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Synthesis of Novel Fluoro Analogues of MKC442 as Microbicides

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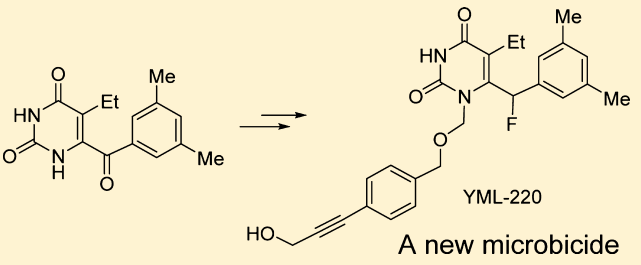
Supporting Information

ABSTRACT: Novel analogues of MKC442 (6-benzyl-1-(ethoxymethyl)-5-isopropylpyrimidine-2,4(1*H*,3*H*)-dione) were synthesized by reaction of 6-[(3,5-dimethylphenyl)-fluoromethyl]-5-ethyluracil (**5**) with ethoxymethyl chloride and formaldehyde acetals. The Sonogashira reaction was carried out on the *N*1-(*p*-iodobenzyl)oxy]methyl derivative of compound **5** using propagyl alcohol to afford compound **12** (YML220). The latter compound was selected for further studies since it showed the most potent and selective activity in vitro against wild-type HIV-1 and non-nucleoside reverse transcriptase inhibitor-, nucleoside reverse transcriptase inhibitor-, and protease inhibitor-resistant mutants and a wide range of HIV-1 clinical isolates. **12** also showed microbicidal activity in long-term assays with heavily infected MT-4 cells.



INTRODUCTION

MKC442 (emivirine) (Chart 1) is one of the first-generation non-nucleoside reverse transcriptase inhibitors (NNRTIs),[1−4] endowed with potent and selective activity against the HIV-1 wild type (wt) but not against NNRTI-resistant mutants. Second-generation NNRTIs were characterized by a much greater resilience to the presence of single-point resistance mutations; among them are DMP266 (efavirenz)[5] (Chart 1) approved for the treatment of HIV/AIDS in combination therapy and TMC125 (etravirine)[6] (Chart 1).

Several attempts have been made to increase the activity of MKC442 against NNRTI-resistant mutants.[7−11] Hopkins et al.[12] synthesized TNK651 (Chart 1), and then El-Brollosy et al.[13] synthesized a series of hybrid analogues of MKC442 and TNK651. Some of these compounds showed activity in the picomolar range against the HIV-1 wt and in the submicromolar range against clinically relevant Y181C and K103N resistant mutants (MKC442-resistant mutants included). Wamberg et al.[14] synthesized hybrid analogues of MKC442 and SJ3366 (Chart 1) with the aim of investigating whether the substitution of the 6-aryl ketone in SJ3366[15] with a 6-arylvinyl group and that of an ethoxymethyl group with various (allyloxy)methyl moieties could lead to improved activity against HIV-1. The new derivatives turned out active against wt HIV-1 in the potency range of DMP266 and moderately active against Y181C and Y181C + K103N resistant mutants.

In the meantime, the NNRTIs emerged as microbicides with the best potential for the prevention of transmission of HIV-1 infections. Among them were TMC120 (dapivirine)[16,17] (Chart 1), which showed favorable pharmacokinetics when delivered through gels or vaginal rings[18,19] and is now being tested in phase III clinical trials,[20] and MC1220[21] (Chart 1), which showed promising potential as a microbicide in rhesus monkeys, no matter whether delivered through liposomes or vaginal rings.[22−24]

The structural similarities among MKC442, TNK651, SJ3366, TMC120, and MC1220 prompted the present study aimed at the synthesis and biological evaluation of a series of fluoro analogues of MKC442. Among them, **12** (YML220) (Scheme 3) emerged as the most potent derivative in cell-based assays against the HIV-1 wt and variants carrying clinically relevant NRTI, NNRTI, and protease inhibitor (PRI) mutations. Viruses resistant to **12**, MC1220, TMC120, and TMC125 were then selected in vitro, and their mutations and cross-resistance patterns were defined. Finally, **12** and structurally related NNRTIs were comparatively evaluated for microbicidal activity in long-term assays and efficacy against clinical isolates belonging to various HIV-1 clades.

CHEMISTRY

Two different routes were explored for the synthesis of 6-[fluoro(3,5-dimethylphenyl)methyl]-5-ethylpyrimidine-2,4-(1*H*,3*H*)-dione (**5**). In the first one, the protected 5-aroyluracil **1**[25] was reduced by sodium borohydride in methanol to the hydroxy compound **2**, which was fluorinated by (diethylamino)sulfur trifluoride (DAST) to give the fluoro

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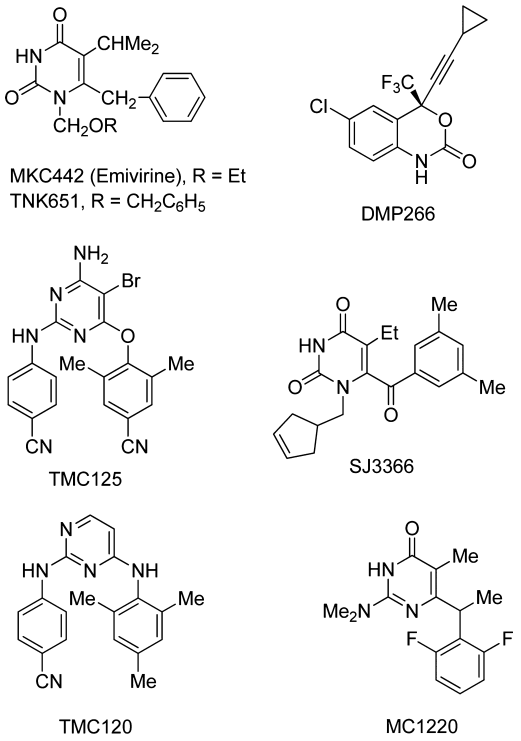
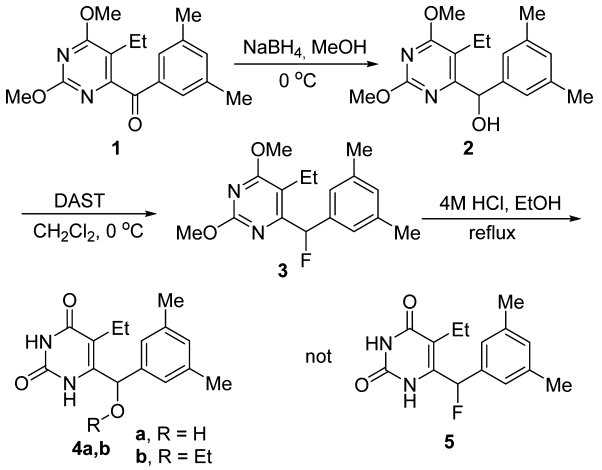
Chart 1. Highly Potent NNRTIs



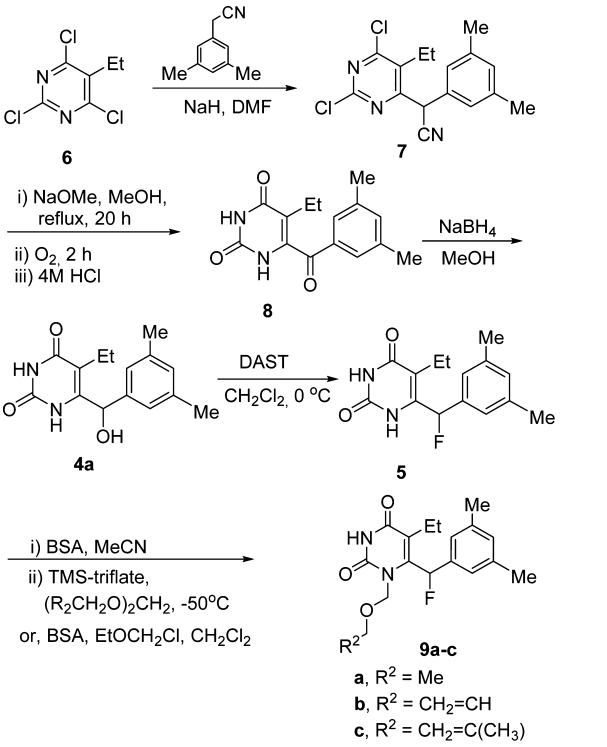
derivative **3**. Deprotection of compound **3**, through acidic hydrolysis using 4 M HCl and ethanol, furnished two different compounds, **4a,b**, in which the fluoro atom was substituted with a hydroxyl and with an ethoxy group, respectively. However, the desired compound **5** was not obtained (Scheme 1).

Scheme 1



In the second route, 5-ethyl-2,4,6-trichloropyrimidine (**6**)[26] was reacted with 3,5-dimethylbenzyl cyanide using sodium hydride in DMF to give compound **7** as previously described by Loksha.[27] Compound **7** was refluxed in methanolic sodium methoxide to replace the chlorine atoms with methoxy groups and then oxidized by a stream of oxygen to achieve the carbonyl group. This was followed by evaporation of methanol and refluxing the residual material in 4 M HCl to give the (3,5-dimethylbenzoyl)uracil derivative **8**. This method can be considered as a novel one-pot synthesis of 6-benzoyluracil derivatives without separation of the intermediates. Reduction of compound **8** to the hydroxy derivative **4a** and subsequent fluorination by DAST afforded the desired compound **5** (Scheme 2).

Scheme 2



Compound **5** was silylated with *N,O*-bis(trimethylsilyl)-acetamide and alkylated by treatment with either ethoxymethyl chloride or a bis(allyloxy)methane,[13] in the presence of trimethylsilyl trifluoromethanesulfonate (TMS triflate) as a Lewis acid catalyst,[28] to give the MKC442 analogues **9a−c** (Scheme 2).

Danel et al.[29] synthesized bis[(4-iodobenzyl)oxy]methane (**10**) according to a previously published method for the preparation of formaldehyde acetals[13,30] (Scheme 3) in which methylene bromide, *p*-iodobenzyl alcohol, KOH, and tetrabutylammonium bromide were refluxed in benzene, and the reaction product was isolated by column chromatography in 71% yield. We synthesized the acetal **10** by coupling 1 equiv of methylene bromide with 2 equiv of *p*-iodobenzyl alcohol using sodium hydride in DMF, and the reaction product was isolated as a solid compound in 96% yield and used without any further purification (Scheme 3).

Compound **11a** (analogue of TNK-651)[12] was synthesized by alkylation with the acetal **10** using the same procedure described for compounds **9b,c**. The N3 coupling product was also observed (compound **11b**). The assignement of N1 versus N3 coupling product was based on comparison with ^{13}C NMR spectra of the corresponding *N*-methyluracils.[31] In support of the assignment, it was found that the enantiotopic protons of the methylene group at N1 in compound **11a** close to the chiral center were found at different ^{1}H NMR chemical shifts (5.00 and 5.38 ppm) as an AB system, whereas those far away from the chiral center at N3 in compound **11b** were recorded as a singlet at 5.45 ppm.

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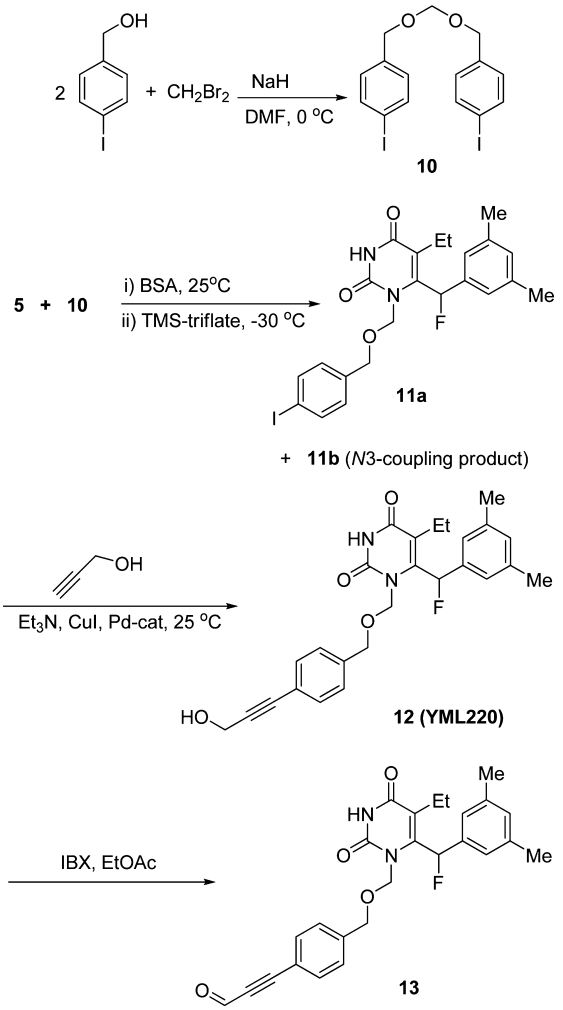
Scheme 3



Sonogashira cross-coupling[32−35] was applied on compound **11a** and propagyl alcohol, using copper(I) iodide and $PdCl_2(PPh_3)_3$ as catalysts, to afford compound **12**. The alcoholic part of compound **12** was oxidized to the aldehyde derivative **13** by treatment with 2-iodoxybenzoic acid (IBX)[36,37] in ethyl acetate (Scheme 3).

■ ANTIVIRAL ACTIVITY

Cytotoxicity and antiviral activity of the newly synthesized compounds were determined in MT-4 cell-based assays. The antiretroviral spectrum was determined using HIV-1 laboratory strains, both the wt and variants carrying clinically relevant NRTI, NNRTI, and PRI mutations and one or more of the following reference inhibitors: MKC442, nevirapine (NVP), MC1220, DMP266, TMC120, TMC125, zidovudine (AZT), and SQV.

Test compounds (Table 1) showed cytotoxicity in the range of 27−100 μM, whereas TMC120, TMC125, and SQV were the most cytotoxic (CC_{50} = 2, 11, and 15, respectively) reference inhibitors. Among the newly synthesized derivatives, **12** was by far the most potent against both wt and mutant HIV-1; in this respect, it turned out to be even significantly more potent than MKC442 and NVP. Interestingly, the potency of **12** turned out to be comparable to that of MC1220, DMP266, TMC120, and TMC125 against wt HIV-1 and comparable to that of TMC120, but over 10-fold superior to that of MC1220, against the NNRTI-resistant mutants.

The above results suggest that the presence of a conjugated system, such as the benzene ring in ether linkage at N1 with the uracil ring, leads to an increased potency against HIV-1 mutants, possibly due to stacking with the aromatic parts of the amino acid residues present in the mutated reverse transcriptase. This is in agreement with the previously published work on TNK-651.[12] The triple bond on the benzene ring can also contribute to stacking, not to mention the importance of the hydroxy group in establishing hydrogen bonding between **12** and an acceptor residue in the non-nucleoside binding site. In fact, when the hydroxyl group was oxidized into a carbonyl group, the corresponding compound **13** showed a significantly lower potency than **12** against both wt HIV-1 and NNRTI-resistant mutants. In all cases, with respect to the compounds synthesized by El-Brollosy et al.[13] and Danel et al.,[38] the presence of a fluoro atom at the methylene group of 6-substituents leads to an increase of potency against HIV-1-resistant mutants.

The comparative genomic analysis of resistant mutants and parental wt HIV-1 (subcultured for the same time period in the absence of drugs) shows (Table 2) that no mutations occur in the *p10*, *p15*, and *p31* genes of the latter, whereas different mutation patterns characterize the RT (*p66/p51*) gene of the former. These findings are consistent with the capability of the above NNRTIs to inhibit the recombinant reverse transcriptase (rRT) of the HIV-1 wt in enzyme assays (results not shown). In particular, mutants resistant to **12** and MC1220 share the mutation Y181C (commonly related to failure of a combination antiretroviral therapy including nevirapine[39] or other NNRTI) associated, in the former mutant, with V106I and, in the latter, with L100I and V179D. It is worth noting that V106I is a polymorphism occurring with similar frequency in untreated and NNRTI-treated patients (HIV Drug Resistance Database, Stanford University, http://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi); however, when in combination with V179D, V106I is believed to be responsible for NNRTI resistance.[40] The L100I mutation has been described, together with another mutation, in patients subjected to a DMP266 treatment and, as a single mutation, in patients subjected to nevirapine or delavirdine treatments.[41] V179D has been described in clinical isolates less commonly than other mutations.[42,43] Both L100I and V179D have also been described in variants selected by treatments with novel NNRTIs, such as capravirine[42] or benzophenones.[44,45]

The susceptibility of the above-mentioned mutants to **12**, NVP, MC1220, DMP266, TMC120, TMC125, AZT, and SQV was comparatively evaluated in MT-4 cells. As reported in Table 3, all resistant mutants show full susceptibility to AZT and SQV, and with the exception of the TMC125-resistant mutant, they show a significant susceptibility to DMP266. Finally, all of them cross-resist to the other NNRTIs tested. A notable exception is represented by the MC1220- and TMC120-resistant mutants, which share the 100I mutation and, interestingly, retain a significant susceptibility to **12**.

As HIV-1 strains have diverged widely in different regions of the world, the antiretroviral efficacy of **12** and reference inhibitors was evaluated in dendritic cells infected with HIV-1 clinical isolates belonging to the subtypes predominant in various continents and including wt strains and variants carrying clinically relevant NRTI mutations. As shown in Table 4, the efficacy of reference compounds in inhibiting the

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Table 1. Cytotoxicity and Antiviral Activity of Test and Reference Compounds against the HIV-1 wt and Variants Carrying Clinically Relevant NRTI, NNRTI, and PRI Mutations[a]

	CC_{50}[b]	EC_{50}[c]						
	MT-4	HIV-1_{IIIB}	DMP266^R (100I, 103R, 179D, 225H)	N119 (181C)	A17 (103N, 181C)	AZT^R (67N, 70R, 215F, 219Q)	MDR (41L, 74V, 106A, 215Y)	SQV^R
				Test Compounds				
9a	85	0.005	6	0.3	2	0.04	ND[d]	9
9b	64	0.004	2	0.2	1.6	0.05	ND	4.2
9c	40	0.008	4	0.4	7	0.07	3.4	3.8
11a	43	0.4	1.4	0.2	2	0.3	1.5	1.2
11b	>100	9	>100	>100	>100	11	>100	23
12	37	0.006	0.1	0.04	0.3	0.01	0.02	0.02
13	27	0.08	2.4	0.4	14	0.1	2.3	5.2
				Reference Compounds				
MKC442	>100	0.03	100	>10	>100	0.08	1.5	0.06
NVP	>100	0.3	>10	>10	>10	0.06	3	0.3
MC1220	≥100	0.002	2	0.8	>20	0.004	0.01	0.01
DMP266	40	0.002	6	0.01	0.08	0.002	0.004	0.004
TMC120	2	0.003	0.2	0.04	0.05	0.001	0.002	0.003
TMC125	11	0.002	0.3	0.01	0.01	0.001	0.001	0.003
AZT	50	0.01	0.01	0.02	0.01	0.2	0.2	0.01
SQV	15	0.02	0.01	0.02	0.01	0.01	0.02	0.09

[a]Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. [b]Compound concentration (μM) required to reduce the proliferation of mock-infected MT-4 cells by 50% as determined by the MTT method. [c]Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity as determined by the MTT method. [d]Not determined.

Table 2. Mutations in the RT Gene of HIV-1 Variants Selected in Vitro for Resistance to 12 and the Reference Inhibitors MC1220, TMC120, and TMC125[a]

selecting drug	mutations in the RT gene	selecting drug	mutations in the RT gene
12	V106I, Y181C	TMC120	L100I, E138G
MC1220	L100I, V179D, Y181C	TMC125	L109M, E138 K, G190E

[a]Resistant variants were selected by serial passages of HIV-1_{IIIB} in the presence of the drugs, whose concentrations were stepwise doubled up to 128 times the initial EC_{50} values. Aliquots of virus solutions (5×10^5 $CCID_{50}$/mL) were subjected to RNA extraction, RT-PCR, and sequencing of the *pol* gene to identify the mutation pattern responsible for drug resistance.

Table 3. Cross-Resistance Profiles of Selected Resistant Mutants (12^R, MC1220^R, TMC120^R, TMC125^R)[a]

	EC_{50}[b]				
compd	HIV-1_{IIIB}	**12**^{R} (106I, 181C)	MC1220^R (100I, 179D, 181C)	TMC120^R (100I, 138G)	TMC125^R (109M, 138K, 190E)
12	0.006	2	0.04	0.01	0.9
NVP	0.3	>20	4	1	>20
MC1220	0.002	0.7	3	2	3
DMP266	0.002	0.06	0.1	0.09	7
TMC120	0.003	0.1	0.1	>20	>20
TMC125	0.002	0.1	0.03	0.07	0.2
AZT	0.01	0.01	0.009	0.01	0.008
SQV	0.02	0.02	0.01	0.02	0.01

[a]Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. [b]Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity as determined by the MTT method.

various clinical isolates was fairly comparable. The uracil-based compound YLM-220 was 10-fold less potent than MC1220 against subtypes A (MP582), CRF01 (MP0044), and CRF02 (MP810). It proved as potent as MC1220 and TMC120 in inhibiting subtypes B (W5269), C (MP1308), F1 (MP411), and G (MP1033) and equipotent with TMC120, but 10-fold more potent than MC1220, against subtype D (MP634). Interestingly, all compounds were very effective (0.0001 μM) against the NRTI-resistant subtype C (MP1315).

We previously described[46] long-term assays (40 days) suitable to evaluate the efficacy of NNRTIs in irreversibly knocking out (KO) in vitro the HIV multiplication under a variety of treatment conditions. Here these assays were used to comparatively determine the maximum nontoxic dose (MNTD) of a continuous treatment with **12**, MC1220, and TMC120, as well as their lowest concentration capable of irreversibly inhibiting the HIV-1 multiplication following a chronic treatment or short-term (4 h) treatments carried out either immediately after or immediately before the HIV-1 infection of MT-4 cells with a high multiplicity of infection (MOI) (5−10 $CCID_{50}$ (50% cell culture infectious dose)/cell).

As far as the MNTDs are concerned (Table 5), the concentrations of **12** and MC1220 allowing full exponential growth of MT-4 cells during a treatment protracted for 40 days (the cells were resuspended at 10^5 cells/mL every 4 days in new drug-containing medium) are 30 and 10 μM, respectively, whereas the TMC120 MNTD is considerably lower (0.3 μM). On the other hand, following a chronic treatment, the most effective compounds (listed in order of decreasing KO potency) are MC1220 (<0.1 μM), TMC120 (0.25 μM), and **12** (1.5 μM). Interestingly, MC1220 shows the same KO potency (<0.1 μM) even when the treatment is limited to the first 2 h postinfection (pi), whereas its KO concentration is 7.5 μM when the treatment is limited to the first hour pi. Vice versa, the KO concentrations of **12** and TMC120 are higher

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Table 4. Comparative Activity of 12 and Reference Compounds against Clinical Isolates Belonging to Different HIV-1 Subtypes[a]

	EC_{50}[b]					EC_{99}[c]				
	B		A	C	C ($NRTI^R$)	CRF01	CRF02	D	F1	G
	IIIB	WS269	MP582	MP1308	MP1315	MP0044	MP810	MP634	MP411	MP1033
Test Compound										
12	0.006	0.01	0.01	0.01	0.0001	0.01	0.1	0.01	0.01	0.01
Reference Compounds										
DMP266	0.002	0.01	ND	0.01	≤0.0001	0.01	0.01	0.01	ND	0.01
MC1220	0.002	0.01	0.001	0.01	≤0.0001	0.001	0.01	0.1	0.01	0.01
TMC120	0.003	ND	ND	0.01	0.0001	0.01	0.01	0.01	0.01	0.01

[a]Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. [b]Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity as determined by MTT assay. [c]Compound concentration (μM) required to achieve 99% inhibition of p24 in supernatants of HIV-1-infected dendritic cell cultures.

Table 5. Maximum Nontoxic Dose (MNTD) and Lowest Knocking Out (KO) Concentration of 12, MC1220, and TMC120 in Long-Term (40 days) Assays with MT-4 Cells[a]

	MNTD[b] (μM)	KO[c] (μM)		
compd	chronic treatment	chronic treatment	4 h treatment pi	4 h treatment bi[d]
12	10	0.5−2.5	>20	>20
MC1220	30	<0.1	<5	<5
TMC120	0.3	0.1−0.5	>20	>20

[a]Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. [b]Maximum compound concentration (μM) capable of allowing the exponential proliferation of mock-infected MT-4 cells as determined by the MTT method. [c]Lowest compound concentration (μM) required to irreversibly knock out the HIV-1 multiplication as determined by the MTT method. [d]Before infection.

than 20 μM. Following a 4 h treatment, no matter whether carried out immediately after or before infection, the sole compound effective in knocking out the HIV-1 multiplcation is MC1220 (<5 μM), whereas **12** and TMC120 fail even at concentrations as high as 20 μM. Overall, the above long-term experiments suggest that MC1220 is not only the least cytotoxic, but also the most potent compound in knocking out the HIV-1 multiplication under any circumstances.

DISCUSSION AND CONCLUSIONS

The attempt to improve the anti-HIV-1 potency and spectrum of MKC442-type NNRTIs was successful. The new fluoro derivatives were synthesized in a few steps from 5-ethyl-2,4,6-trichloropyrimidine with good overall yields and costs comparable to those of other NNRTI microbicides (i.e., TMC120 and MC1220). Among the title compounds, **12** emerged as the most potent derivative, and structure−activity relationship (SAR) studies point to the importance of the benzene ring conjugated in ether linkage at N1 with the uracil ring, to the presence of both a triple bond and a hydroxy group on the benzene ring, and, finally, to the fluorine substitution on the benzylic carbon.

In short-term experiments, the potency of **12** against the HIV-1 wt and variants bearing 103 and 181 resistance mutations is comparable to those of second-generation NNRTIs. Notewhorthy, **12**, followed by TMC125, proves to be the most active NNRTI against the MC1220- and TMC120-resistant mutants, which share the L100I mutation. Unfortunately, the latter favorable properties are counteracted by the lower potency of **12** (when compared to other NNRTIs) against NRTI- and PRI-resistant mutants.

Compound **12** proves active against a wide panel of clinical isolates belonging to the B, C, D, and F subtypes and recombinant forms and, like the other NNRTIs tested, shows huge potency against the C NRTI-resistant clinical isolate. At the moment, the dramatic activity of the NNRTIs against the clade C HIV-1 variant carrying NRTI-resistant mutations remains unclear, especially as far as **12** is concerned; in fact, it proved to be the NNRTI with the lowest potency against AZT^R and MDR strains.

Like other NNRTI-resistant mutants, **12**-resistant variants show full susceptibility to AZT and SQV, maintain some susceptibility to DMP266, and show cross-resistance to the other members of the NNRTI class.

The microbicidal potential of NNRTIs derives from their capability, unique among antiretroviral agents, to knock out the HIV multiplication. In this respect, also **12** has some potential: in fact, following a chronic treatment, its KO concentration is comparable to that of TMC120 and MC1220. However, following short-term (4 h) treatments either before or after infection, **12** behaves like TMC120, proving totally ineffective. Under these conditions MC1220 shows a KO concentration of 2 μM, thus proving to be the sole microbicide with some potential for treatments to be started immediately after an infection is believed to have occurred.

Nevertheless, unlike short-lasting gels that must be applied soon before sexual intercourse, vaginal rings might provide continuous and sustained protection that affords coital independence and minimizes the risk of nonadherence to protocols. In conclusion, this work favorably supports the continued development of antiretroviral compounds as topical microbicides against HIV-1 transmission, even though surprisingly few have been reported so far.

EXPERIMENTAL SECTION

General Procedures. NMR spectra were recorded on a Varian Gemini 2000 NMR spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C with TMS as the internal standard. MALDI mass spectra were recorded on a 4.7 T Ultima (IonSpec, Irvine, CA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Melting points were determined on a Büchi melting point apparatus. Elemental analyses were performed at the H.C. Ørsted Institute, University of Copenhagen. Silica gel (0.040−0.063 mm) used for column chromatography and analytical silica gel TLC plates 60 F_{254} were purchased from Merck. Solvents for chromatography were purchased

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as HPLC grade or distilled prior to use. Reactions were in general carried out under an argon atmosphere. CH_3CN was dried over 3 Å molecular sieves. The purity of the synthesized compounds was determined to be ≥95% by elemental analysis (C, H, N) and/or by ^1H NMR. ^1H NMR spectra are included in the Supporting Information.

(3,5-Dimethylphenyl)(5-ethyl-2,6-dimethoxypyrimidin-4-yl)-methanol (2). Using an ice cooling bath, sodium borohydride (170 mg, 4.5 mmol) was added portionwise to a stirred solution of compound **1** (1.2 g, 4 mmol) in methanol (20 mL). The reaction mixture was left to reach room temperature gradually with stirring for 1 h. Acetic acid (1 mL) was added followed by addition of water (40 mL). The solid product formed was filtered off and dried to afford 0.81 g of the pure compound **2**: yield 67%; mp 85−87 °C; ^1H NMR ($CDCl_3$) δ 0.80 (t, 3H, J = 7.5 Hz, CH_3CH_2), 2.27 [s, 6H, $(CH_3)_2Ar$], 2.36−2.43 (m, 2H, CH_3CH_2), 3.99, 4.06 (2 s, 6H, 2 OCH_3), 5.13 (br s, 1H, OH), 5.65 (s, 1H, CHOH), 6.89 (s, 3H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ 12.43 (CH_3CH_2), 17.39 (CH_3CH_2), 21.23 [$(CH_3)_2Ar$], 54.21, 54.73 (2 OCH_3), 71.60 (CHOH), 113.09 (C5), 125.10, 129.61, 138.12, 142.04 (C_{arom}), 162.38 (C6), 166.08 (C2), 170.23 (C4); EI MS m/z 302 (86, M^+), 183 (100); HRMS (MALDI) m/z calcd for $C_{17}H_{22}N_2NaO_3$ (MNa^+) 325.1523, found 325.1512.

4-[(3,5-Dimethylphenyl)fluoromethyl]-5-ethyl-2,6-dimethoxypyrimidine (3). A solution of DAST (0.8 mL, 6 mmol) in methylene chloride (2 mL) was added dropwise at −5 °C to a solution of compound **2** (1.51 g, 5 mmol) in dichloromethane (10 mL) under nitrogen. The solution was stirred and left to reach room temperature gradually during 4 h. The reaction was quenched by addition of 2 mL of a saturated solution of sodium carbonate with stirring. The solvents were evaporated under reduced pressure; water (20 mL) was added to the residual material, the resulting mixture stirred for 1 h, and the precipitate then filtered off and dried. The precipitate was purified by silica gel column chromatography using petroleum ether/ether (PE/E) (2:1, v/v) as an eluent to afford 1.2 g of compound **3** (79%) as an oil: ^1H NMR ($CDCl_3$) δ 0.99 (t, 3H, J = 7.5 Hz, CH_3CH_2), 2.30 [s, 6H, $(CH_3)_2Ar$], 2.51−2.60 (m, 2H, CH_3CH_2), 3.98, 3.99 (2s, 6H, 2OCH_3), 6.44 (d, $J_{H,F}$ = 47.4 Hz, CHF), 6.95 (s, 1H, H_{arom}), 7.05 (s, 2H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ 13.56 (CH_3CH_2), 17.58 (d, J = 2.3 Hz, CH_3CH_2), 21.19 [$(CH_3)_2Ar$], 54.05, 54.51 (2OCH_3), 92.65 (d, J = 177.0 Hz, CHF), 114.77 (C5), 124.00 (d, J = 5.9 Hz, C_{arom}), 130.11 (d, J = 1.5 Hz, C_{arom}), 137.52 (d, J = 21.3 Hz, C_{arom}), 137.90 (C_{arom}), 162.90 (C2), 163.14 (d, J = 21.3 Hz, C4), 170.56 (C6); HRMS (MALDI) m/z calcd for $C_{17}H_{21}FNaN_2O_2$ (MNa^+) 327.1479, found 327.1480.

Synthesis of Compounds 4a,b from Compound 3. Compound **3** (1.1 g, 3.6 mmol) was refluxed in a mixture of 4 M HCl (30 mL) and ethanol (20 mL) for 16 h. The solvents were evaporated under reduced pressure until 10 mL, and the solid product formed was filtered off, washed with water, and dried. TLC showed the existence of two products which were separated by silica gel column chromatography using CH_2Cl_2/EtOAc (3:1, v/v) as the eluent to furnish compounds **4a** and **4b**.

*Data for 6-[(3,5-dimethylphenyl)hydroxymethyl]-5-ethylpyrimidine-2,4(1H,3H)-dione (**4a**):* yield 19%; mp 230−232 °C; ^1H NMR (DMSO-d_6) δ 0.80 (t, 3H, J = 7.3 Hz, CH_3CH_2), 2.23−2.33 [m, 8H, CH_3CH_2, $(CH_3)_2Ar$], 5.62 (s, 1H, CH), 6.25 (br s, 1H, OH), 6.93 (s, 1H, H_{arom}), 7.07 (s, 2H, H_{arom}), 10.01 (s, 1H, NH), 11.03 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 13.14 (CH_3CH_2), 16.97 (CH_3CH_2), 68.68 (CH), 109.63 (C5), 124.05, 129.04, 137.25, 140.72 (C_{arom}), 150.54 (C6), 150.98 (C2), 164.55 (C4); EI MS m/z 274 (100, M^+). Anal. Calcd for $C_{15}H_{18}N_2O_3$ (274.32): C, 65.68; H, 6.61; N, 10.21. Found: C, 65.66; H, 6.53; N, 10.08.

*Data for 6-[(3,5-dimethylphenyl)ethoxymethyl]-5-ethylpyrimidine-2,4(1H,3H)-dione (**4b**):* yield 10%; mp 162−164 °C; ^1H NMR (DMSO-d_6) δ 0.83 (t, 3H, J = 7.3 Hz, CH_3CH_2), 1.21 (t, 3H, J = 7.0 Hz, CH_3CH_2O), 2.27−2.33 (m, 8H, CH_2CH_3 and $(CH_3)_2Ar$), 3.46−3.52 (m, 2H, OCH_2CH_3), 5.40 (s, 1H, CH), 6.96 (s, 1H, H_{arom}), 7.08 (s, 2H, H_{arom}), 10.26 (s, 1H, NH), 11.07 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 13.14 (CH_3CH_2), 14.85 (CH_3CH_2O), 16.92 (CH_3CH_2), 20.87 [$(CH_3)_2Ar$)], 64.34 (CH_3CH_2O), 76.40 (CH), 111.08 (C5), 124.20, 129.39, 137.35, 138.22 (C_{arom}), 148.63 (C4), 150.60 (C2), 164.31 (C6); EI MS m/z 302 (82, M^+), 241 (100). Anal. Calcd for $C_{17}H_{22}N_2O_3$ (302.37): C, 67.53; H, 7.33; N, 9.26. Found: C, 67.52; H, 7.39; N, 9.06.

2-(2,6-Dichloro-5-ethylpyrimidin-4-yl)-2-(3,5-dimethylphenyl)acetonitrile (7). Sodium hydride (3.24 g, 0.135 mol, 55% suspension in paraffin oil) was added portionwise at 0 °C to a solution of 5-ethyl-2,4,6-trichloropyrimidine (9.36 g, 0.045 mol) and 3,5-dimethylbenzyl cyanide (6.53 g, 0.045 mol) in dry DMF (75 mL) using a $CaCl_2$ drying tube. The reaction mixture was stirred and left to reach room temperature gradually for 6 h (TLC; PE/E, 5:1). The mixture was poured into ice-cold water (150 mL) with stirring, and extracted with ether (3 × 50 mL). The ether phases were dried using $MgSO_4$ and evaporated under reduced pressure. The residual material was treated with methanol (20 mL) and left at −5 °C overnight. The solid product formed was filtered off, washed with cold methanol, and dried to afford 11.7 g of compound **7** (81%): mp 108−110 °C. ^1H NMR ($CDCl_3$) δ 1.01 (t, 3H, J = 7.5 Hz, CH_3CH_2), 2.31 [s, 6H, $(CH_3)_2Ar$], 2.75 (q, 2H, J = 7.5 Hz, CH_3CH_2), 5.34 (s, 1H, CHCN), 6.98 (s, 3H, H_{arom}); ^{13}C NMR ($CDCl_3$) δ 11.91 (CH_3CH_2), 21.21 (CH_3CH_2), 21.68 [$(CH_3)_2Ar$], 42.58 (CHCN), 116.94 (CN), 125.43, 130.86, 131.66, 139.45 (C_{arom}), 132.04 (C5), 157.57 (C2), 163.98 (C4), 164.58 (C6).

6-(3,5-Dimethylbenzoyl)-5-ethylpyrimidine-2,4(1*H*,3*H*)-dione (8). Sodium (2.9 g, 0.13 mol) was dissolved in anhydrous methanol (100 mL). Compound **7** (9.7 g, 0.032 mol) was added, and the mixture was refluxed for 20 h. A stream of oxygen was pumped through the solution at room temperature for 2 h (TLC), and the solvent was concentrated to 20 mL under reduced pressure. HCl (4 M) (100 mL) was added, and the reaction mixture was refluxed for 24 h and cooled. The solid product formed was filtered off, washed with water, and dried to afford 7.6 g of compound **5** (86%): mp 266−268 °C (lit.[21] mp 249−250 °C).

Synthesis of 4a from Compound 8. Sodium borohydride (1.36 g, 0.036 mol) was added portionwise at 0 °C to a stirred solution of compound **8** (7.48 g, 0.0285 mol) in methanol (75 mL). The reaction mixture was stirred and left to reach room temperature for 2 h. Water (100 mL) was added to the reaction mixture, and the solid product formed was filtered off, washed with water, and dried to afford 7.6 g of compound **4a** (98%), which was identical to compound **4a** synthesized from compound **3**.

6-[(3,5-Dimethylphenyl)fluoromethyl]-5-ethylpyrimidine-2,4(1*H*,3*H*)-dione (5). A solution of DAST (6 mL, 0.038 mol) in 10 mL of CH_2Cl_2 was added dropwise at −5 °C to a solution of compound **4a** (6.85 g, 0.025 mol.) in dichloromethane (60 mL) under nitrogen. The solution was stirred and left to reach room temperature for 4 h. The reaction was quenched by addition of 10 mL of a saturated solution of sodium carbonate with stirring. The solvents were evaporated under reduced pressure, water (100 mL) was added to the residual material, and the resulting mixture was stirred for 1 h. Filtration afforded 6.8 g (99%) of the crude compound **5**, which can be used directly (TLC) or purified by silica gel column chromatography using CH_2Cl_2/EtOAc (1:1, v/v) as the eluent to afford 5.5 g of compound **5** (82%): yield 82%; mp 168−170 °C; ^1H NMR (DMSO-d_6) δ 0.81 (t, 3H, J = 7.1 Hz, CH_3CH_2), 2.23−2.35 [m, 8H, CH_3CH_2 and $(CH_3)_2Ar$], 6.59 (d, 1H, $J_{H,F}$ = 45.3 Hz, CHF), 7.06 (s, 1H, H_{arom}), 7.12 (s. 2H, H_{arom}), 10.80 (s, 1H, NH), 11.20 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 13.21 (CH_3CH_2), 17.08 (CH_3CH_2), 88.08 (d, J = 174.0 Hz, CH-F), 111.23 (d, J = 2.9 Hz, C5), 124.23 (d, J = 5.6 Hz, C_{arom}), 130.68 (d, J = 2.4 Hz, C_{arom}), 135.64 (d, J = 21.3 Hz, C_{arom}), 137.88 (C_{arom}), 145.58 (d, J = 21.3 Hz, C4), 150.58 (C2), 164.13 (C6); EI MS m/z 276 (100, M^+).

6-[(3,5-Dimethylphenyl)fluoromethyl]-1-(ethoxymethyl)-5-ethylpyrimidine-2,4(1*H*,3*H*)-dione (9a). Compound **5** (0.14 g, 0.5 mmol) was stirred in dry CH_2Cl_2 (10 mL) under N_2, and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (0.27 mL, 1.1 mmol) was added. After complete silylation (clear solution, after 10 min), ethoxymethyl chloride (0.071 mL, 1.5 mmol) was added dropwise to the mixture at room temperature and the resulting mixture stirred for 3 h. The reaction was quenched by addition of 5% aqueous sodium bicarbonate solution (1 mL) with stirring, and then water (10 mL) and methylene

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chloride (10 mL) were added to the reaction mixture. The two layers were separated, and the organic layer was dried using sodium sulfate. The solvent was removed under reduced pressure, and the residual material was purified by silica gel column chromatography using EtOAc/CH_2Cl_2 (1:1, v/v) as the eluent to afford 140 mg of compound **9a**: yield 84%; mp 196−198 °C; ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, J = 7.5 Hz, CH_3CH_2), 1.05 (t, 3H, J = 6.9 Hz, CH_3CH_2O), 2.26 [s, 6H, $(CH_3)_2Ar$], 2.32−2.42 (m, 2H, CH_3CH_2), 3.42−3.58 (m, 2H, CH_3CH_2O), 4.89 (d, J = 11.1 Hz, $OCH_{2a}N$, 5.30 (d, J = 10.8 Hz, $OCH_{2b}N$), 6.71 (d, 1H, J = 47.1 Hz, CHF), 6.86 (s, 2H, H_{arom}), 6.96 (s, 1H, H_{arom}), 9.57 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.65 (CH_3CH_2), 14.83 (CH_3CH_2O), 19.24 (d, J = 4.2 Hz, CH_3CH_2), 21.31 [$(CH_3)_2Ar$], 64.98 (CH_3CH_2O), 73.19 (OCH_2N), 88.43 (d, J = 176.5 Hz, CHF), 118.75 (C5), 123.68 (d, J = 4.8 Hz, C_{arom}), 131.09 (d, J = 2.5 Hz, C_{arom}), 135.00 (d, J = 20.5 Hz, C_{arom}), 138.78 (C_{arom}), 146.59 (d, J = 20.0 Hz, C6), 151.53 (C2), 163.45 (C4); EI MS m/z 334 (3, M^+), 59 (100). Anal. Calcd for $C_{18}H_{23}FN_2O_3 \cdot 0.25H_2O$ (334.39): C, 63.80; H, 6.84; N, 8.27. Found: C, 63.64; H, 6.96; N, 8.27.

Synthesis of Compounds 9b,c. Compound **4a** (140 mg, 0.5 mmol) was stirred in dry CH_3CN (10 mL) under N_2, and BSA (0.27 mL, 1.1 mmol) was added. The mixture became clear after being stirred at room temperature for 10 min. The reaction mixture was cooled to −50 °C, and TMS triflate (0.09 mL, 0.5 mmol) was added followed by dropwise addition of the appropriate acetal (1 mmol). The reaction mixture was stirred at room temperature for 3 h, quenched with an ice-cold saturated solution of $NaHCO_3$ (1 mL), and evaporated under reduced pressure. The residue was extracted with Et_2O (3 × 20 mL), and the combined organic fractions were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was chromatographed on a silica gel column using EtOAc/CH_2Cl_2 (1:1) as the eluent to afford compounds **9b** and **9c**.

*Data for 1-[(allyloxy)methyl]-6-[(3,5-dimethylphenyl)-fluoromethyl]-5-ethylpyrimidine-2,4(1H,3H)-dione (**9b**):* yield 80%; mp 106−108 °C; ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, J = 7.2 Hz, CH_3CH_2), 2.26 [s, 6H, $(CH_3)_2Ar$], 2.35−2.44 (m, 2H, CH_3CH_2), 3.98−4.02 (m, 2H, $OCH_2CH{=}CH_2$), 4.89 (d, 1H, J = 11.1 Hz, $OCH_{2a}N$), 5.07−5.20 (m, 2H, $CH_2{=}CH$), 5.30 (d, 1H, J = 11.1 Hz, $OCH_{2b}N$), 5.66−5.79 (m, 1H, $CH_2{=}CH$), 6.71 (d, 1H, $J_{H,F}$ = 46.8 Hz, CHF), 6.85, (s, 2H, H_{arom}), 6.96 (s, 1H, H_{arom}), 9.28 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.74 (d, J = 2.4 Hz, CH_3CH_2), 19.21 (d, J = 4.2 Hz, CH_3CH_2), 70.55 ($OCH_2C{=}C$), 72.98 (NCH_2O), 88.45 (d, J = 177.0 Hz, CHF), 117.73 ($CH_2{=}CH$), 118.82 (C5), 123.65 (d, J = 4.9 Hz), 131.16 (d, J = 2.0 Hz, C_{arom}), 133.44 (C_{arom}), 134.89 (d, J = 20.8 Hz, C_{arom}), 138.87 ($CH_2{=}CH$), 146.44 (d, J = 19.3 Hz, C6), 151.45 (C2), 163.24 (C4); HRMS (MALDI) m/z calcd for $C_{19}H_{23}FNaN_2O_3$ (MNa^+) 369.1585, found 369.1583. Anal. Calcd for $C_{19}H_{23}FN_2O_3$ (346.4): C, 65.88; H, 6.69; N, 8.09. Found: C, 65.72; H, 6.75; N, 7.97.

*Data for 6-[(3,5-dimethylphenyl)fluoromethyl]-5-ethyl-1-[[(2-methylallyl)oxy]methyl]pyrimidine-2,4(1H,3H)-dione (**9c**):* yield 83%; mp 136−138 °C; ^{1}H NMR ($CDCl_3$) δ 1.04 (t, 3H, J = 9.8 Hz, CH_3CH_2), 1.68 (s, 3H, $CH_3C{=}C$), 2.33 [s, 6H, $(CH_3)_2Ar$], 2.42−2.51 (m, 2H, CH_3CH_2), 3.99 (s, 2H, $OCH_2C{=}C$), 4.86, 4.92 (2 s, 2H, $CH_2{=}C$), 4.94 (d, 1H, J = 12.0 Hz, $OCH_{2a}N$), 5.40 (d, 1H, J = 10.8 Hz, $OCH_{2b}N$), 6.81 (d, 1H, $J_{H,F}$ = 47.1 Hz, CHF), 6.93 (s, 2H, H_{arom}), 7.03 (s, 1H, H_{arom}), 9.53 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.69 (d, J = 2.1 Hz, CH_3CH_2), 19.19 (d, J = 3.8 Hz, CH_3CH_2), 19.33 ($CH_3C{=}C$), 21.31 [$(CH_3)_2Ar$], 73.09 ($OCH_2C{=}C$), 73.58 (OCH_2N), 88.42 (d, J = 176.8 Hz, CHF), 112.57 ($CH_2{=}C$), 118.81 (C5), 123.64 (d, J = 4.7 Hz, C_{arom}), 131.14 (d, J = 2.1 Hz, C_{arom}), 134.92 (d, J = 20.3 Hz, C_{arom}), 141.08 (C_{arom}), 138.85 ($CH_2{=}C$), 146.48 (d, J = 19.7 Hz, C6), 151.47 (C2), 163.37 (C4); HRMS (MALDI) m/z calcd for $C_{20}H_{25}FNaN_2O_3$ (MNa^+) 383.1741, found 383.1751. Anal. Calcd for $C_{20}H_{25}FN_2O_3$ (360.42): C, 66.65; H, 6.99; N, 7.77. Found: C, 66,68; H, 7.04; N, 7.65.

Bis[(4-iodobenzyl)oxy]methane (10). To a solution of 4-idodobenzyl alcohol (9.4 g, 0.04 mol) and methylene bromide (1.4 mL, 0.02 mol) in dry DMF (25 mL) in a $CaCl_2$ drying tube was added portionwise sodium hydride (1.92 g of 55% suspension in paraffin oil, 0.044 mol) at 0 °C. The mixture was stirred and left to reach room temperature for 3 h. The reaction mixture was poured into ice-cold water with stirring. The solid product formed was filtered off, washed with water, and dried to give 9.26 g of the acetal **10**: yield 96%; mp 78−80 °C (lit.[26] mp 78−80 °C).

Synthesis of Compounds 11a,b. BSA (9.7 mL, 0.04 mol) was added to a stirred solution of compound **4** (5g, 0.018 mol) in dry CH_3CN (100 mL) under nitrogen after 15 min. The mixture was cooled to −50 °C. TMS triflate (9.8 mL, 0.054 mol) was added to the reaction mixture followed by the addition of the acetal **10** (14 g, 0.03 mol). The reaction was stirred and left to reach room temperature overnight. The reaction was quenched by addition of 10 mL of a saturated aqueous solution of sodium carbonate. The solvents were removed under reduced pressure. Then water (30 mL) and methylene chloride (30 mL) were added to the residual material, and the two layers were separated. The CH_2Cl_2 layer was dried over magnesium sulfate and evaporated under reduced pressure. The residual material was chromatographed on a silica gel column using CH_2Cl_2/EtOAc (10:1, v/v) as the eluent to afford compounds **11a** and **11b**.

*Data for 6-[(3,5-dimethylphenyl)fluoromethyl]-5-ethyl-1-[[(4-iodobenzyl)oxy]methyl]pyrimidine-2,4(1H,3H)-dione (**11a**):* yield 42%; semisolid; ^{1}H NMR ($CDCl_3$) δ 1.05 (t, 3H, J = 7.2 Hz, CH_3CH_2), 2.45 [s, 6H, $(CH_3)_2Ar$], 2.46 (q, 2H, J = 7.2 Hz, CH_3CH_2), 4.53 (s, 2H, CH_2Ar), 5.00 (d, J = 10.8 Hz, $OCH_{2a}N$), 5.38 (d, J = 10.8 Hz, $OCH_{2b}N$), 6.77 (d, $J_{H,F}$ = 46.8 Hz, CHF), 6.88 (s, 2H, H_{arom}), 6.98 (d, 2H, J = 8.1 Hz, H_{arom}), 7.01 (s, 1H, H_{arom}), 7.62 (d, 2H, J = 8.1, H_{arom}), 9.38 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.79 (CH_3CH_2), 19.19 (CH_3CH_2), 21.33 [$(CH_3)_2Ar$], 70.92 (CH_2Ar), 73.18 (NCH_2O), 88.39 (d, J = 177.6 Hz, CHF), 118.90 (C5), 123.51 (d, J = 4.8 Hz, C_{arom}), 131.19 (d, J = 1.9 Hz, C_{arom}), 134.76 (d, J = 20.7 Hz, C_{arom}), 93.42, 129.50, 137.44, 137.55, 138.93 (C_{arom}), 146.19 (d, J = 13.6 Hz, C6), 151.48 (C2), 163.12 (C4); HRMS (MALDI) m/z calcd for $C_{23}H_{24}FIN_2NaO_3$ (MNa^+) 545.0708, found 545.0681.

*Data for 6-[(3,5-dimethylphenyl)fluoromethyl]-5-ethyl-3-[[(4-iodobenzyl)oxy]methyl]pyrimidine-2,4(1H,3H)-dione (**11b**):* yield 16%; mp 114−116 °C; ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H, J = 7.4 Hz, CH_3CH_2), 2.29−2.36 [m, 8H, CH_3CH_2 and $(CH_3)_2Ar$], 4.64 (s, 2H, CH_2Ar), 5.45 (s, 2H, NCH_2), 6.35 (d, J_{HF} = 46.4 Hz, CHF), 6.98 (s, 2H, H_{arom}), 7.06 (s, 1H, H_{arom}), 7.10 (d, 2H, J = 8.0 Hz, H_{arom}), 7.61 (d, 2H, J = 8.0, H_{arom}), 8.44 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 12.99 (CH_3CH_2), 18.30 (CH_3CH_2), 21.26 [$(CH_3)_2Ar$], 70.21 (CH_2Ar), 71.62 (NCH_2O), 88.11 (d, J = 176.8 Hz, CHF), 112.66 (C5), 124.36 (J = 5.7 Hz, C_{arom}), 134.46 (d, J = 21.1 Hz, C_{arom}), 131.95 (d, J = 2.9 Hz, C_{arom}), 93.14, 129.47, 137.33, 137.58, 139.133(C_{arom}), 143.53 (d, J = 20.4 Hz, C6), 150.98 (C2), 163.30 (C4); HRMS (MALDI) m/z calcd for $C_{23}H_{24}FIN_2NaO_3$ (MNa^+) 545.0708, found 545.0681.

6-[(3,5-Dimethylphenyl)fluoromethyl]-5-ethyl-1-{[[4-(3-hydroxyprop-1-ynyl)benzyl]oxy]methyl}pyrimidine-2,4(1*H*,3*H*)-dione (12). By a balloon with a long needle immersed in the solution, a stream of argon was flushed through a solution of compound **11a** (2.15 g, 0.004 mol) and propagyl alcohol (2.3 mL, 0.04 mol) in dry triethylamine (30 mL) for 0.5 h. Then the reaction mixture was transferred by a syringe with a long needle to another flask containing copper(I) iodide (45 mg, 5% mol) and $PdCl_2(PPh_3)_3$ (85 mg, 3% mol) under argon. The reaction mixture was stirred under argon for 3 h. The triethylamine was evaporated under reduced pressure and coevaporated with chloroform (3 × 30 mL). The residual material was purified by silica gel column chromatography using CH_2Cl_2/MeOH (10:1, v/v) to afford 1.3 g of compound **12** (72%): mp 110−112 °C; ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, J = 7.2 Hz, CH_3CH_2), 2.23 [s, 6H, $(CH_3)_2Ar$], 2.40 (q, 2H, J = 7.2 Hz, CH_3CH_2), 4.42 (s, 2H, CH_2OH), 4.52 (s, 2H, CH_2Ar), 4.94 (d, 1H, J = 11.1 Hz, $OCH_{2a}N$), 5.35 (d, 1H, J = 11.1 Hz, $OCH_{2b}N$), 6.71 (d, 1H, J = 46.8 Hz, CHF), 6.82 (s, 2H, H_{arom}), 6.94 (s, 1H, H_{arom}), 7.11 (d, 2H, J = 8.1 Hz, H_{arom}), 7.30 (d, 2H, J = 8.1 Hz, H_{arom}), 9.36 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.79 (CH_3CH_2), 19.18 (CH_3CH_2), 21.30 [$(CH_3)_2Ar$], 51.55 (CH_2OH), 71.19 (CH_2Ar), 73.24 (NCH_2O), 85.34 ($C{\equiv}CCH_2$), 87.48 ($C{\equiv}CCH_2$), 88.40 (d, J = 178.1 Hz, CH_2F), 118.89 (C5), 123.54 (J = 4.8 Hz, C_{arom}), 131.19 (d, J = 2.1 Hz, C_{arom}), 134.75 (d, J = 20.7 Hz, C_{arom}), 122.05, 127.47, 131.66, 137.54, 138.93 (C_{arom}), 146.17

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(d, J = 19.5 Hz, C6), 151.47 (C2), 163.19 (C4); HRMS (MALDI) m/z calcd for $C_{26}H_{27}FN_2NaO_4$ (MNa^+) 473.1847, found 473.1855.

3-{4-[((6-((3,5-Dimethylphenyl)fluoromethyl)-5-ethyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methoxy)methyl]-phenyl}propiolaldehyde (13). A mixture of compound **12** (100 mg, 0.22 mmol) and IBX (186 mg, 0.66 mmol) in ethyl acetate (3 mL) was heated at 80 °C for 12 h. The reaction mixture was left to reach room temperature and then evaporated under reduced pressure. The crude material was purified by silica gel column chromatography using $EtOAc/CH_2Cl_2$ (1:1, v/v) as the eluent to afford 70 mg of compound **13**: yield 70%; semisolid; ^1H NMR ($CDCl_3$) δ 1.06 (t, 3H, J = 7.4 Hz, CH_3CH_2), 2.30 [s, 6H, $(CH_3)_2Ar$], 2.49 (q, 2H, J = 7.4 Hz, CH_3CH_2), 4.63 (s, 2H, CH_2Ar), 5.07 (d, 1H, J = 11.1 Hz, $OCH_{2a}N$), 5.41 (d, 1H, J = 11.1 Hz, $OCH_{2b}N$), 6.49 (d, 1H, J = 46.8 Hz, CHF), 6.90 (s, 2H, H_{arom}), 7.01 (s, 1H, H_{arom}), 7.26 (s, 2H, J = 8.3 Hz, H_{arom}), 7.54 (d, 2H, J = 8.3 Hz, H_{arom}), 9.41 (s, 1H, CHO), 9.52 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 13.80 (CH_3CH_2), 19.16 (CH_3CH_2), 21.31 [$(CH_3)_2Ar$], 70.88 (CH_2Ar), 73.35 (NCH_2O), 88.38 (d, J = 178.4 Hz, CHF), 88.50 (C≡CCHO), 94.87 (C≡CCHO), 118.68, 119.02, 127.53, 131.19, 133.27, 138.97, 140.91 (C_{arom}), 134.77 (d, J = 20.1 Hz, C_{arom}), 123.39 (d, J = 5.1 Hz, C5), 145.97 (d, J = 19.2 Hz, C6), 151.59 (C2), 163.13 (C4), 176.72 (CHO); HRMS (MALDI) m/z calcd for $C_{26}H_{25}FN_2NaO_4$ (MNa^+) 471.1691, found 471.1668.

Antiviral Assay Procedures. The compounds were solubilized in DMSO at 100 μM and then diluted in culture medium.

Virus and Cells. MT-4, C8166, and H9/IIIB cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect kit (Gibco). Human immunodeficiency virus type 1 (HIV-1_{IIIB} strain) was obtained from supernatants of persistently infected H9/IIIB cells. The HIV-1 stock solutions had titers of 4.5×10^6 $CCID_{50}$/mL. The K103R + V179D + P225H mutant ($DMP266^R$) was derived from an HIV-1_{IIIB} strain passaged in MT-4 cells in the presence of increasing efavirenz concentrations (up to 2 μM). The Y181C mutant (NIH N119) was derived from an AZT-sensitive clinical isolate passaged initially in CEM and then in MT-4 cells in the presence of nevirapine (10 μM). The double mutant K103N + Y181C (NIH A17) was derived from the HIV-1_{IIIB} strain passaged in H9 cells in the presence of BI-RG 587 (1 μM). EFV^R, N119, and N117 stock solutions had titers of 4.0×10^7, 1.2×10^8, and 2.1×10^7 $CCID_{50}$/mL, respectively.

HIV Titration. Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope scoring of syncytia after a 4 day incubation. Virus titers were expressed as $CCID_{50}$ per milliliter.

Short-Term Cytotoxicity Assays. Cytotoxicity assays were carried out in parallel with antiviral assays. Exponentially growing cells derived from human hematological tumors [CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4)] were seeded at an initial density of 1×10^5 cells/mL in 96-well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of the test compounds. Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[47]

Long-Term Cytotoxicity Assays. MT-4 cells, resuspended in growth medium at a density of 1×10^5 cells/mL, were incubated at 37 °C in the absence or presence of various concentrations of the test compounds, alone or in combination. One-tenth of the culture, in the absence or presence of the same concentrations of the test compounds, was transplanted every 3–4 days into new flasks at low cell density to allow continuous exponential growth. Cell viability was determined at each passage with the MTT method.

Anti-HIV Assays. The activity of the test compounds against the multiplication of wt HIV-1 and EFV^R, N119, and N117 variants in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, 50 μL of culture medium containing 1×10^4 cells was added to each well of flat-bottom microtiter trays containing 50 μL of culture medium without or with serial concentrations of the test compounds. Then 20 μL of HIV suspensions (containing the appropriate amount of $CCID_{50}$ to cause complete cytopathogenicity at day 4) was added. After incubation at 37 °C, the cell viability was determined by the MTT method. The activity of the test compounds against clinical isolates was evaluated in ex vivo dendritic cells using the end point determination of p24 antigen (PerkinElmer).

Selection of Drug-Resistant Mutants. Drug-resistant variants were selected by serial passages of HIV-1_{IIIB} in the presence of stepwise doubling drug concentrations, starting from a cell culture infected with an MOI of 0.01 and treated with a drug concentration equal to the EC_{50}. Usually, the amount of virus obtained after each passage was sufficient to determine the infection of the next cell culture which, after infection and washing, was incubated with a double amount of the selecting drug. Drug-resistant viruses were selected up to drug concentrations 128-fold greater than the EC_{50}. Resistant virus preparations were subjected to RNA extraction, RT-PCR, and genome sequencing to identify the mutation patterns responsible for resistance.

Molecular Analysis of Resistant Viruses. Viral RNAs from the wt and drug-resistant mutants were obtained using the QIAamp viral RNA minikit (Qiagen), starting from 140 μL of cell-free viral suspensions containing about 5×10^5 $CCID_{50}$/mL, to determine the nucleotide sequence of the *pol* region of the HIV-1 genome.

Reverse transcription was carried out according to the manufacturer's protocol using the Superscript II enzyme (Invitrogen) and outR2 (5′- CTTACCTCTTATGCTTGTGCTGATATTGAAAGA-3′) as the primer. cDNAs were amplified by four PCRs using Platinum Pfx polymerase (Invitrogen) and using the following primers:

- INF (5′-TGAAAGATTGTACTGAGAGACAGG-3′)/MR3 (5′-CCCTTCCTTTTCCATTTCTG-3′)
- MF1 (5′-GACCTACACCTGTCAACATA-3′)/INR (5′-TCTATTCCTACTAAAAATAGTATTTTCCTGATTCC-3′)
- MF3 (5′-ATATGCAAGAATGAGGGGTG-3′)/IR3 (5′-GTTCAGCCTGATCTCTTACCTGTC-3′);
- IF2 (5′-GCGGGAATCAAGCAGGAATTTGGA-3′)/IR1 (5′-TCGTAACACTAGGCAAAGGTGGCT-3′).

PCR fragments contained 664, 1762, 1133, and 917 bp, respectively. All PCR amplifications consisted of an initial denaturation of 3 min, 34 cycles of denaturation at 94 °C for 30 s, annealing at 51.5 °C for 30 s, extension at 68 °C for 1.5 min, and final extension at 68 °C for 5 min.

PCR fragments were purified using the QIAquick PCR purification kit (Qiagen) and analyzed using the cycle-sequencing method (CIBIACI service of the University of Firenze). Both DNA strands were sequenced with specific primers. Comparative analysis of the chromatograms allowed the identification of the mutations responsible for resistance to the various drugs.

Long-Term Assays. In vitro long-term assays were carried out as previously described.[41] The KO concentrations of the test compounds were determined under conditions of chronic treatments, treatments limited to the first 4 h pi, or treatments limited to 4 h before infection (bi).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR spectra of the compounds studied. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Y.M.L. and E.B.P. contributed to chemistry and R.L., G.S., G.G., G.C., and P.L.C. to virology.

Notes

P.L.C. is a named inventor on granted patents for MC1220 (U.S. Patents 6.635.636 and 6.545.007).

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■ ABBREVIATIONS USED

BSA, *N*,*O*-bis(trimethylsilyl)acetamide; DAST, (diethylamino)-sulfur trifluoride; DMF, *N*,*N*-dimethylformamide; EFV, efavirenz, HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; IBX, 2-iodobenzoic acid; NEV, nevirapine; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; SQV, saquinavir; TLC, thin-layer chromatography; TMS, trifluomethanesulfonate

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