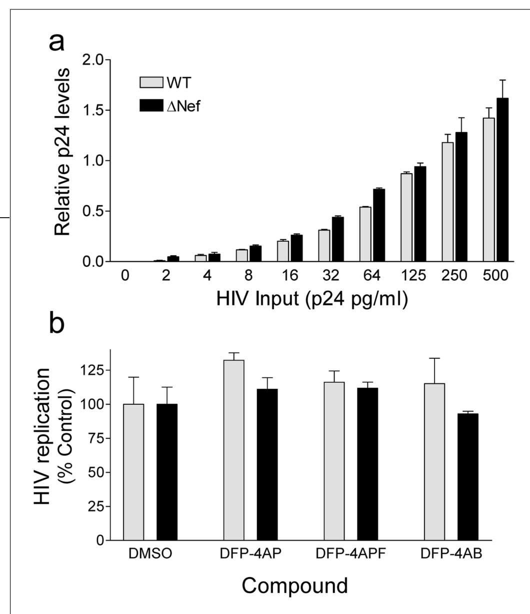

Figure 6. DFP analogs fail to inhibit HIV-1 replication in Jurkat cells. (a) HIV-1 replication is Nef-independent in Jurkat T-cells. Cells were infected in 96-well plates (200 μL final volume) with wild-type HIV strain NL4−3 (WT) or a mutant that fails to express Nef (ΔNef) over the range of viral titers shown. Relative HIV p24 levels were determined by ELISA 5 days later. (b) Jurkat cells were infected with equal titers of wild-type HIV (WT) or the Nef-defective mutant (ΔNef) in the presence of 5 μM of the DFP analogs (Figure 3). HIV p24 release was determined by ELISA 5 days later. Data are presented as percent of p24 release observed in the absence of compound ± SD.

To explore the Nef-dependence of the antiretroviral actions of the DFP analogs further, we performed viral replication assays in Jurkat T-cells. Unlike U87MG cells, replication of HIV is independent of Nef in this cell line (Figure 6, panel a). Wild-type and Nef-defective HIV replication was then tested in Jurkat cells in the presence of each compound at 5 μM, a concentration sufficient to block wild-type HIV replication in U87MG cells (Figure 5). A shown in Figure 6, panel b, none of the DFP analogs showed antiretroviral activity against wild-type or Nef-defective HIV, providing further support that these compounds act through a novel Nef-dependent mechanism to block HIV replication. No cytotoxicity was observed in Jurkat cells at this concentration (Supplementary Figure S5).

Summary and Conclusions. The majority of current drug therapies for HIV target either the viral reverse transcriptase and protease enzymes or interfere with virus-host cell fusion (*32*). While these compounds, especially in combination, have dramatically reduced the morbidity and mortality of HIV-induced disease, the emergence of drug-resistant viruses and the lack of an effective vaccine underscore the need for new anti-HIV agents (*33*). Work presented in this report supports the concept that Nef, an HIV accessory protein essential for AIDS progression, is a valid target for anti-HIV drug discovery. Cou-

pling Nef to one of its well-known host cell target proteins (Hck) enabled HTS for inhibitors of this critical HIV accessory factor, and identified diphenylfuropyrimidines as potential new leads for anti-HIV drug development. Including Hck in the assay not only provides an enzymatic activity easily adaptable to HTS, but may also induce relevant conformations of both Hck and Nef essential for small molecule inhibitor binding and function. This idea is supported by our observation that DFP-4AP, as well as two active analogs (DFP-4AB; DFP-4APF), all demonstrated enhanced potency and efficacy in the kinase assay when Nef was present. Interestingly, the Nef-dependent antiretroviral effects of diphenylfuropyrimidines may be unique among Src-family kinase inhibitors, as the potent, broad-spectrum SFK inhibitor dasatinib (*34*) had no effect on HIV replication in the U87MG system (data not shown). The compounds identified in this screen should be valuable tools to dissect the nature of Nef interactions with Src-family kinases and possibly other host cell factors that interact with Nef in HIV-infected cells. Moreover, combining other HIV accessory factors with relevant host cell binding partners may represent a general strategy for enabling antiretroviral drug discovery.

METHODS AND MATERIALS

Recombinant Protein Expression and Purification. For the initial screen, recombinant Hck-YEEI and Nef (SF2 allele) were expressed in Sf9 insect cells as N-terminal His-6 fusion proteins and purified as described elsewhere (*19*). For subsequent experiments, recombinant His-6-tagged Nef was expressed in *E. coli* (*35*).

Chemical Syntheses. All reactions were conducted in oven-dried (120 °C) glassware under a nitrogen atmosphere. All chemicals were purchased from Aldrich Chemical or Fisher Scientific. Tetrahydrofuran (THF) was distilled over CaH_2 prior to use. Dimethylformamide (DMF) was purchased as anhydrous and transferred under dry nitrogen. 5,6-Diphenylfuro[2,3-*d*]pyrimidin-4-amine (DFP-4-amine) was prepared according to the reported procedure (*36*). ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a Bruker Avance system in $CDCl_3$ using $CHCl_3$ (^{1}H δ 7.26) and $CDCl_3$ (^{13}C δ 77.00) as internal references. Gas chromatography–mass spectrometry (GC-MS) was carried out on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 30 m HP-5 (5% phenyl methylsilicone) Hewlett-Packard capillary column and a Hewlett-Packard 5971 mass selective detector in the electron ionization (EI) mode. High resolution mass spectrometry (HRMS) was performed on an Applied Biosystems 4700 MALDI-TOF-MS using α-cyano-4-hydroxycinnamic acid as the matrix.

3-(5,6-Diphenylfuro[2,3-d]pyrimidin-4-ylamino)propan-1-ol (DFP-4-aminopropanol). Potassium *t*-butoxide (134 mg, 1.2 mmol) was added to a solution of DFP-4-amine (287 mg, 1.0 mmol) in 4 mL of THF at 0 °C and the mixture stirred for 5 min. 3-Bromopropanol (167 mg, 1.2 mmol) was added and the mixture stirred for 18 h at RT. The mixture was concentrated by rotary evaporation, diluted with ethyl acetate, washed with 4 M NH_4Cl and brine, dried over $MgSO_4$, filtered, and concentrated. Purification of the residue by flash column chromatography (silica gel, 2:1 ethyl acetate–hexanes) gave DFP-4-aminopropanol (41.4 mg, 12% yield) as a pale-orange solid: mp 152–153 °C; ^{1}H NMR δ 8.39 (1 H, s), 7.57–7.45 (7 H, m), 7.29–7.23 (3 H, m), 4.91 (1 H, s), 3.71 (1 H, br s), 3.61–3.57 (2 H, m), 3.56 (2 H, t, $J = 5.6$ Hz), 1.67–1.62 (2 H, m); ^{13}C NMR δ 164.7, 158.0, 153.9, 146.9, 132.3, 129.8, 129.7, 129.4, 129.0, 128.5, 126.3, 114.8, 103.0, 58.8, 37.5, 32.6; MS (EI) *m/z* 345 ($M^{+\bullet}$), 326, 77; HRMS (MALDI-TOF) calculated for $C_{21}H_{20}N_3O_2$ $[M+H]^+$ *m/z* 346.1556, found 346.1563.

4-(5,6-Diphenylfuro[2,3-d]pyrimidin-4-ylamino)butan-1-ol (DFP-4-aminobutanol). 4-Bromobutan-1-ol (459 mg, 3 mmol) was mixed with dihydropyran (336 mg, 4 mmol) and freshly recrystallized *p*-toluenesulfonic acid monohydrate (7.1 mg, 0.037 mmol) in 2 mL of dichloromethane and the mixture was stirred at RT for 14 h. The resulting mixture was diluted into 20 mL of dichloromethane, then washed with 20 mL of 5% aqueous sodium bicarbonate and 20 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give 2-(4-bromobutoxy)tetrahydro-2*H*-pyran (711 mg, quantitative yield) as a colorless oil.

DFP-4-amine (290.6 mg, 1.01 mmol) in 2 mL of DMF was treated with NaH (48.5 mg, 1.21 mmol) and the mixture was stirred at RT for 2 h. The mixture was treated with 2-(4-bromobutoxy)tetrahydro-2*H*-pyran (450 mg, 1.9 mmol) and stirred at RT for 14 h. The mixture was diluted with 20 mL of H_2O and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with 60 mL of brine, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography (SiO_2, 4:1 hexanes-ethyl acetate) to give 5,6-diphenyl-*N*-(4-(tetrahydro-2*H*-pyran-2-yloxy)butyl)furo[2,3-*d*]pyrimidin-4-amine (274 mg, 99% yield) as a sticky, clear liquid.

5,6-Diphenyl-*N*-(4-(tetrahydro-2*H*-pyran-2-yloxy)butyl)furo[2,3-*d*]pyrimidin-4-amine (150 mg, 0.34 mmol) was dissolved in 10 mL of CH_3OH and treated with freshly recrystallized *p*-toluenesulfonic acid monohydrate (2 mg, 0.01 mmol). The mixture was stirred at 45 °C for 2 h. The mixture was concentrated and purified by flash column chromatography (SiO_2, ethyl acetate) and recrystallization from dichloromethane/hexanes to give DFP-4-aminobutanol (122 mg, quantitative yield) as a pale-yellow solid: mp 117–119 °C; ^{1}H NMR δ 8.39 (1 H, br), 7.48–7.37 (7 H, m), 7.18 (3 H, br s), 4.75 (1 H, NH), $J = 4.9$ Hz), 3.67 (2 H, m, app t), 3.46 (2 H, app quintet), 2.08 (1 H, br s), 1.58–1.50 (2 H, m), 1.5–1.46 (2 H, m); ^{13}C δ 164.6, 157.5, 153.9, 146.5, 132.3, 129.7, 129.5, 129.3, 128.8, 128.4, 128.3, 126.2, 114.8, 103.0, 61.9, 40.9, 29.4, 25.8; HRMS (MALDI-TOF) calculated for $C_{22}H_{22}N_3O_2$ $[M+H]^+$ *m/z* 360.1712, found 360.1707.

N-(3-(Furan-2-yl)propyl)-5,6-diphenylfuro[2,3-d]pyrimidin-4-amine (DFP-4-amino-propylfuran). NaH (48.5 mg, 1.21 mmol) was added to a solution of DFP-4-amine (289 mg, 1.01 mmol) in 2 mL of DMF and the mixture was stirred at RT for 2 h. A 6:1 (*v*/*v*) mixture of 2-(3-bromopropyl)furan (ca. 350 mg, ca. 1.85 mmol) and 1,2-dibromoethane (ca. 50 mg, ca. 50 mg, ca. 0.25 mmol) in 1 mL of DMF was added, and the mixture was stirred at RT for 14 h. The mixture was concentrated on a rotary evaporator, diluted with ethyl acetate, washed with 4 M NH_4Cl and brine, dried over $MgSO_4$, filtered, and concentrated. Purification of the residue by flash column chromatography (silica gel, 10:1 hexanes-ethyl acetate) gave DFP-4-aminopropylfuran (171.6 mg, 68% yield) as a pale-yellow oil: ^{1}H δ 8.39 (1 H, br), 7.48–7.37 (7 H, m), 7.25–7.23 (4 H, m), 6.22 (1 H, d, $J = 4$ Hz), 5.87 (1 H, s), 4.68 (1 H, br s, NH), 3.42 (2 H, app t),

2.52 (2 H, app t,), 1.77 (2 H, m), 1.58–1.50 (2 H, m), 1.5–1.46 (2 H, m); ^{13}C δ 164.8, 157.6, 154.8, 154.2, 146.5, 141.0, 132.6, 129.8, 129.7, 129.5, 128.9, 128.5, 128.4, 126.3, 114.8, 110.1, 105,2, 103.2, 39.9, 27.7, 24.9; MS (EI) m/z 395 (M–H), 341, 301 (base peak), 286, 273, 216, 201, 189, 94, 81, 77, 53; HRMS (MALDI-TOF) calculated for $C_{25}H_{22}N_3O_2$ $[M+H]^+$ m/z 396.1712, found 396.1718.

***In vitro* Kinase Assay and Chemical Library Screening.** Protein-tyrosine kinase assays were performed in 384-well plates using the Z′-lyte kinase assay system and Tyr2 peptide substrate (Invitrogen) as described elsewhere (*19*). Chemical libraries were purchased from ChemDiv, Inc. and included a kinase-directed library (2500 compounds) a phosphatase-directed library (2500 compounds) and a diversity set (5040 compounds). Library screens were conducted in 384-well plates in a final volume of 10 μL per well. Compounds were added to each well (10 μM final), followed by a preformed complex of Hck-YEEI (10 ng/well) and Nef (1:20 molar ratio) plus the substrate peptide (2 μM). Reactions were initiated by the addition of ATP (50 μM final) and incubated at RT for 35 min. Reactions were developed and terminated as per the manufacturer's protocol and fluorescence ratios were calculated as described in the text and elsewhere (*19*).

HIV Replication Assays. Virus stocks were prepared by transfection of 293T cells (ATCC) with the wild-type and ΔNef recombinant viral genomes (NL4–3 strain) and amplified in the T-cell line, SupT1 (NIH AIDS Research and Reference Reagent Program) (*37*). Viral replication was assessed in the U87MG astroglioma cell line (*28, 29*) engineered to express the HIV-1 coreceptors CD4 and CXCR4 or in the Jurkat T-cell line (Clone E6–1; NIH AIDS Research and Reference Reagent Program). Viral replication was monitored by measuring p24 gag protein levels in the culture supernatant by standard ELISA-based techniques. Test compounds were added to the culture 1 h prior to infection with HIV, and DMSO was used as the carrier solvent at a final concentration of 0.1%.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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