Characterisation of novel anti-HIV/tuberculosis natural product analogues

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KEY MESSAGES

1. 10-chloromethyl-11-demethly-12-oxo-calanolide A (F18) is an effective antiretroviral compound *in vitro*.
2. F18 remains effective against HIV-1 strains that contain various mutations rendering them resistant to non-nucleoside reverse-transcriptase inhibitors.

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Introduction

Non-nucleoside reverse-transcriptase inhibitors (NNRTIs) are one of the key components of antiretroviral drug regimens against human immunodeficiency virus type 1 (HIV-1) replication. (+)-calanolide A, which is purified from the rainforest plant *Calophyllum*, is an anti-HIV-1/tuberculosis drug. In our previous study, a library of (+)-calanolide A was constructed and one novel compound 10-chloromethyl-11- demethly-12-oxo-calanolide A (F18) with improved antiviral activity was identified. This study investigated antiviral breadth, drug-resistance profile, and underlying mechanism of action of F18.

Methods and results

This study was conducted from March 2010 to February 2012.

In vitro selection of F18-resistant virus

HTLV-1 transformed human T-cell leukaemia (MT-2) cells were infected with 10^4 $TCID_{50}$ $HIV\text{-}1_{NL4\text{-}3}$ wild-type virus and cultured in 2-fold increasing concentrations of F18 ranging from 0.14 nM to 20 μM for 120 days. Virus replication was monitored based on syncytium formation. Ten resistant strains were selected (Table 1). Genotypic analysis showed that seven of ten variants contained a single mutation of amino acid Leu to Ile at position 100 in the RT gene. In addition, mutations of V292I, K103N, Y188H, V106I, T139R, and P225H also emerged in three other strains following F18 treatment.

To confirm their resistance and cross-resistance profile, these ten variants were assessed in MT-2 cells in the presence of F18, nevirapine (NVP), efavirenz (EFV), and etravirine (ETR). All mutant viruses were resistant to F18 (Table 1). The mutant viruses with mutations L100I or Y188H retained high sensitivity to the other three NNRTIs. K103N-containing virus resulted in more than a 350-fold change in EC_{50} to EFV and NVP, but was sensitive to ETR, whereas the HIV-1 variant with V106I/WT and T139R showed moderate resistance to EFV, NVP, and ETR. Therefore, F18-resistant HIV-1 mutations occurred in *in vitro* long-term culture of MT-2 cells infected with wild-type virus in the presence of F18, and these mutations conferred some cross-resistance to other NNRTIs.

Antiviral activity of F18 against NNRTI-resistant pseudovirus

To confirm the relevance of these *in vitro*-induced resistant mutations, a panel of seven NNRTI-resistant site-directed mutagenesis (SDM)-generated pseudoviruses for a GHOST (3)-CCR5 cell-based assay was established. Mutations that were induced by *in vitro* F18 selection were engineered into a clean $HIV\text{-}1_{NL4\text{-}3R\text{-}E\text{-}Luc}$+ pseudovirus backbone by SDM. Sensitivity of these mutant viruses was determined by infection with GHOST (3)-CCR5 cells in the presence of F18, NVP or (+)-calanolide A, and by measuring the degree of inhibition compared with infection with a wild-type virus (Fig 1a). Virus constructed with the minor Y188H mutation displayed a >100-fold higher level of resistance to F18 or (+)-calanolide A, compared with NVP, whereas virus containing the dominant

TABLE 1. Selection scheme and genotypic analysis of the reverse transcriptase of mutant HIV-1 strains that emerged under dose-escalating treatment of HIV-1_{NL4-3} wild-type virus with F18

Strain No. (No. of days in cell culture)	F18 concentration at which the selection initiated and the resistant strain selected	Mutation(s) in HIV-1 RT*	Fold change†			
			F18	NVP	EFV	ETR
1,4,6,7,8,9,10						
0	0.14 nM					
84	312 nM	L100I				
128	20 μM	**L100I**	>128	1.67	2.05	1.37
2						
0	0.14 nM					
91	625 nM	Y188H/WT				
105	2.5 μM	Y188H				
128	20 μM	Y188H	>128	3.75	0.004	0.34
3						
0	0.14 nM					
98	1.25 μM	T139R/WT				
120	10 μM	V106I/WT, T139R				
128	20 μM	V106I/WT, T139R	121	36.5	18.06	20.15
5						
0	0.28 nM					
91	1.25 μM	P225H				
98	2.5 μM	K103N/WT, P225H/WT				
105	5 μM	K103N				
112	10 μM	K103N, P225H/WT				
120	20 μM	K103N, P225H/WT, V292I	>128	>357	568.45	2.79

* The complete protease and reverse transcriptase genes were sequenced. The predominant mutation is underlined and in bold

† Calculated as the ratio between the EC_{50} of the compound for the mutant strain and the HIV-1_{NL4-3} wild-type strain obtained in the same experiment at the end of culture. EC_{50} of F18, NVP, EFV, and ETR against HIV-1_{NL4-3} wild-type virus is 41.0±2.0 nM, 42.0±1.7 nM, 0.1±0.003 nM, and 0.1±0.002 nM, respectively. Each result is the mean for a single experiment conducted in triplicate

mutation (7 of out 10 strains) L100I induced by F18 *in vitro* resulted in a 30- and 10-fold change in EC_{50} to F18 and (+)-calanolide A, respectively (Table 1). NVP susceptibility was noted with L100I mutant viruses. The novel mutation T139R found by F18 selection *in vitro* showed cross-resistance to all three compounds NVP, F18, and (+)-calanolide A. To a lesser extent, viruses with K101E and K103N mutations also displayed cross-resistance to F18, NVP, and (+)-calanolide A. Interestingly, virus with double mutations K103N and P225H exhibited high resistance to NVP with more than a 100-fold change in EC_{50}, but resistance to F18 and (+)-calanolide A was abolished when compared with the single mutation K103N.

As cross-resistant mutations may exist among various NNRTIs, susceptibility of ten additional HIV-1 NNRTI-resistant SDMs to F18, NVP, and (+)-calanolide A, alone or in combination, was determined (Fig 1b). These mutations were previously reported based on the current NNRTIs in clinical use. One of the most prevalent mutations among antiretrovial-therapy-experienced patients was Y181C, and virus with this mutation was sensitive to F18 with an EC_{50} of 1.0 nM and a reduced susceptibility to NVP for more than a 100-fold difference in EC_{50}. Double mutant with K103N/Y181C, Y188L and V106A/G190A/F227L mutants were cross-resistant to the antiviral effects of the three NNRTIs.

Antiviral activity of F18 in combination with some US-FDA-approved antiretrovirals

Given the unique antiviral features of F18, its efficacy when used in combination with each of eight US-FDA-approved drugs using PBMC assays was determined. There were no antagonistic effects for any combinations of F18 plus another drug against HIV-1_{NL4-3} infected PBMCs (Table 2). Most combinations showed a slightly or highly synergistic effect as calculated by the Prichard and Shipman MacSynergy II software.

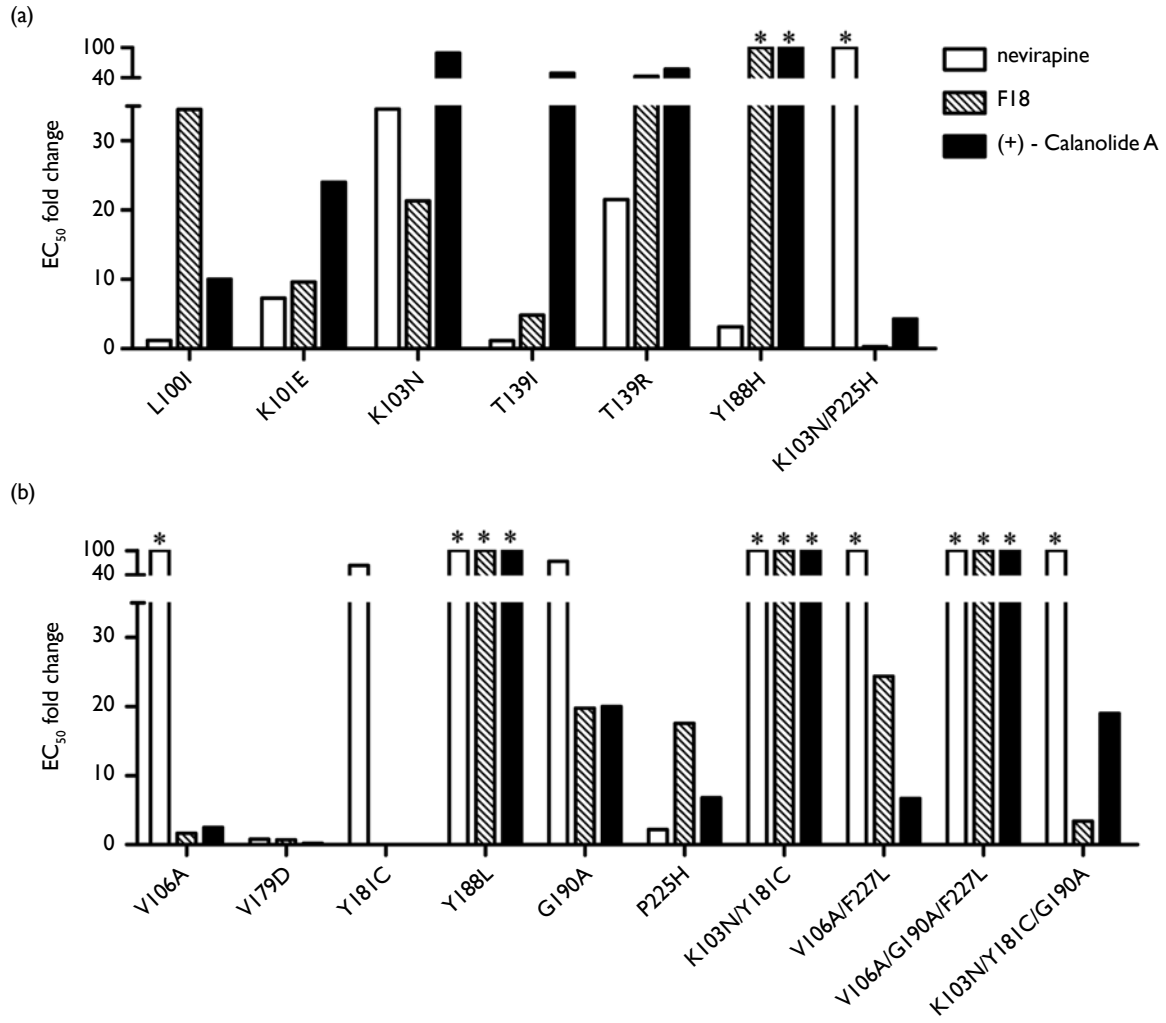

FIG. Resistance of constructed HIV_{NL4-3} SDMs to different non-nucleoside reverse-transcriptase inhibitors (NNRTIs): GHOST (3)-CCR5 cells were infected in the presence of NVP, F18, or (+)-calanolide A with NNRTI mutant pseudoviruses constructed by SDM for (a) mutations identified from Table 1, (b) mutations with resistance to different classes of antiretroviral drugs. The average EC_{50} fold changes are shown for triplicate experiments.
* EC_{50} is not reached at the concentration tested

Discussion

One of the major obstacles to the development of NNRTIs is the extensive cross-resistance within this class of antiretrovirals. F18 displayed a unique profile of cross-resistance. In contrast to NVP, F18 remained effective against HIV-1 SDMs containing NNTRI-resistant mutations T139I, Y181C, V179D, V106A, K103N/P225H, and K103N/Y181C/G190A. From our F18-resistance induction study, F18 did not readily induce NVP-resistant or (+)-calanolide A-resistant viruses, but the resultant dominant L100I mutation attributed to F18-resistance, which is similar to another (+)-calanolide A stereoisomer–dihydrocostatolide.[1] This finding suggests that F18 may function more similarly to dihydrocostatolide than (+)-calanolide A. In addition, due to structural similarity, F18 and (+)-calanolide A shared a similar profile against most NNRTI-cross resistant viruses (Fig). These two drugs also displayed their unique characteristics. For example, F18 was a potent inhibitor against (+)-calanolide A-induced T139I mutant. Conversely, (+)-calanolide A inhibited F18-induced L100I mutant more effectively. These results suggest that the major binding moiety of F18 engages HIV-1 RT at a different binding motif towards L100 when compared with T139 usage of (+)-calanolide A. In addition, most of the resistant viruses including L100I selected by F18 can be inhibited by NVP, EFV, and ETR, indicating a low level of cross-resistance between F18 and these three

TABLE 2. Antiviral effect of F18 in combination with eight US-FDA-approved antiretroviral compounds against HIV-1_{NL4-3} in PBMC assays

Drug	Mean synergy volume*	Antiviral effect
Nucleoside reverse transcriptase inhibitors		
Zidovudine	87.62	Slightly synergistic
Lamivudine	17.32	Additive
Stavudine	27.71	Additive
Didanosine	297.47	Highly synergistic
Non-nucleoside reverse transcriptase inhibitors		
Nevirapine	73.1	Slightly synergistic
Efavirenz	4.01	Additive
Protease inhibitors		
Nelfinavir	116.85	Highly synergistic
Integrase inhibitors		
Raltegravir	54.12	Slightly synergistic

* Positive values represent the synergistic interaction between F18 and other antiretroviral drugs

FDA-approved NNRTIs (Table 1). We conclude that the drug-resistance profile of F18 is distinctly different from that of other NNRTIs,[2-4] possibly due to a distinct binding motif of F18 to HIV-1 RT that differs to other NNRTIs.

Since the discovery of NNRTIs, combination therapy of at least three antiretrovirals has become the gold standard for clinical treatment of HIV-1 patients in the last 20 years. To avoid issues of drug-drug interaction, we tested the combined anti-HIV activity of F18 with each of eight commonly used antiretrovirals. As a natural product-derived small molecule, F18 had no antagonistic effect when used in two-drug combination against both wild-type and drug-resistant viruses (Table 2). Interestingly, the lack of antagonistic effects between F18 and NVP or EFV provided further evidence that F18 does not share identical binding motifs to HIV-1 RT with either NNRTI.

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