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Bioorganic & Medicinal Chemistry 12 (2004) 6437–6450
Bioorganic & Medicinal Chemistry

Optimization of unique, uncharged thioesters as inhibitors of HIV replication

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Received 9 July 2004; revised 16 September 2004; accepted 16 September 2004
Available online 6 October 2004

Abstract—A combinatorial chemistry approach was employed to prepare a restricted library of N-substituted *S*-acyl-2-mercaptobenzamide thioesters. It was shown that many members of this chemotype display anti-HIV activity via their ability to interact with HIV-1, HIV-2, SIV-infected cells, cell-free virus, and chronically and latently infected cells in a manner consistent with targeting of the highly conserved HIV-1 NCp7 zinc fingers. Compounds were initially screened using two different in vitro antiviral assays and evaluated for stability in neutral buffer containing 10% pooled human serum using a spectrophotometric assay. These data revealed that there was no significant correlation between thioester stability and antiviral activity, however, a slight inverse correlation between serum stability and *virucidal* activity was noted. Based on the virucidal capability and the ability to select lead compounds to inhibit virus expression from latently infected TNFα-induced U1 cells, we next determined if these compounds could prevent HIV cell-to-cell transmission. Several thioesters demonstrated potent inhibition of HIV cell-to-cell transmission with EC_{50} values in the 80–100 nM range. Thus, we have optimized a series of restricted thioesters and provided evidence that serum stability is not required for antiviral activity. Moreover, selected compounds show potential for development as topical microbicides.

1. Introduction

An estimated 42 million people[1,2] worldwide are infected with the human immunodeficiency virus (HIV), and total infections could exceed 70 million by the year 2010. Development and optimization of therapeutic anti-HIV strategies continue to be an essential element in controlling this pandemic. Significant progress has been achieved in the treatment of people infected with the HIV virus through the introduction of highly active antiretroviral therapy (HAART) and other combination therapies consisting of nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs),[3,4] and protease inhibitors.[5,6] Unfortunately, issues of patient compliance, evolving novel toxicities, emergence of multi-drug resistance, and the persistence of virus in tissue reservoirs despite prolonged suppression of replication negatively impacts the progress made in dealing with the HIV/AIDS pandemic. Therefore, despite our continued success at controlling virus replication, there is still the need to identify and develop new antiviral targets.

The p7 nucleocapsid protein (NCp7) of HIV-1 is an excellent target for the development of new antiviral agents, based on its conservation and broad range of function in virus replication. The highly conserved NCp7 protein of HIV contains two copies of the zinc finger motif, $Cys(X)_2Cys(X)_4His(X)_4Cys$ (CCHC).[7–10] NCp7 plays pivotal roles during both early[3,4] and late phases[5] of HIV-1 replication, being required for the functioning of the reverse transcriptase,[4a] integrase,[4b] and protease enzymes.[5] Intact Zn fingers are also critical for the selection and packaging of the RNA genome into

Keywords: Thioester; HIV NCp7; Zinc finger.
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doi:10.1016/j.bmc.2004.09.032

maturing virions.[11] Mutations in the conserved Zn chelating and/or nonchelating residues have been shown to result in loss of nucleocapsid-mediated functions, rendering viruses such as MuLV[12,13] and HIV-1[14,15] noninfectious. Thus, the central role and absolute requirement for an intact nucleocapsid protein with its Zn fingers, during HIV replication, makes this protein an attractive target for drug development.

Diverse sets of electrophilic compounds that react with cysteine thiolates in the NCp7 protein or NCp7 protein precursors ($Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$) have been identified. Although different in chemical composition, all lead molecules result in the ejection of Zn^{2+} ions bound within the structural Zn finger motifs of the NC protein. Described NCp7 inhibitors, such as 3-nitrosobenzamide (NOBA),[16] 2,2′-dithiobisbenzamides (DIBAs),[17–19] and their benzisothiazolone derivatives (BITA),[20] cyclic 2,2′-dithiobisbenzamides (SRR-SB3),[21] 1,2-dithiane 4,5-diol-1,1-dioxide (dithiane),[22] and azodicarbonamide (ADA)[23] inhibited the replication of all laboratory and clinical isolates of HIV-1, HIV-2, and SIV tested. ADA and selected DIBA chemotypes were shown to be selective for the NCp7 protein Zn finger over cellular Zn fingers, while NOBA was more promiscuous.[24] ADA was later advanced to Phase-I clinical trials, and shown to be moderately active (0.5–1.0 log reduction in virus load) in late stage (CD4 counts <50), highly treatment experienced patients.[23] However, despite this interesting array of Zn finger-reactive chemotypes and the development done on the individual compounds, there is still significant work required to ultimately identify a successful NCp7 inhibitor.

To accomplish our goal of developing drugable Zn finger inhibitors, further development of the DIBA chemotype was performed, leading us to the pyridinioalkanoyl thioesters (PATEs).[25] These compounds replaced the disulfide character of the inhibitor with a thiol blocked by a pyridinioalkanoyl group. Although PATES inhibited HIV replication in both acutely and chronically HIV-infected cell models, a significant improvement in antiviral activity was not obtained (50% inhibitory concentration 1–5 μM) over other Zn finger inhibitor chemotypes; however, reduced in vitro cytotoxicity, improved solubility, and lack of susceptibility to glutathione reduction represented significant improvements in this inhibitor class. The thioesters retained the NCp7 as their antiviral target as demonstrated by their virucidal activity to cell-free virions, ejection of Zn^{2+} from purified NCp7 protein, and cross-linking NCp7 and its precursors in infected cells via disulfide bonds.[25] Further, molecular modeling indicated that these thioesters associate with the NCp7 protein in a manner consistent with interaction with Cys49[26] in the C-terminal zinc finger loop to mediate displacement of the coordinated Zn^{2+}. The PATEs, however, still were not optimal drug candidates, since many congeners were hygroscopic oils and were difficult to purify. In an attempt to correct these deficiencies, identify new potential Zn finger-reactive chemotypes, and increase antiviral potency, we explored other acylthiols such as thiolcarbamates and thiolcarbonates.[27] These compounds were synthesized and some

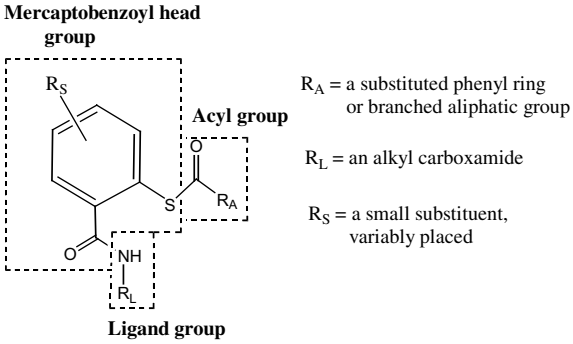


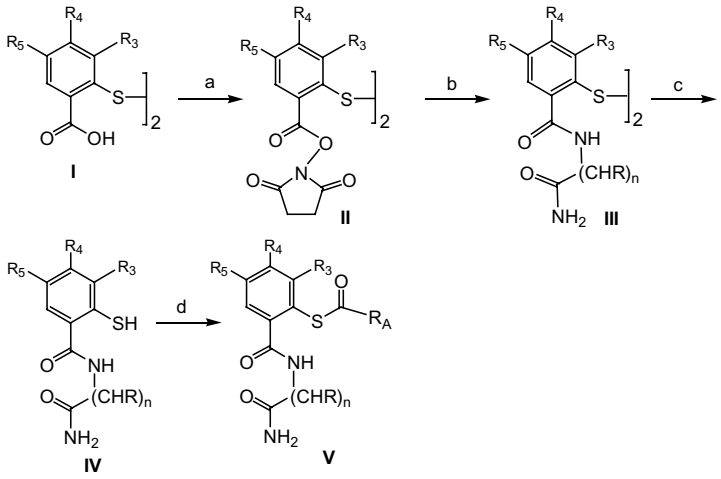
Figure 1. Combinatorial template for thioesters.

were found to possess increased antiviral potency, but as new inhibitor classes, they proved to be highly unstable both in buffered saline solutions and human serum, readily decomposing mainly to free thiols (unpublished observations). Although progress was being made toward finding more potent, soluble, and easily purified Zn finger-reactive compounds, the chemotypes were drifting away from our hypothetically ideal thioester with a lower rate of free thiol formation in serum-containing solutions.

We next designed and tested a series of uncharged, *S*-acyl-2-mercaptobenzamide thioester derivatives that share the general features of the antiviral 2-mercaptobenzoyl template (see, Fig. 1). In order to maximize the potential for identifying optimal compound configurations, we employed a combinatorial chemistry approach to explore three independent domains associated with the thioester chemotype (see Fig. 1, R_A, R_L, and R_S). Using this model as a skeleton for synthetic variation, we prepared a library of several hundred compounds and screened them for antiviral activity, cellular toxicity, and relative stability in solutions containing human serum in order to determine the relationship between serum stability and antiviral efficacy. Lead compounds were further evaluated in an additional battery of antiviral assays to verify maintenance of the established NCp7 Zn finger inhibitor spectrum of action. Finally, we expanded the scope of antiviral evaluation to include identification of compounds with the potential for development as topical microbicide candidates by assessing their ability to prevent cell-to-cell transmission of virus. These efforts resulted in the identification of several novel lead thioester derivatives with properties that have promising features for their development as systemic antiviral therapies or topical microbicides for the inhibition of or prevention of HIV transmission.

2. Chemistry

The synthesis (Scheme 1) of 2,2′-dithiobis(*N*-substituted benzamides) (**III**) was accomplished via coupling of an amino acid amide with the intermediate, *N*,*N*′-disuccinimidyl-2,2′-dithiosalicylate (**II**), prepared from 2,



Scheme 1. Reagents and conditions: (a) *N*-hydroxysuccinimide/DIC/THF-2-PrOH/25 °C; (b) $H_2N(CH)_nC(=O)NH_2$/DMF/25 °C; (c) TCEP·HCl/Et_3N/DMF–H_2O(9:1)/25 °C; (d) R_ACOCl/DMA/25 °C.

2′-dithiosalicylic acid (**I**), *N*-hydroxysuccinimide, and 1,3-diisopropylcarbodimide (DIC). The disulfide benzamides were reduced to thiols (**IV**) using tris-(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) in 90% DMF in water under nitrogen. The 2-mercaptobenzamide thioesters (**V**) were prepared by the acylation of **IV** with an acid chloride in dimethylacetamide (DMA). To obtain a library of thioesters on the *S*-acyl-2-mercaptobenzamide template, we introduced a variety of substituents and structures in the acyl, ligand and the mercaptobenzoyl head groups in order to study certain structure–activity relationships (Table 2). We targeted changes that would produce compounds that are uncharged at neutral pH and are readily crystallized solids. The products were characterized by NMR spectroscopy, melting point, thin layer chromatography, and occasionally mass spectroscopy. Data for representative compounds are given in the Experimental section.

3. Results and discussion

3.1. In vitro antiviral assays

Compounds in our library of thioesters that fall under the generic structure of Figure 1 were initially screened in an algorithm developed to identify new potential inhibitors of Zn finger function. Compounds were concomitantly optimized with respect to antiviral activity during both early (pre-integration) and late (post-integration) phases of infection. Thioester stability in serum was also evaluated. In order to assess antiviral activity during early and late phases of HIV replication by candidate compounds, two antiviral assays were employed. The first antiviral assay involved the inhibition of acute HIV infection of CEM-SS cells by the laboratory-adapted RF strain of HIV-1 using cytoprotection as an endpoint[16b,22] to assess inhibition of virus replication early in the replication cycle (acute infection assay (AIA)). In the second assay, the effects of inhibitors on late stage events in HIV replication occurring during post-integration viral expression were determined, using the proviral activation assay (PAA).[28,29] The PAA measures the ability of compounds to reduce virus expression derived from the integrated provirus in LPS-activated spleen cells taken from Tg166 HIV-1 transgenic mice. Activities were expressed as the concentration of compound (EC_{50}) achieving 50% cytoprotection or inhibition, respectively. In conjunction with each assay, cytotoxicity was determined as the concentration (IC_{50}) of test compound eliciting 50% cell death in uninfected target cells using mitochondrial reduction of the tetrazolium dyes XTT or MTS, respectively.

3.2. Optimization of thioesters

Optimization of the thioester structures was further explored in relation to the stability of compounds toward alterations promoted by serum components in buffered solutions containing 10% pooled, normal human serum. Spectrophotometric methods were developed which followed the hydrolysis of the thioester linkage as revealed by the appearance of a broad thiolate absorption band near 334 nm in the presence of serum. For all compounds, the change in absorbance versus time was plotted, and the value corresponding to the half-maximal change ($t_{1/2}$) at room temperature (23 °C ± 2 °C) was determined and used as an estimation of the *relative* stability. Lower rates of reaction occurring at 23 °C, in 10% serum, paralleled rates obtained at 37 °C in whole serum (data not shown), and provided rates of change that were more easily measured in screening a large number of compounds. With this data it was feasible to rank the stability of the various thioesters. Additional details are given in the Experimental section. Hydrolysis of the thioester was confirmed to be serum-dependent by the observation that thioesters were stable in phosphate buffered saline (PBS) for up to 24 h with no significant change in absorbance spectra detected, demonstrating that alterations are mediated by serum components, presumably thioesterases.

The library of compounds synthesized using the restricted template in Figure 1 yielded compounds with

a broad range of antiviral activity in both the AIA and PAA assays. This combinatorial plan was carried out to explore structure–activity relationships. The several hundred thioesters that were synthesized and screened were combinations taken from a repertoire of 40 acyl ($R_AC{=}O$) groups, 6 R_S substitutions on the mercaptobenzoyl substructure and 19 'ligand' (NHR_L) groups. The majority of combinations represented in our restricted library displayed anti-HIV activity in the AIA and PAA assays, since the Figure 1 template was based on successful hits from earlier exploratory investigations.[25–30] It is interesting to note that a closely related thioester in which the sulfur atom was linked *meta*, rather than *ortho*, to the benzoyl carbonyl was essentially inactive. It was discovered that good anti-HIV activity was compatible with nearly all of the acyl groups and most of the halogen substitutions on the benzoyl ring (at R_4- and R_5-positions). Much more constraint was required in the ligand structure in order to achieve optimal activity ($EC_{50} < 8\,\mu M$): Consistently, the best results in terms of antiviral potency and cytotoxicity were obtained using ligands that were simple amino acid primary amides, namely, those of glycine, β-alanine, and D- or L-alanine.

3.3. Structure–activity boundaries

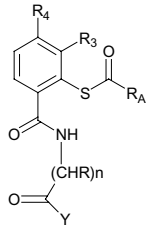
A number of structural changes resulted in screening parameters out of the desirable range of antiviral potency ($EC_{50} > 8\,\mu M$). These modifications are listed in Table 1 and fall into six groups. In Group I are benzoyl acyl moieties that are substituted at both the 2- and 6-positions. The mechanism resulting in reduced potency appears to be primarily steric hindrance preventing access to the thioester bond, since these substituents are either electron donating or withdrawing types. Generally, 2,6-difluorobenzoyl thioesters were active (e.g., compound **52**, Table 2), since fluorine atoms are essentially isosteric with hydrogen. Steric hindrance may also be introduced by substituents, R_3, on the opposite side of this reactive bond as listed in Group II. Side chains (R) on the amino acid amide 'ligands' tend to reduce antiviral activity (Group III): the bulkier the side chain, the greater is the effect. In earlier studies on pyridinioalkanoyl thioesters reported by Song et al. it was shown that the amides of L-isoleucine, L-phenylalanine, and L-glutamine were inactive.[30] Also, in this same study, it was found that amino acid ester ligands were inactive. Group IV compounds illustrate how certain combinations of electronic influences may be unfavorable for activity. Group V items show how quite specific associations of a steric factor (D-alanine as opposed to an L-alanine side chain) with acyl groups bearing electron withdrawing functions can reduce or eliminate activity. Finally, Group VI compounds are two examples of other changes to the ligand moiety: to a glycine *N*-*n*-hexylamide group and the combination of R_A = 3-pyridyl, usually an activity-promoting group, with a sulfa drug as ligand. Other acyl groups associated with this latter ligand were found in active thioesters including some PATEs.[25] Thus, the value of screening restricted combinatorial libraries as an optimization strategy is demonstrated by these and other examples (not reported) from this study, whereby the influence on activity of certain combinations of structural features is not easily predicted or explained.

3.4. Identification of potential therapeutically useful thioesters

In order to identify the most promising compounds from the combinatorial library, we employed the three-parameter assessment described above (AIA, PAA, and serum stability). The efficacy algorithm is based on the hypothesis that improved pharmacokinetic properties, namely better oral bioavailability and protection of the active thioester species as it traverses cell and organ membranes/absorptive surfaces, would correlate with in vitro and in vivo antiviral efficacy. Our principal approach to identifying optimized congeners was therefore based upon good antiviral potency (low EC_{50}, $<5\,\mu M$), low cytotoxicity (high IC_{50}, $>125\,\mu M$), and an arbitrarily defined high stability in serum ($t_{1/2} > 100$ min). Using these parameters we selected 12 compounds for evaluation (Table 2). Table 2 shows that there is little if any correlation of thioester stability with either AIA or PAA efficacy or cytotoxicity within this data set. In fact, plots of EC_{50} or IC_{50} versus $t_{1/2}$ for the entire library yielded scattergrams with correlation coefficients (r) of only 0.15 (AIA) and 0.02 (PAA) for EC_{50}. A similar lack of correlation was obtained plotting therapeutic index (IC_{50}/EC_{50}) versus $t_{1/2}$. The failure to demonstrate a direct correlation between susceptibility of the thioester to hydrolysis and antiviral efficacy and cytotoxicity suggests that the formation in solution of a free thiol should not be used as the single determining factor for selection of a lead Zn finger inhibitor.

Although decomposition to a free thiol under in vivo conditions could be considered disadvantageous for candidate compounds, our previous promising results with compound **19** (Table 3) support the lack of correlation between antiviral activity and stability observed in the above in vitro studies. Compound **19** has been previously advanced to antiviral efficacy[29] testing in vivo using the HIV-1 transgenic model system. This compound was shown to reduce replication and inactivate the virus in a manner consistent with targeting the NCp7 Zn finger and was relatively nontoxic (maximum tolerated dose of 160 mg/kg). Furthermore, this compound has been well tolerated in pigtail macaques (Simon Barratt-Boyes, U. Pitt, personal communication). These data suggest that the linkage of thioester stability with optimal zinc finger inhibitor activity in vivo might not lead to optimal compound selections. In order to identify additional lead compounds, we therefore took a more conservative approach. The data used to generate the three-parameter selection process (AIA, PAA, and serum stability) was reassessed, and the weight of the serum stability ($t_{1/2}$) was reduced to a secondary factor, with optimal EC_{50} and IC_{50} being the primary determinants for lead selection. Fourteen promising compounds were selected from our database where $t_{1/2} \leqslant 100$ min and they are listed in Table 3. Again, we did not observe any significant correlation between

Table 1. Minor modifications to active compounds that significantly diminish or abrogate anti-HIV activity (EC_{50})

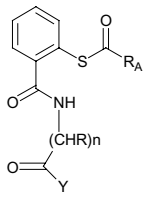


Compd #	R_3	R_4	R	*n*	R_A	Y	EC_{50}[a]	IC_{50}[a]	$t_{1/2}$[b]
Group I									
70	H	H	H	2	2,6-ClC_6H_3	NH_2	NA	>200	145
							16.9	286	
24	H	H	H	2	2,4,6-MeC_6H_2	NH_2	NA	>200	422
							55	>500	
69	H	H	H	2	2,6-$MeOC_6H_3$	NH_2	—	—	150
							15.2	141	
Group II									
75	MeO	H	H	1	CH_3	NH_2	NA	>200	233
							>100	720	
61	Me	H	H	2	C_6H_5	NH_2	NA	116	421
							15.7	376	
68	Me	H	H	2	2-MeC_6H_4	NH_2	43.9	111	1038
							17.7	77	
73	MeO	H	H	2	3-Pyridyl	NH_2	81.8	>200	117
							50.2	392	
Group III									
191	H	H	C_2H_5	1	$C(CH_3)_3$	NH_2	8.8	120	292
							11.7	253	
159	H	H	$CH(Me)_2$ (L)	1	$C(CH_3)_3$	NH_2	24.9	115	154
							14.4	118	
171	H	H	$CH(Me)_2$ (L)	1	$C(CH_3)_2C_2H_5$	NH_2	13.1	122	82
							7.8	85.6	
207	H	H	$CH_2CH(Me)_2$ (L)	1	$C(CH_3)_2C_2H_5$	NH_2	NA	11.6	219
							7.3	100	
214	H	H	n-C_3H_7 (L)	1	2,6-FC_6H_3	NH_2	11.4	162	38
							9.7	183	
203	H	H	$CH_2CH(Me)_2$ (L)	1	3,4-$MeOC_6H_3$	NH_2	11.4	58.3	148
							33.3	59	
213	H	H	$CH_2CH(Me)_2$ (L)	1	3,4,5-$MeOC_6H_2$	NH_2	9.6	113	265
							39.1	146	
Group IV									
114	H	H	H	1	2-F,6-CF_3–C_6H_3	NH_2	17.9	60.4	426
							2.8	20.5	
124	H	H	H	1	5-F,2-$CF_3C_6H_3$	NH_2	69.3	>200	28.8
							>100	161	
25	H	Cl	H	2	3-Pyridyl	NH_2	31.8	573	8
							15.2	91	
Group V									
128	H	H	Me (D)	1	2,6-FC_6H_3	NH_2	25.7	85	165
							22.2	>500	
120	H	H	Me (D)	1	3,4,5-$MeOC_6H_2$	NH_2	NA	>200	500
							>100	776	
Group VI									
62	H	H	H	1	2-MeC_6H_4	NHC_6H_{13}	NA	123	165
							5.8	55	
153	H	H	H	1	3-Pyridyl	Figure 1 R_L = 4-$H_2NSO_2C_6H_4CH_2$	24.9	115	154
							14.4	118	

[a] Upper EC and IC values for each compound result from the acute infection assay (AIA) while the lower values are derived from the proviral activation assay (PAA). Units are in μM.
[b] Units are in minutes.

EC_{50} (AIA or PAA) and stability ranking ($t_{1/2}$). Thus, in general, a variety of steric and electronic factors important for stability of the thioester linkage do not significantly impact antiviral potency or cellular toxicity.

Table 2. Compounds conjointly optimal for high antiviral activity (low EC_{50}), low cellular toxicity (high IC_{50}), and relatively high serum stability ($t_{1/2}$ > 100 min)



Compd #	R	*n*	R_A	Y	EC_{50}[a]	IC_{50}[a]	TI[b]	$t_{1/2}$[c]
35	H	2	$C(CH_3)_3$	NH_2	2.7	158	58	335
					4.5	237	43.5	
103*	Me (L)	1	$C(CH_3)_3$	NH_2	3.5	157	45.1	461
					2.1	>500	>192	
52*	H	2	2,6-FC_6H_3	NH_2	4.8	535	111	121
					3.7	473	128	
111	Me (L)	1	2,6-FC_6H_3	NH_2	3.8	145	38.2	106
					4.3	>500	>116	
119*	Me (L)	1	2-$MeOC_6H_4$	NH_2	2.6	>200	>76.9	221
					4.8	>500	>104	
174	Me (D)	1	2,3-$MeOC_6H_3$	NH_2	3.6	>200	>55.6	237
					3.8	150	39.4	
122*	H	1	2,3-$MeOC_6H_3$	NH_2	2.4	134	57	132
					4.8	>500	>104	
126	H	2	2,3,4-$MeOC_6H_2$	NH_2	3.6	>200	>55.5	147
					2.4	247	103	
145	Me (D)	1	2,3,4-$MeOC_6H_2$	NH_2	1.6	>200	>125	298
					2.8	237	84.6	
146*	Me (D)	1	2,4,5-$MeOC_6H_2$	NH_2	2.9	>200	>69	125
					2	248	124	
10	H	2	3,4,5-$MeOC_6H_2$	NH_2	3.8	>200	>53	165
					4.3	>250	>58	
89*	Me (L)	1	3,4,5-$MeOC_6H_2$	NH_2	2.1	>200	>95.2	146
					4.8	>500	>104	

* Selected for further study.

[a] Upper EC and IC values for each compound result from the acute infection assay (AIA) while the lower values are derived from the proviral activation assay (PAA). Units are in μM.

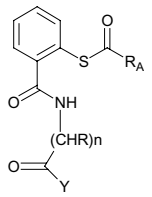
[b] Therapeutic index (TI) = IC_{50}/EC_{50}. Upper values for each compound result from the AIA while the lower values are derived from the PAA.

[c] Units are in minutes.

3.5. Further antiviral activities of selected thioester lead compounds

To further evaluate promising compounds, we selected six lead thioesters from Table 2 for further study and direct comparison to the previously in vitro- and in vivo-evaluated compound **19** (see Table 3). The structures of these seven compounds are presented in Figure 2. As expected of NCp7-targeted inhibitors, the lead compounds inhibited the replication of a broad range of retroviruses (HIV-1, HIV-2, and SIV) (see Table 4). The inhibitors were also active against a clinical HIV-1 isolate from a highly therapy-experienced patient expressing resistance mutations to both reverse transcriptase inhibitors (AZT, 3TC, ddI, d4T, nevarapine, and foscarnet) and protease inhibitors (nelfinavir, indinavir, and saquinavir) (Table 4). Although a 10- and 210-fold drop in antiviral efficacy were observed for compounds **52** and **89**, respectively, versus the wild type B subtype HT/92/596 strain of HIV, the resulting EC_{50} was still in the low micromolar range, indicating sensitivity to the compounds, in contrast to the failure of either indinavir or AZT to inhibit HIV MDR769 replication. Contrary to antiviral activity in PBMC, antiviral efficacy in monocyte/macrophages was more variable, with EC_{50}s ranging from less than 0.01 (compound **89**) to 37 μM (compound **146**). The broad range in EC_{50}s did not correlate with serum stability or virucidal activity, suggesting that there are additional factors influencing antiviral potency in monocytes/macrophages. The six lead thioesters were also assessed for direct virucidal activity on cell-free virus and their ability to inhibit TNF-α-induced virus expression from promonocytic U1 cells (Table 4). This latter assay is similar to the PAA assay in that all virus expression derives from an integrated provirus.[31] It is interesting to note that the thioesters, as a class, are less virucidal than AT-2 (4- to 70-fold less potent) but are more effective at inhibiting acute infection of PBMCs (unpublished observations JAT). Interestingly, we observed a slight inverse correlation ($r = 0.83$) of virucidal activity to thioester stability. This suggests that interaction with and ejection of Zn^{2+} from the closely packed NCp7 Zn fingers in the nucleocapsid of HIV is dependent upon the ability of the compound to readily form a free thiol. This could suggest that the processes by which the inhibitors interact with NCp7 Zn fingers in the virion and NCp7 Zn fingers in the infected cell is qualitatively different. Finally, compounds **52** and **89** as representatives

Table 3. Compounds conjointly optimal for high antiviral activity (low EC_{50}), low cellular toxicity (high IC_{50}), and moderate to low serum stability ($t_{1/2}$ < 100 min)



Compd #	R	*n*	R_A	Y	EC_{50}[a]	IC_{50}[a]	TI[b]	$t_{1/2}$[c]
8[*]	H	2	C_6H_5	NH_2	3.3	676	205	22
					4	>250	>63	
123[*]	H	1	C_6H_5	NH_2	3.8	>200	>52.6	27
					2.2	>500	>227	
53	H	2	2,4-FC_6H_3	NH_2	4.9	446	91	22
					4.5	496	110	
55[*]	H	2	2,4,6-FC_6H_2	NH_2	0.8	>200	>250	33
					4	>500	>125	
9[*]	H	2	2-$MeOC_6H_4$	NH_2	2.7	790	298	80
					3.1	>250	>81	
96	Me (D)	1	2-$MeOC_6H_4$	NH_2	2.2	>20	>909	54
					4.7	508	108	
141	Me (L)	1	2,5-$MeOC_6H_3$	NH_2	2.5	>20	>80	90
					4.4	279	63.4	
85	H	1	2-$CH_3CO_2C_6H_4$	NH_2	2.8	>200	>71.4	4.5
					2.2	>500	>227	
19	H	2	3-Pyridyl	NH_2	2.9	461	16	49
					5.3	198	37	
140	Me (L)	1	3-Pyridyl	NH_2	2.6	>20	>76.	9
					5.3	273	51.5	
51	H	1	3-Pyridyl	NH(n-C_3H_7)	5.4	219	41	30
					3.4	>500	>147	
30	H	2	4-Pyridyl	NH_2	2.1	728	343	7
					4.8	249	51.9	
142	Me (L)	1	4-Pyridyl	NH_2	1.5	>20	>13	7.5
					4.6	304	66.1	
131	H	1	4-Pyridyl	NH_2	2.2	>20	>91	15.4
					5.7	254	44.6	

[*] Selected for further study.
[a] Upper EC and IC values for each compound result from the acute infection assay (AIA) while the lower values are derived from the proviral activation assay (PAA). Units are in μM.
[b] Therapeutic index (TI) = IC_{50}/EC_{50}. Upper values for each compound result from the AIA while the lower values are derived from the PAA.
[c] Units are in minutes.

of the average serum stability for lead compounds, were selected, with 19 as a control, to determine whether inclusion of excess human AB serum (50%, heat inactivated) in the PBMC antiviral assay would significantly alter the antiviral efficacy of the compounds. Both compounds and the control maintained antiviral activity in 50% AB serum, although a significant decrease (26-fold), in antiviral activity (EC_{50}: 0.1–2.6 μM), was observed for **89** (data not shown).

The virucidal activity of the thioesters, as well as their ability to inhibit the replication of cell-free virus and decrease the release of virus from infected cells, suggests that they could potentially be candidates for development as topical microbicides.[32] Since the mechanism of sexual HIV transmission is unknown and could possibly entail transmission via both cell-free virions and direct transfer of virus between cells (cell-to-cell transmission), we next determined whether the compounds could prevent transmission of HIV from chronically infected CEM-SS cells to uninfected CEM-SS cells (Table 5). In this assay CEM-SS cells, chronically infected with the laboratory-adapted HIV-1 isolate designated SK-1, were mixed with uninfected CEM-SS cells. Twenty-four hours after initiation of co-culture, a reduction in syncytia was assessed as a measure of inhibition of virus-mediated fusion; and 72 h after initiation, virus replication (supernatant RT activity) was measured to determine cell-to-cell transmission. Table 5 shows that the selected thioesters inhibited cell-to-cell transmission of HIV with EC_{50}s ranging from 0.08 to 7 μM. For all compounds, except **103** and **119**, inhibition of virus replication was 4.5- to 11-fold more potent than inhibition of syncytium formation, suggesting that the compounds work primarily after initiation of cell-to-cell contact to prevent virus transmission. This is consistent with previous observations demonstrating inhibition of HIV replication by targeting NCp7 function occurring during reverse transcription.[33] Compounds **103** and **119** did not demonstrate any differential between inhibition of

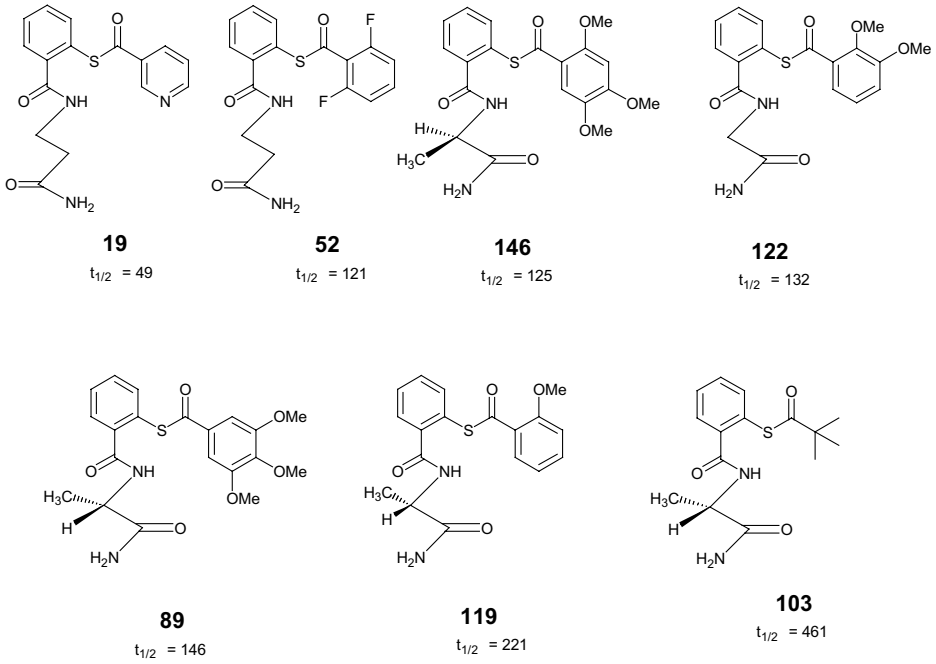


Figure 2. Structures of compounds from Tables 2 and 3 selected for further evaluation.

Table 4. Other antiviral activities of selected HIV-inhibitory thioesters arranged in order of increasing serum stability

Assay*		19	52	146	122	89	119	103	AZT	AT-2	IDV
PBMC/HIV1[a]	EC_{50}	1.1	0.5	0.8	1	0.02	2	1.5	0.007	—	—
B subtype	IC_{50}	>100	>100	>100		>100		>100	>1	—	—
HT/92/596	TI	>91	>192	>122	>105	>5000	>143	>68	>143	—	—
PBMC/MDR769[a]	EC_{50}	1.2	4.9	0.3	0.8	4.2	7.4	5.8	0.6	—	—
	IC_{50}	>100	>100	>100	>100	>100	>100	>100	>1	—	—
	TI	>83	>20	>400	>125	>24	>14	>17	>1.6	—	—
PBMC/HIV2[a]	EC_{50}	0.5	4	8.5	5.6	5.9	5.3	2.6	0.002	—	—
CDC310342	IC_{50}	>100	>100	>100	>100	>100	>100	>100	>1	—	—
	TI	>200	>25	>12	>19	>17	>19	>38	>500	—	—
PBMC/SIV[a]	EC_{50}	1.7	1	11.4	0.8	4.7	1.3	4.7	0.018	—	—
Mac251	TC_{50}	56.3		60.3	56.3	40.4	56.3	64.8	>1	—	—
	TI	33	56.3	5.3	70.4	8.6	43	14	>55.6	—	—
Monocytes[a]	EC_{50}	5	3.1	36.9	2	<0.01	20.5	16.1	0.01	—	—
HIV BaL	IC_{50}	>100	>100	>100		>100	>100	>100	>1	—	—
	TI	>20	>32	>2.7	>51	>10000	>5	>6	>100	—	—
U1[b]	EC_{50}	5	11.9	21.6	13.2	7.4	14	8.6	—	—	0.24
TNF-α induced	IC_{50}	>100	>100	>100	>100	82.9	>100	>100	—	—	>1
	TI	>20	>8	>4.6	>7.6	11	>7	>12	—	—	>4.2
Virucidal Activity[c] HIV IIIB	EC_{50}	9	19	35	11	12	11	155	—	2.2	—
Serum stability[d]	$t_{1/2}$	49	121	125	132	146	221	461	—	—	—
Cobalt ejection[e]	mAU	35.9	23.4	15.6	14.5	15.4	14.9	2.5	—	—	—

IDV: indinavir; AT-2: Aldrithiol-2.

* Additional assay details can be found in the Experimental section.

[a] Viral replication in PBMC and monocytes/macrophage cultures were determined on day 7 post-infection by measuring supernatant reverse transcriptase (RT) activity or p24 antigen expression by ELISA. Units are in μM.

[b] U1 cells were TNFα induced and expression of supernatant RT determined at day 3 post-treatment. Units are in μM.

[c] Reduction in infectivity of HIV-1 IIIB following a 4 h incubation with PATEs. Residual infectivity was determined in HeLa CD4 LTR β-gal cells by chemiluminescence. Units are in μM.

[d] Stability experiments of thioesters were performed at room temperature with thioesters suspended in 10% human serum. Values represent the time (min) it took for the absorbance (334 nm) to undergo 50% of its eventual change.

[e] Cobalt was substituted for zinc to compare relative metal ion ejection rates of the compounds. Values represent milli-absorbance units (mAU).

Table 5. Inhibition of cell-to-cell transmission using selected thioester compounds*

		19	52	146	122	89	119	103	DS (μg/mL)
Virus replication	EC_{50}	0.08	0.09	2	0.5	0.1	6	7	0.4
Syncytia formation	EC_{50}	0.6	1	9	6	0.9	5	5	0.8

DS: Dextran sulfate (μg/mL).

* CEM-SS cells chronically infected with the SK-1 strain of HIV-1 were assessed for supernatant RT or syncytia formation. Units are in μM unless otherwise stated. Additional details can be found in the Experimental section.

syncytium formation and virus replication, (EC_{50} supernatant RT: 7.0 and 6.0 μM, respectively), and their potency of inhibition of acute infection was 3- to 4.5-fold higher (EC_{50} 2.0 and 1.5 μM, respectively). Thus, compounds **103** and **119** do not appear to be optimal candidates for inhibition of cell-to-cell transmission of HIV. It is interesting to note that all compounds inhibited syncytium formation to some extent. Lifson and coworkers has shown that cell-free virus treated with the zinc finger inhibitor, Aldrithiol-2, can still mediate virus fusion.[34] Therefore, the reduction in syncytium formation should not arise from inactivation of cell-free virus released during the 24 h incubation, but rather via actions on NCp7 Zn fingers within the cell. Other zinc finger inhibitor chemotypes have been shown to result in extensive intracellular cross-linking of NCp7 and its gag precursors,[5,24,35,36] resulting in accumulation of unprocessed gag proteins in the cell and inhibition of virus release. Interestingly, the observed 4.5- to 11-fold greater efficacy at preventing virus replication demonstrated by the compounds does not correlate with activity in the latent infection U1 assay system (Table 4). This may reflect the fact that virus derived from co-culture of uninfected and infected cells is typically elicited at the sites of close contact between the cells[37] versus the more generalized virus production that occurs within U1 cells following TNF-α induction.

4. Conclusions

Using a combinatorial library strategy we have generated a diversity set of several hundred congeners of a thioester-based chemotype under consideration for development as a HIV replication inhibitor. Our initial approach was to correlate antiviral activity in systems that would allow targeting of early (pre-integration) and late (post-integration) phases of virus replication with pharmacological parameters deemed applicable to the thioester class as a whole. We choose as one of our primary pharmacological parameters the stability of the thioester in serum, based on the hypothesis that free thiols represent an active group that would act to enhance nonspecific interaction of the compounds with other proteins, leading to loss of efficacy and increased systemic toxicity in vivo. However, experimental evidence showed that there was no significant correlation between the stability of the thioester in serum and antiviral activity. This observation is partially supported by our previous analysis of an earlier thioester, which was shown to be relatively nontoxic and efficacious in vivo. The failure to demonstrate any significant correlation with thioester stability led us to redesign the lead selection algorithm and re-evaluate the combinatorial library for lead thioesters. This strategy led to the identification of an additional 14 lead compounds with low to moderate serum stability. One compound from this set (#19) was further studied along with six, more stable thioesters from the previous set (see Fig. 2). These seven compounds showed a broad range of action against wild type retroviruses (HIV-1, HIV-2, and SIV), activity against a multi-drug-resistant clinical isolate, activity in monocytes/macrophages, virucidal activity, and the ability to inhibit virus expression from cells latently infected with HIV.

Further characterization identified a subset of the seven compounds with in vitro antiviral activity compatible with their development as topical microbicides namely, the inhibition of acute and late stages of HIV replication, virucidal activity, and cell-to-cell transmission. These results have implications not only for the development of the thioesters for a new therapeutic modality (topical microbicides), but taken in the context of serum stability suggest antiviral activity may be the result of a complex interaction with the host cell that may or may not proceed through a thioester intermediate. Preliminary investigation has suggested that formation of a conjugate with cellular acyl-CoA can act as an intermediate in the reaction with the Zn^{2+} coordinating cysteines of the NCp7 protein (manuscript in preparation). These recent findings suggest that the thioester may act as a 'prodrug' for the active compound.

These studies in toto demonstrate that a combinatorial approach to designing better Zn finger inhibitors is possible and that the algorithm used to select lead compounds must employ sufficient flexibility to be able to eliminate noncorrelative activity parameters.

5. Experimental

5.1. Cell lines and viruses

The U1 and CEM-SS cell lines were obtained from the AIDS Research and Reference Reagent Program (Bethesda, MD) and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamate (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). The HIV clinical B subtype isolate 92/HT/596, HIV-2 CDC310342, and the SIV MAC251 strain were also obtained from the AIDS Research and Reference Reagent Program. The multi-drug-resistant MDR-769 strain was a kind gift from Dr. Tom Merrigan (Stanford University Palo Alto CA.) This virus was derived from a highly experienced patient and expresses resistance to reverse transcriptase inhibitors (AZT,

3TC, ddI, d4T, nevarapine, foscarnet) and protease inhibitors (nelfinavir, indinavir, and saquinavir).

5.2. Proviral activation assay (PAA)

The transgenic (Tg) line 166, containing the full length wild type NL4-3 viral clone that was transfected into mice on an FVB/N background, was derived and maintained as previously described.[38,39] Age- and sex-matched Tg mice between 8 and 12 weeks of age were used in all experiments. The in vitro proviral activation assay (PAA) using HIV-Tg spleen cells was performed as described previously[28,29] and measures the inhibition of HIV-p24 induction by LPS stimulation. All compounds were initially dissolved in DMSO at a concentration of 100 mM, then serially diluted in complete medium containing 100 ng/mL LPS. Controls included cells not stimulated with LPS or untreated, LPS-stimulated. The highest concentration of DMSO used in the cultures never exceeded 0.1%.

Cultures (5×10^6 cells/mL) were incubated at 37 °C with 5% CO_2 in RPMI 1640 (Life Technologies) medium supplemented with 10% FCS (HyClone), 10 mM HEPES (Life Technologies), 2 mM glutamine (NIH Stock), 100 U/mL penicillin, 100 μg/mL streptomycin (NIH Stock), and 5.5×10^{-5} M 2-ME (Life Technologies). Supernatants were removed after a 3-day culture period and assayed for HIV-p24 or stored frozen at −20 °C. The HIV-p24 antigen was quantified by ELISA using a commercial kit (Beckman-Coulter, Miami, FL). To determine cell viability in the presence of thioesters, nontransgenic mice were subcutaneously injected in the nape of the neck with 3 mLs of sterile 3.5% BioGel P-100 (BioRad, Hercules, CA). Inflammatory cells (>99% macrophages) were recovered from the gel matrix 6 days post-injection separated using a 70-μ cell strainer (Becton Dickinson). Cells were washed, suspended in complete media, and plated at a density of 2×10^5 cells/well in 96 well plates. After an initial overnight incubation at 37 °C with 5% CO_2 to allow cells to adhere, increasing concentrations of thioesters (or DMSO vehicle control) in complete medium were added to wells in duplicate. Cells were then incubated for 72 h at 37 °C with 5% CO_2 after which WST-1 reagent (Roche, Indianapolis, IN) was added to each well and allowed to incubate for an additional 4 h. Color change was determined spectrophotometrically by measuring the absorbance at 450 nm and subtracting the absorbance detected at 650 nm.

5.3. Acute infection assay (AIA)

The HIV cytoprotection assay has been previously described[16b,22] and uses the laboratory-adapted RF strain of HIV-1 and CEM-SS cells. Antiviral activity was determined by measurement of cell survival (cytoprotection) following a 6-day incubation with the test agents. Cell viability at assay termination was measured using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide) dye reduction. AZT was used as a positive control for all assays with each assay performed in duplicate with triplicate determinations per condition. Typical intra-triplicate variation was less 10%.

5.4. Virucidal assay

Cell-free virus inactivation assays were performed as previously described.[30] Briefly, a known titer of the IIIB strain of HIV-1 was preincubated for 4 h at 37 °C with test materials and excess compound removed by centrifugation at 18,000 *g* for 2 h at 4 °C. The virus pellets were suspended in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamate, 100 U/mL penicillin, and 200 μg/mL streptomycin and placed on HeLa CD4 LTR β-gal cells for 48 h. At 48 h, the media was removed and β-galactosidase enzyme expression determined by chemiluminescence per manufacturers instructions (Gal-screen™, Tropix, Bedford, MA, USA). Compound toxicity was monitored on a sister plate using CellTiter96® reagent (Promega Corp. Madison, WI, USA), a single solution formazan-based dye reduction system similar to XTT for measuring cell survival and viability.

5.5. Antiviral assays

Human peripheral blood monocytes were isolated from hepatitis and HIV sero-negative donors by Ficoll hypaque gradient centrifugation as previously described.[40] Antiviral assays were performed with 6 day cultured monocytes–macrophages. All antiviral evaluations were performed in triplicate in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamate (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).

5.6. Infection of PBMCs and monocyte macrophages

Peripheral blood mononuclear cells isolated from hepatitis and HIV sero-negative donors by Ficoll hypaque gradient centrifugation as previously described.[40] Monocytes were further purified by adherence to plastic and washing to remove unadhered lymphocytes. PBMCs were activated for 3 days with phytohemagglutinin-P (PHA-P)/IL-2 and subsequently infected. Monocytes were cultured for 7 days in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamate (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). All antiviral evaluations were in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamate (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). HIV replication in PBMC and monocyte macrophage cultures were determined day 7 post-infection by measurement of supernatant reverse transcriptase (RT) activity[41] or p24 antigen expression by ELISA (Coulter Diagnostics). Cell viability was determined by XTT dye reduction. All determinations were performed in triplicate with the standard deviation within triplicates was less than 10%. AZT was used as a positive control for all assays.

5.7. Cell-to-cell transmission assay

Assessment of the inhibition of cell-to-cell transmission of HIV was performed using uninfected CEM-SS cells

and CEM-SS cells chronically infected with the SK-1 strain of HIV (CEM-SK-1). CEM-SK-1 cells were cultured in RPMI 1640 supplemented as described above. Chronically infected CEM-SK-1 cells were co-cultivated in serial logarithmic dilutions with uninfected $CD4^+$ cells at 5×10^5 cells per well. Syncytium formation was first detected at 6 h post-co-cultivation and quantified at 24 h in the flat-bottom 96-well microtiter plate with an Opelco CK40 inverted microscope. Cell-to-cell transmission was also evaluated in this assay by quantification of the burst of virus production, which occurs between 24 and 48 h after the co-cultivation of the infected and uninfected cells. Virus production was quantified using a reverse transcriptase (RT) assay.[41] Toxicity quantification involved the XTT-based evaluation as described above. Dextran sulfate was used as a positive control for the assay.

5.8. Inhibition of HIV replication in latently infected cells

The effect of compounds on virus expression from TNF α-induced, latently infected cells was performed using U1 cells obtained from the AIDS Research and Reference Reagent Program (Bethesda, MD, USA). The cells were maintained in RPMI 1640 supplemented as described above. U1 cells are derived from the histocytic leukemia cell line U937, and contain a single integrated (HIV IIIB) cytokine inducible provirus.[31] Fifty thousand U1 cells were induced with 10 ng/mL of TNFα (R&D Systems, Minneapolis, MN, USA) in the presence of the test compounds. Cultures were incubated for 3 days, and virus expression measured by supernatant RT activity, and cytotoxicity determined by CellTiter96® dye reduction.

5.9. Stability assay of thioesters

Stability experiments of thioesters were performed in 10% pooled, normal human AB serum (JGemini Bio-Products, Woodland, CA). A 25 mM stock solution of thioester in methanol was prepared, and 50 μL of the stock solution of thioester was added to 4.45 mL of PBS containing 10% pooled normal human serum. After mixing this medium with the test compound in a cuvette, we followed the absorbance changes near 334 nm at room temperature (~23 °C) where the released thiolate has its maximum absorbance. Aqueous components were pre-equilibrated with nitrogen to minimize oxidation of thiolate that otherwise tended to occur at a slow rate. Serum absorbance was low at this wavelength and was blanked out. From these time curves the hydrolytic half-life of a compound was ascertained giving numbers that allowed ranking different compounds on a relative stability scale.

5.10. Cobalt ejection assay

The full length 55 amino acid NCp7 protein was synthesized using the Applied Biosystem 431A peptide synthesizer. Cobalt ejection was monitored spectrophotometrically by measuring the absorbance (642 nm) of the metal coordinated finger (300 μM) in the presence and absence of compounds (300 μM) at 25 °C. The *net* extent of cobalt ejection was determined 60 min after the addition of the compound by allowing for the spontaneous loss of cobalt that occurs during that same period. All compounds were compared in a common reference frame by first normalizing the absorbance values to their respective values at zero time then subtracting the resulting ratio for protein with thioester from the ratio of protein in the absence of compound. The difference obtained was then multiplied by the zero time value of protein without compound, as expressed below:

$$(A^a_{60}/A^a_0 - A^c_{60}/A^c_0)A^a_0 \times 1000$$

where A is absorbance at 642 nm at 0 time or 60 min. Superscript a applies to the coordinated protein alone; superscript c applies to the coordinated protein plus thioester. Although all compounds were capable of ejecting cobalt from NCp7, a higher numerical value (AU) suggests faster cobalt ejection.

6. Organic synthesis

Most organic reagents and intermediates were obtained from Sigma and Aldrich (St Louis, MO, USA). Solvents and other chemicals, from various suppliers, were research grade. Melting points were determined in open capillary tubes on a Büchi 510 melting point apparatus and are uncorrected. Proton magnetic resonance (NMR) spectra were recorded on a 300 MHz Perkin–Elmer spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane. Fast atom bombardment (FAB) mass spectra were recorded on a Jeol JMS-SX102 spectrometer. TLC was carried out on silica gel plates (Merck) using chloroform/methanol (9:1) as eluent.

6.1. *N*,*N*′-(2,2′-Dithiobisbenzoyl)-bisalaninamide/bisglycinamide

To a solution of 2,2′-dithiosalicylic acid (2.0 mmol) in a mixture of tetrahydrofuran (THF) (14 mL) and 2-propanol (6.0 mL) was added *N*-hydroxysuccinimide (4.4 mmol) and 1,3-diisopropylcarbodiimide (4.1 mmol). The solution was stirred at room temperature for 4 h. The light yellow precipitate was filtered off and washed with 2-propanol (20 mL) in portions, yielding *N*,*N*′-disuccinimidyl-2,2′-dithiosalicylate (89%). A solution of this compound (1.0 mmol) in *N*,*N*-dimethylformamide (DMF) (5.0 mL) was added to the corresponding amino acid amide hydrochloride (2.2 mmol) and triethylamine (2.0 mmol). This solution was stirred at room temperature for 4–6 h. After completion of the reaction, the solvent was removed under vacuum, and the residue was treated with 20% hot ethanol and washed with hot water, yielding the corresponding 2,2′-dithiobisbenzamide. In an alternative procedure, the amino acid amide hydrochloride was replaced by the corresponding free amine, freshly obtained from the *N*-α-benzyloxycarbonyl amino acid amide by treatment with hydrogen (1 atm) over 10% Pd on charcoal in methanol followed by removal of solvent.

6.2. *N*-(2-Mercaptobenzoyl)-alaninamide/glycinamide

To a solution of the 2,2′-dithiobisbenzamide (1.0 mmol) in 14 mL of 90% DMF (10% water) was added tris-(2-carboxyethyl)phosphine hydrochloride (1.5 mmol) and triethylamine (3.0 mmol). The reaction mixture was stirred at room temp under N_2 overnight. The solvent was removed under vacuum, and the residue was treated with ice-cold water (5 mL) for several hours, collected by filtration, washed with a small volume of ice-water, and dried in vacuo.

6.3. *S*-Acyl-2-mercaptobenzamide

The desired acid chloride (1.5 mmol) was added to a solution of the 2-mercaptobenzamide (1.0 mmol) in dimethylacetamide (DMA) (7.5 mL). In the case of compounds **19**, **30**, **142**, **140**, **51**, and **131**, triethylamine (2.8 mmol) was added to start the reaction, since the acid chlorides are also hydrochloride salts and HCl is evolved. The pH of the mixture is further adjusted to 3.3 (monitored via moistened test strips). The resulting mixture was stirred at room temperature under nitrogen overnight. The pH was then raised to approximately 6.5 before workup to ensure that the product was isolated in uncharged form. The solvent was removed under vacuum, and the remaining residue was treated with ether for several hours. The precipitate thus obtained was filtered off, washed with ether and water, and crystallized from acetonitrile or dichloromethane–ether (1:4). Prior to crystallization, several compounds, such as **19**, required additional purification on a silica gel column developed with chloroform–methanol 9:1 v/v.

6.4. β-Ala-NH_2 thioesters

6.4.1. *N*-[2-(Trimethylacetylthio)benzoyl]-β-alaninamide (35). Yield, 64.6%; mp 124 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (s, 9H, $3CH_3$), 2.28–2.31 (m, 2H, CH_2), 3.32–3.35 (m, 2H, CH_2), 6.82 (s, 1H, NH), 7.35 (s, 1H, NH), 7.4–7.48 (m, 4H, Ar-H), 8.20 (t, 1H, NH); MS (FAB) *m*/*z* 309.2 (MH^+).

6.4.2. *N*-[2-(2,4-Difluorobenzoylthio)benzoyl]-β-alaninamide (53). Yield, 23%; mp 173–174 °C; ^{1}H NMR (DMSO-d_6) δ 2.33 (t, 2H, CH_2), 3.38 (q, 2H, CH_2), 6.85 (s, 1H, NH), 7.26–7.40 (m, 3H, Ar-H, NH), 7.50–7.72 (m, 4H, Ar-H), 7.90 (m, 1H, Ar-H), 8.42 (t, 1H, NH); MS (FAB) *m*/*z* 365.10 (MH^+).

6.4.3. *N*-[2-(2,4,6-Trifluorobenzoylthio)benzoyl]-β-alaninamide (55). Yield, 20%; mp 165–167 °C; ^{1}H NMR (DMSO-d_6) δ 2.31 (t, 2H, CH_2), 3.38 (q, 2H, CH_2), 6.80 (s, 1H, NH), 7.36 (s, 1H, NH), 7.55–7.70 (m, 4H, Ar-H), 7.75–8.0 (m, 2H, Ar-H), 8.50 (t, 1H, NH); MS (FAB) *m*/*z* 383.10 (MH^+).

6.4.4. *N*-[2-(Benzoylthio)benzoyl]-β-alaninamide (8). Yield, 66.8%; mp 135 °C; ^{1}H NMR (DMSO-d_6) δ 2.30 (t, 4H, $2CH_2$), 6.83 (s, 1H, NH), 7.34 (s, 1H, NH), 7.52–7.63 (m, 6H, Ar-H), 7.72–7.74 (m, 1H, Ar-H), 7.97 (d, 2H, Ar-H), 8.39 (t, 1H, NH); MS (FAB) *m*/*z* 329.2 (MH^+).

6.4.5. *N*-[2-(2-Methoxybenzoylthio)benzoyl]-β-alaninamide (9). Yield, 58.4%; mp 92 °C; ^{1}H NMR (DMSO-d_6) δ 2.29–2.32 (m, 4H, $2CH_2$), 3.89 (s, 3H, OMe), 6.84 (s, 1H, NH), 7.08 (t, 1H, Ar-H), 7.23 (s, 1H, Ar-H), 7.49–7.54 (m, 5H, Ar-H), 7.66 (d, 1H, Ar-H), 8.16 (t, 1H, NH); MS (FAB) *m*/*z* 359.2 (MH^+).

6.4.6. *N*-[2-(2,3,4-Trimethoxybenzoylthio)benzoyl]-β-alaninamide (126). Yield, 50.2%; mp 182–184 °C; ^{1}H NMR (DMSO-d_6) δ 2.64–2.31 (m, 4H, $2CH_2$), 3.79 (s, 3H, OMe), 3.81 (s, 6H, 2OMe), 6.82 (s, 1H, NH), 6.97 (d, 2H, Ar-H), 7.33 (s, 1H, NH), 7.47–7.54 (m, 4H, Ar-H), 8.29 (t, 1H, NH); MS (FAB) *m*/*z* 419.2 (MH^+).

6.4.7. *N*-[2-(3,4,5-Trimethoxybenzoylthio)benzoyl]-β-alaninamide (10). Yield, 69.3%; mp 183 °C; ^{1}H NMR (DMSO-d_6) δ 2.08 (t, 2H, CH_2), 2.22 (t, 2H, CH_2), 3.76 (s, 3H, OMe), 3.86 (t, 6H, OMe), 6.84 (s, 1H, NH), 7.20 (s, 2H, Ar-H), 7.36 (s, 1H, NH), 7.52–7.60 (m, 4H, Ar-H), 8.36 (t, 1H, NH); MS (FAB) *m*/*z* 419.2 (MH^+).

6.4.8. *N*-[2-(2,6-Difluorobenzoylthio)benzoyl]-β-alaninamide (52). Yield, 54.9%; mp 190–193 °C; ^{1}H NMR (DMSO-d_6) δ 2.33 (t, 2H, CH_2), 3.38 (t, 2H, CH_2), 6.85 (s, 1H, NH), 7.26–7.40 (m, 3H, Ar-H, NH), 7.50–7.72 (m, 5H, Ar-H), 8.42 (t, 1H, NH); MS (FAB) *m*/*z* 365.1 (MH^+).

6.4.9. *N*-[2-(Isonicotinoylthio)benzoyl]-β-alaninamide (30). Yield, 57.4%; mp 197 °C; ^{1}HNMR (DMSO-d_6) δ 2.26–2.32 (m, 4H, CH_2), 6.82 (s, 1H, NH), 7.33 (s, 1H, NH), 7.52–7.64 (m, 4H, Ar-H), 7.83 (d, 2H, py-H), 8.43 (t, 1H, NH), 8.86 (s, 2H, py-H); MS (FAB) *m*/*z* 366.2 (MH^+).

6.4.10. *N*-[2-(Nicotinoylthio)benzoyl]-β-alaninamide (19). Yield, 57.4%; mp 150–152 °C; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 4H, $2CH_2$), 6.82 (s, 1H, NH), 7.35 (s, 1H, NH), 7.52–7.64 (m, 4H, Ar-H), 7.70–7.75 (m, 1H, py-H), 8.41–8.46 (m, 2H, py-H), 8.91 (t, 1H, NH), 9.11 (s, 1H, py-H); MS (FAB) *m*/*z* 366.2 (MH^+).

6.5. D-Ala-NH_2 thioesters

6.5.1. *N*-[2-(2-Methoxybenzoylthio)benzoyl]-D-alaninamide (96). Yield, 66.8%; mp 165 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, CH_3), 3.39 (s, 3H, OMe), 4.26–4.33 (m, 1H, CH), 7.03 (s, 1H, NH), 7.25–7.36 (m, 4H, Ar-H, NH), 7.51–7.65 (m, 5H, Ar-H), 8.31 (d, 1H, NH); MS (FAB) *m*/*z* 359.2 (MH^+).

6.5.2. *N*-[2-(2,3,4-Trimethoxybenzoylthio)benzoyl]-D-alaninamide (145). Yield, 66.9%; mp 145 °C; ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 1H, CH_3), 3.79 (s, 3H, OMe), 3.95 (s, 3H, OMe), 4.28–4.33 (m, 1H, CH), 6.96 (d, 1H, Ar-H), 6.99 (d, 1H, Ar-H), 7.05 (s, 1H, NH), 7.25 (s, 1H, NH), 7.51–7.55 (m, 4H, Ar-H), 8.22 (d, 1H. NH); MS (FAB) *m*/*z* 419.2 (MH^+).

6.5.3. *N*-[2-(2,4,5-Trimethoxybenzoylthio)benzoyl]-D-alaninamide (146). Yield, 62.1%; mp 154 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (d, 3H, CH_3), 3.71 (s, 3H, OMe), 3.90 (s, 3H, OMe), 3.96 (s, 3H, OMe), 4.27–4.32 (m, 1H, CH), 6.83 (s, 1H, Ar-H), 7.07 (s, 1H, NH), 7.23 (s, 2H, NH, Ar-H), 7.51–7.58 (m, 4H, Ar-H), 8.11 (d, 1H, NH); MS (FAB) *m*/*z* 419.2 (MH^+).

6.6. L-Ala-NH_2-thioesters

6.6.1. *N*-[2-(Trimethylacetylthio)benzoyl]-L-alaninamide (103). Yield, 71.2%; mp 128 °C; ^{1}H NMR (DMSO-d_6) δ 1.22–1.28 (m, 12H, $4CH_3$), 4.30–4.35 (m, 1H, CH), 7.10 (s, 1H, NH), 7.24 (s, 1H, NH), 7.40–7.57 (m, 4H, Ar-H), 8.19 (d, 1H, NH); MS (FAB) *m*/*z* 309.2 (MH^+).

6.6.2. *N*-[2-(2-Methoxybenzoylthio)benzoyl]-L-alaninamide (119). Yield, 66.9%; mp 159 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, CH_3), 3.89 (s, 3H, OMe), 4.29–4.34 (m, 1H, CH), 7.07–7.10 (m, 2H, Ar-H, NH), 7.21–7.23 (m, 2H, Ar-H, NH), 7.55–7.69 (m, 6H, Ar-H), 8.25 (d, 1H, NH); MS (FAB) *m*/*z* 359.2 (MH^+).

6.6.3. *N*-[2-(2,3-Dimethoxybenzoylthio)benzoyl]-L-alaninamide (174). Yield, 69.5%; mp 175–180 °C; ^{1}H NMR (DMSO-d_6) δ 1.22 (d, 3H, CH_3), 3.82 (s, 3H, OMe), 3.87 (s, 3H, OMe), 4.31–4.38 (m, 1H, CH), 7.05 (s, 1H, NH), 7.20–7.33 (m, 4H, Ar-H, NH), 7.37–7.60 (m, 4H, Ar-H), 8.30 (d, 1H, NH); MS (FAB) *m*/*z* 389.2 (MH^+).

6.6.4. *N*-[2-(2,5-Dimethoxybenzoylthio)benzoyl]-L-alaninamide (141). Yield, 69.5%; mp 120 °C; ^{1}H NMR (DMSO-d_6) δ 1.22 (s, 3H, CH_3), 3.75 (s, 1H, OMe), 3.85 (s, 1H, OMe), 4.29–4.36 (m, 1H, CH), 7.05 (s, 1H, NH), 7.19–7.26 (m, 4H, Ar-H, NH), 7.52–7.61 (m, 4H, Ar-H), 8.23 (d, 1H, NH); MS (FAB) *m*/*z* 389.2 (MH^+).

6.6.5. *N*-[2-(3,4,5-Trimethoxybenzoylthio)benzoyl]-L-alaninamide (89). Yield, 59.7%; mp 167–169 °C; ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 3H, CH_3), 3.77 (s, 3H, OMe), 3.86 (s, 6H, OMe), 4.28–4.32 (m, 1H, CH), 7.05 (s, 1H, NH), 7.20 (s, 2H, Ar-H), 7.27 (s, 1H, NH), 7.56–7.58 (m, 4H, Ar-H), 8.32 (d, 1H, NH); MS (FAB) *m*/*z* 419.2 (MH^+).

6.6.6. *N*-[2-(2,6-Difluorobenzoylthio)benzoyl]-L-alaninamide (111). Yield, 68.6%; mp 205–210 °C; ^{1}H NMR (DMSO-d_6) δ 1.27 (s, 3H, CH_3), 4.28–4.40 (m, 1H, NH), 7.07 (s, 1H, NH), 7.27–7.32 (m, 3H, Ar-H, NH), 7.59–7.68 (m, 5H, Ar-H), 8.48 (d, 1H, NH); MS (FAB) *m*/*z* 365.1 (MH^+).

6.6.7. *N*-[2-(Nicotinoylthio)benzoyl]-L-alaninamide (142). Yield, 60.7%; mp 175–178 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (d, 3H, CH_3), 4.30–4.38 (m, 1H, CH), 7.04 (s, 1H, NH), 7.26 (s, 1H, NH), 7.59–7.64 (m, 4H, Ar-H), 8.30 (d, 2H, Py-H, NH), 8.32 (d, 1H, Py-H), 8.87–8.89 (m, 1H, Py-H), 9.07 (s, 1H, Py-H); MS (FAB) *m*/*z* 330.2 (MH^+).

6.6.8. *N*-[2-(Isonicotinoylthio)benzoyl]-L-alaninamide (140). Yield, 61.5%; mp 180 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 3H, CH_3), 4.28–4.33 (m, 1H, CH), 7.04 (s, 1H, NH), 7.26 (s, 1H, NH), 7.60–7.65 (m, 4H, Ar-H), 7.81 (d, 2H, Py-H), 8.40 (d, 1H, NH), 8.85 (d, 2H, Py-H); MS (FAB) *m*/*z* 330.2 (MH^+).

6.7. Glycinamide thioesters

6.7.1. *N*-[2-(Nicotinoylthio)benzoyl]glycine *N*′-*n*-propylamide (51). Yield, 32%; mp 105.5–107 °C; ^{1}H NMR (DMSO-d_6) δ 0.80 (t, 3H, CH_3), 1.30 (m, 2H, CH_2), 3.0 (q, 2H, CH_2), 4.85 (d, 2H, CH_2), 7.40–7.80 (m, 6H, Ar-H, Py-H, NH), 8.30 (d, 1H, py-H), 8.40 (t, 1H, NH), 8.82 (d, 1H, Py-H), 9.10 (s, 1H, Py-H); MS (FAB) *m*/*z* 358.20 (MH^+).

6.7.2. *N*-[2-(2-Acetoxybenzoylthio)benzoyl]glycinamide (85). Yield, N.D.; mp 144–145 °C; ^{1}H NMR (DMSO-d_6) δ 2.24 (s, 3H, CH_3), 4.85 (d, 2H, CH_2), 7.15 (s, 1H, NH), 7.20–7.80 (m, 8H, Ar-H, NH), 8.0 (d, 1H, Ar-H), 8.48 (t, 1H, NH); MS (FAB) *m*/*z* 373.10 (MH^+).

6.7.3. *N*-[2-(Benzoylthio)benzoyl]glycinamide (123). Yield, 66.7%; mp 175–180 °C; ^{1}H NMR (DMSO-d_6) δ 3.74 (d, 2H, CH_2), 7.09 (s, 1H, NH), 7.23 (s, 1H, NH), 7.56–7.73 (m, 7H, Ar-H), 7.96 (d, 2H, Ar-H), 8.52 (t, 1H, NH); MS (FAB) *m*/*z* 315.2 (MH^+).

6.7.4. *N*-[2-(2,3-Dimethoxybenzoylthio)benzoyl]glycinamide (122). Yield, 69.4%; mp 175 °C; ^{1}H NMR (DMSO-d_6) δ 3.75 (d, 2H, CH_2), 3.81 (s, 1H, OMe), 3.87 (s, 3H, OMe), 7.09 (s, 1H, NH), 7.20 (s, 1H, NH), 7.22–7.31 (m, 3H, Ar-H), 7.54–7.65 (m, 4H, Ar-H), 8.46 (t, 1H, NH); MS (FAB) *m*/*z* 375.1 (MH^+).

6.7.5. *N*-[2-(Isonicotinoylthio)benzoyl]glycinamide (131). Yield, 63.4%; mp 182–184 °C; ^{1}H NMR (DMSO-d_6) δ 3.75 (d, 2H, CH_2), 7.09 (s, 1H, NH), 7.26 (s, 1H, NH), 7.60–7.72 (m, 4H, Ar-H, Py-H), 7.80–7.83 (m, 2H, Py-H), 8.58 (t, 1H, NH), 8.86 (d, 2H, Py-H); MS (FAB) *m*/*z* 316.2 (MH^+).

Acknowledgements

This project was funded in part by the Intramural AIDS Targeted Antiretroviral Program (IATAP) to and a CRADA with Achillion. Part of this work was performed at Gene Logic (Gaithersburg, MD).

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