Design, Synthesis and Biological Evaluation of a Series of Thioamides as Non-Nucleoside Reverse Transcriptase Inhibitors

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Abstract: A series of thioamides were designed as bio-isosteres to the non-nucleoside reverse transcriptase inhibitor trovirdine by replacement of the thiourea NH groups with methylene groups. Eight thioamides were synthesized and *in vitro* tested for inhibitory effects on the activity of HIV-1 reverse transcriptase wild and mutant types. Three of the 8-thioamides exhibited enzyme inhibitory activities with IC_{50} values below 100 μM. While compound (**2**) exhibited activity against the mutant strain L100I with IC_{50} of 70.1 μM, compound (**4**) showed activity against the mutant strain K103N with IC_{50} of 92.7 μM, and compound (**8**) with activity against the wild type enzyme with IC_{50} of 8.9 μM. Each of the three thioamides could serve as a lead compound for further activity optimization.

Key Words: Non-nucleoside reverse transcriptase inhibitors, PETT bio-isosteres, thioamides.

INTRODUCTION

The reverse transcriptase enzyme (RT) inhibitors constitute an important class of drugs for AIDS therapy. Thirteen of the twenty-one anti-retroviral drugs currently approved by FDA for the treatment of HIV infection target the reverse transcriptase enzyme. Majority of these drugs belong to the class of non-nucleoside inhibitors [1-4]. The Phenethylthiazolylthiourea (PETT) analogs [5], Fig. (**1**), represent a potent class of non-nucleoside reverse transcriptase inhibitors (NNRTI) that acts by a non-competitive mechanism [6]. The structure activity relationships of thiourea [7-10] and urea [11, 12] derivatives have been extensively studied. Recently, the bioisosteric substitution of the thiourea and urea functionalities with sulfamide, cyanoguanidine, and guanidine has also been reported [13]. Trovirdine, Fig. (**1**), is one of the many potent NNRT inhibitors developed through systematic structure modifications of the PETT series [11]. Although modern AIDS therapy uses a combination of drugs to suppress the appearance of drug-resistant virus strains [14-16], evolution of enzyme resistant strains still poses major problem in the management of AIDS. One of the many single mutations is K103N, a lysine to asparagine at amino acid 103 in viral reverse transcriptase enzyme, negates the activity of all currently approved non-nucleoside reverse transcriptase inhibitors [17].

RATIONALE OF DESIGN

Efforts are continuing towards discovery and development of new compounds that would prevent the rapid development of resistant strains or more importantly that are active against drug-resistant strains. In the current research, we have studied the effects of the bio-isosteric replacement of the thiourea moiety of the drug trovirdine with thioamide functionality on activity against wild type and resistant strains of the RT enzyme. The structural similarities and differences of the PETT series, trovirdine, and the current thioamide compounds are illustrated in Fig. (**1**). The structures of compounds (**4**) & (**8**) of Fig. (**1**) have the bio-isosteric methylene group in opposite directions in relationship to the thiocarbonyl group. This modification aimed to verify the importance of the compound capability to form the high activity rigid structures as proposed for trovirdine and other PETT series [7] as illustrated between brackets in Fig. (**1**). The design of the new thioamides aimed also to include variable length for the linker aliphatic chain separating the thioamide functionality from the pyridine or the phenyl ring.

SYNTHESIS

The title compounds were synthesized according to one of two general methods. Thioamides (**3**), (**5**), (**6**), (**7**), and (**8**) (Table **1**) were prepared by the general method outlined in Scheme (**1**) that required the synthesis of the corresponding amides as intermediates. The amides were obtained through the conversion of carboxylic acids to the corresponding acid-chloride followed by treatment with the appropriate amine to give the intermediate amides. Treatment of the amides with Lawesson's reagent in boiling toluene/benzene (6:1) yielded the required thioamides (**3**), (**5**), (**6**), (**7**), and (**8**) in yields ranging from 57 to 84 % as detailed under materials and methods.

Attempts to prepare the thioamides (**1**), (**2**), and (**4**) applying the general method outlined in scheme (**1**) general method have failed, probably because of the presences of active methylene groups in the acid part of the amide. These compounds were prepared by an alternate method that involved the reaction of lithiated 2-Picoline with the corresponding isothiocyante as outlined in scheme (**2**).

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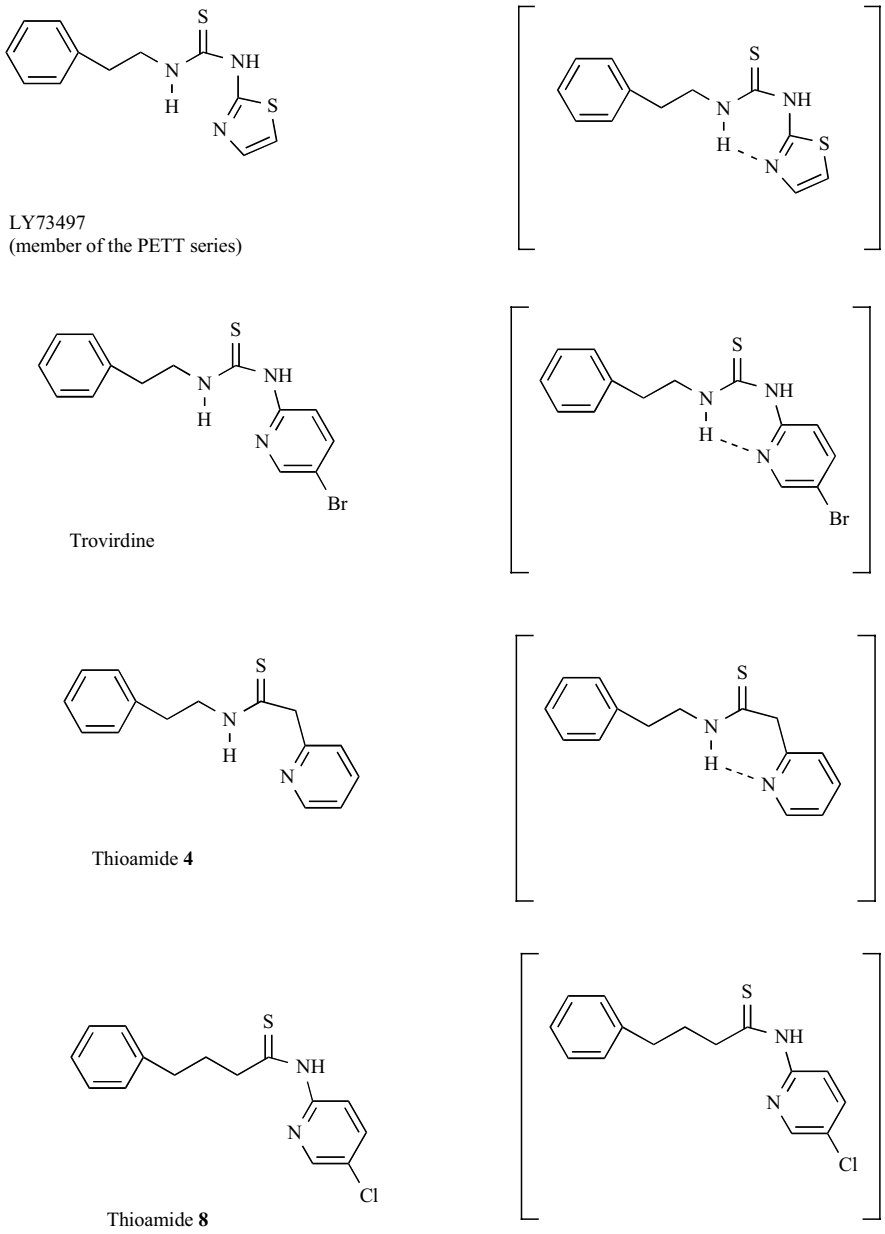
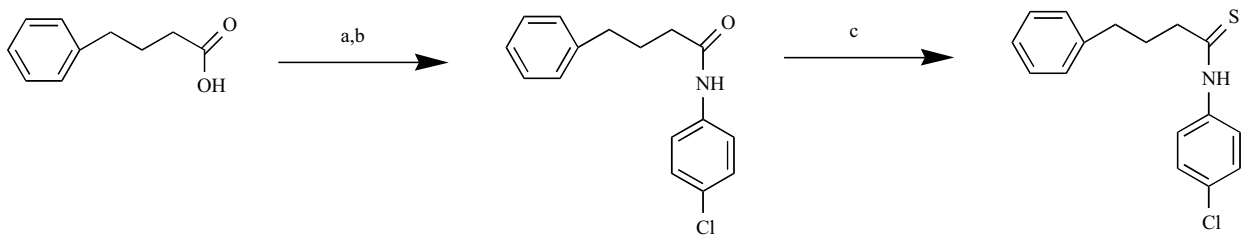


Fig. (1). Structural relationships of PETT, Trovirdine, and the thioamides 4 &8 and the potential of forming high-activity rigid conformations.

Lithiation of 2-picoline was conducted by reacting 2-picoline with LDA (formed in-situ by the reaction of butyl lithium and diisopropylamine in dry THF at –15 °C). The electrophilic attack of picolyl-lithium on the appropriate isothiocyanate gave directly the corresponding thioamide final yields range of 10-26% as detailed under the physical data section. The low yields of this method is attributed to the formation of several side products, with the dimeric structure shown in scheme (**2**) as the main side reaction product. The latter was isolated by flash chromatography and characterized by ^{1}NMR, IR, and mass specrto-photometry. This contaminant is believed to be resulting from further reaction of the target compound with another molecule of isothiocyanate. Structures of the synthesized compounds and intermediates; were confirmed by IR, ^{1}HNMR, and elemental analysis as detailed under materials and methods.

BIOLOGICAL EVALUATION

The eight thioamide compounds were submitted to the Southern Research Institute, Fredrick, for in-vitro biological testing using HIV-1 RT enzyme assay [21]. The antiviral activity was determined on the wild type and on K103N, L100I, and Y181I constructed variants of HIV-1 RT, using UC-38 (compound (**9**), Table **1**) as a reference compound.



Scheme 1. Synthesis of thioacetamides *via* amide intermediates: (a)$SOCl_2$, $CHCl_3$, reflus, 30 minutes; (b) 5-chloro-2-aminopyridine, triethylamine, dioxane, reflux, 1 Hr; (c) Lawesson's reagent, toluene: benzene (6:1).

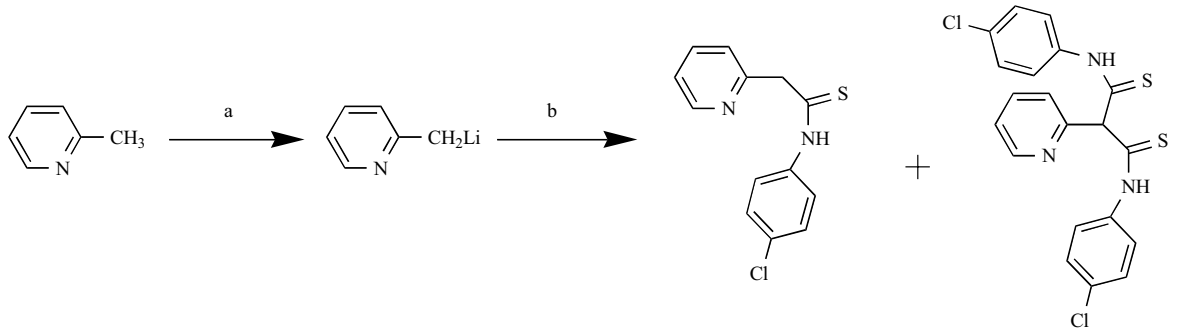
Compounds that with IC_{50} values of less than 100 μM were further subjected for evaluation in cell culture assays.

RESULTS AND DISCUSSION

Table **1** summarizes the IC_{50} values of the eight compounds and its activities against wild type enzyme and the three most common mutant strains. It is worthy to indicate that; in order to assess the importance of the sulfur atom, the precursor amides for compounds (**3**), (**5-8**) were also tested under the same assay conditions, but none showed any activity against neither the wild type nor the mutant strains. While most of the new compounds showed activities with IC_{50} values higher than 100 μM, three compounds (**2**, **4,** and **8)** exhibited activities with IC_{50} values less than 100 μM. Compound (**2**) showed moderate activity against purified L100I mutant RT enzyme, with an IC_{50} value of 70.1 μM. Compound (**4**) showed activity against the purified K103N mutant form of RT enzyme, with an IC_{50} value of 92.7 μM. Compound (**8**) showed selective activity only against the wild type RT with an IC_{50} value of 8.9 μM. The inhibitory effect manifested by the three thioamides is presumed to be through a non-competitive mode of enzyme inhibition as the case is with all other NNRTI. Compounds listed in (table **1**) can be classified into two groups; those molecules that would possibly form the 6-membered rigid conformation like those shown in (Fig. **1**) in brackets such as compounds (**1**), (**2**) and (**4**), and those that do not form that rigid conformation such as compound (**3**), and compounds (**5-8**). It is widely accepted that compound capable of forming the cyclic conformation are generally more active than those that do not [7]. To our surprise the thioamide (**8**), that does not have the capacity to form the rigid conformation, was the only derivative that showed activity against the wild type RT enzyme (with an IC_{50} of 8.9 μM). On the other hand, compound (**4**) that showed no activity against the wild type; found to be active against the mutant K103N with an IC_{50} of 92.7 μM. The mutant K103N is reported to be responsible for 20-50 fold increase in resistance to most if not all the available NNRTIs [17,18], and even is regarded to be sufficient by itself to cause virologic failure with each of the NNRTIs [19,20]. Compound (**5**), which is identical to compound (**4**) but with positions of the pyridyl group and the phenyl ring swabbed, showed no inhibitory activity, probably due to its incapability to form the rigid cyclic conformation. Interestingly, reducing the ethyl linker of compound (**4**) to a methylene group in compound (**2**) resulted in an activity against the L100I variant of RT with an IC_{50} of 70.1 μM. Further elimination of the methylene group (as in compound (**1**)) resulted in loss of activity. None of three thioamides with IC_{50} values below 100 μM (compounds (**2**), (**4**)& (**8**)) were found to have considerable activity when further tested in the in-vitro cell based XTT assay. The results of cell-based assay are presented in the (table **2**). We speculate that the low *in vitro* activity of these compounds contributed to such poor activity in the cell assay.

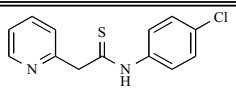
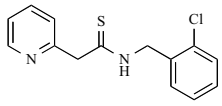
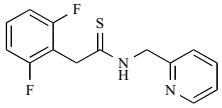
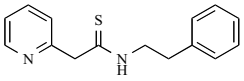
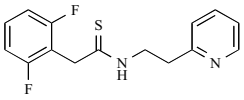
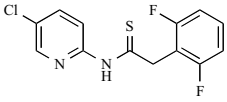
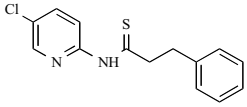
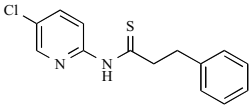
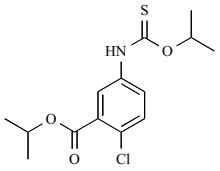
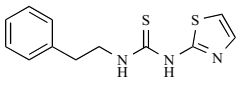
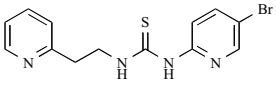
CONCLUSIONS

In conclusion, a number of thioamides were designed as bio-isosteric analogs for the drug trovirdine, synthesized and tested as non-nucleoside reverse transcriptase inhibitors. Three of the new analogs, namely compounds (**2**), (**4**) & (**8**), showed moderate activities against the wild type and resistant strains of the RT enzyme. The activity of compound (**4**) against mutant strain K103N is promising and constitutes a good starting point for further structural modifications through varying the aromatic rings and its substitution pattern to optimize activity against such devastating mutant strain.



Scheme 2. The isothiocyanate method: (a) BuLi, dry THF, DIPA, 1 Hr, -15 ^oC; (b) 4-chloro-phenylisothiocyanate, -15 ^oC to 22 ^oC.

Table 1. RT Inhibition Assay Results[a] for Thioamides

Entry	Compound	IC_{50} μM[b] WT	IC_{50} μM[b] K103N	IC_{50} μM[b] L100I	IC_{50} μM[b] Y181I
1		>100	>100	>100	>100
2		>100	>100	>100	>100
3		>100	>100	70.1	>100
4		>100	92.7	>100	>100
5		>100	>100	>100	>100
6		>100	>100	>100	>100
7		>100	>100	>100	>100
8		8.9	>100	>100	>100
9	 UC-38	0.024	45.5	27.4	71.3
10	 LY 73497	0.9[c]	ND[d]	ND[d]	ND[d]
11	 Trovirdine	0.017[e]	>100[f]	0.43[e]	2.5[e]

[a] The assay used rC/dG as the template/primer and dGTP as the substrate as described in reference [20].

[b] Values are means of two experiments. [c] IC_{50} value obtained from reference [7]. [d] Not determined. [e] IC_{50} value obtained from reference [23]. [f] data from reference [24] .

Table 2. Cell Based XTT Assay[a] for Compounds 2, 4 and 8

Compound	CEMSS/RF EC_{50} (μM)[b]	CEMSS/RF TC_{50} (μM)[c]	TI[d]
AZT	0.005	>1	>219
8	>200	46.7	--
4	>200	>200	--
2	>200	21.1	--
LY 73497[e]	1.3	>380	>292
Trovirdine[e]	0.02	87	4350

[a] The HIV inhibitory activities of the compounds were evaluated as described in ref. [22] by microtiter anti-HIV assays with CEM-SS cells. Quantification was performed with the tetrazolium dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2M-tetrazolium-5-carboxanilide (XTT). [b] The EC_{50} is the 50% inhibitory concentration for cytopathicity of HIV-1_{RF} in CEM-SS cells. [c] Toxicity data are reported as the quantity of drug required to reduce cell viability by 50% (TC_{50}). [d] Therapeutic index (TC_{50}/EC_{50}). [e] Inhibition values for LY 73497 and trovirdine obtained from ref. [7].

MATERIALS AND METHODS

Synthesis

The Thioamides 3,5,6,7 and 8 were Prepared According to Scheme 1 and Structures were Confirmed with the Following Analytical Data

N-(pyridin-2-ylmethyl)-2-(2,6-difluorophenyl)thioacetamide (3)

Yield: 62 % of the amide intermediate, mp 141-142° IR (KBr), ν 3290, 1652, 1592, 1558, 1470, 1061; ^{1}H NMR ($CDCl_3$): δ 3.70 (2H, s), 4.5 (2H, d, *J* = 5 Hz), 6.65-7.30 (5H, m), 7.60 (1H, dt, *J* = 2, 18 Hz), 8.45 (1H, dd, *J* = 1, 5 Hz). Anal. Calcd for $C_{14}H_{12}F_2N_2O$: C, 64.12; H, 4.61; N, 10.68. Found: C, 64.19; H, 4.36; N, 10.57 Yield: 57 % of the thioamide **3**, mp 119-120°. IR (KBr), 3160, 1622, 1592, 1553, 1487, 1135; ^{1}H NMR ($CDCl_3$): $CDCl_3$, δ 4.10 (2H, s), 4.8 (2H, d, *J* = 5 Hz), 6.65-7.30 (5H, m), 7.55 (1H, dt, *J* = 2, 18 Hz), 8.20 (1H, dd, *J* = 1, 5 Hz). Anal. Calcd for $C_{14}H_{12}F_2N_2S$: C, 60.42; H, 4.35; N, 10.07. Found: C, 60.62; H, 4.17; N, 10.02.

N-[(2-pyridin-2-yl)ethyl]-2-(2,6-difluorophenyl)thioacetamide (5)

Yield: 78 % of the amide intermediate, mp 125-125.5°C. IR ν 3290, 2925, 1642, 1590, 1553; ^{1}H NMR ($CDCl_3$): δ 2.95 (2H, t, *J* = 12 Hz), 3.60 (2H, s), 3.65 (2H, q, *J* = 18 Hz), 6.70-7.20 (5H, m), 7.4-7.70 (1H, dt, *J* = 2, 16 Hz), 8.2-8.4 (1H, dd, *J* = 1, 4 Hz). Anal. Calcd for $C_{15}H_{14}F_2N_2O$: C, 65.21; H, 5.11; N, 10.14. Found: C, 65.29; H, 4.99; N, 10.08. Yield: 49 % of the thioamide **5**, mp 100-101° IR (KBr) ν 3198, 2920, 1625, 1587, 1556, 1128, 784; ^{1}H NMR ($CDCl_3$): δ 2.95 (2H, t, *J* = 16 Hz), 4.0 (2H, q, *J* = 18 Hz), 4.1 (2H, s), 6.70-7.2 (5H, m), 7.3-7.60 (1H, dt, *J* = 2, 16 Hz), 7.95-8.1(1H, dd, *J* = 1, 4 Hz). Anal. Calcd for $C_{15}H_{14}F_2N_2S$: C, 61.63; H, 4.83; N, 9.58. Found: C, 61.57; H, 4.64; N, 9.40.

N-(5-chloropyridin-2-yl)-2-(2,6-difluorophenyl)thioacetamide (6)

Yield: 67 % of the amide intermediate, mp 138-139° IR (KBr) ν 3337, 1668, 1525, 1468, 1374, 1013; ^{1}H NMR ($CDCl_3$) δ 3.9 (2H, s), 6.60-7.35 (3H, m), 7.50 (1H, dd, *J* = 2, 10 Hz), 8.1 (1H, s), 8.2 (1H, d, *J* = 8 Hz), 9.0 (1H, s). Anal. Calcd for $C_{13}H_9ClF_2N_2O$: C, 55.24; H, 3.21; N, 9.91. Found: C, 55.38; H, 3.03; N, 9.72.Yield: 61 % of the thioamide **6** mp 115.5-116.5° IR (KBr) ν 3237, 1629, 1577, 1530, 1470, 1368; ^{1}H NMR ($CDCl_3$): δ 4.2 (2H, s), 6.60-7.35 (3H, m), 7.50 (1H, dd, *J* = 2, 10 Hz), 8.1 (1H, d, *J* = 2 Hz), 8.95 (1H, d, *J* = 8 Hz), 9.80 (1H, s). Anal. Calcd for $C_{13}H_9ClF_2N_2S$: C, 52.27; H, 3.04; N, 9.38. Found: C, 52.40; H, 2.85; N, 9.10.

N-(5-chloropyridin-2-yl)-3-phenylthiopropanamide (7)

Yield: 55 % of the amide intermediate, mp 127.5-128.5° IR (KBr) 3257, 1693, 1580, 1526, 1375, 1126; ^{1}H NMR ($CDCl_3$): δ 2.85 (2H, t, *J* = 16 Hz), 3.0 (2H, t, *J* = 15 Hz), 7.15 (5H, s), 7.45 (1H, dd, *J* = 2, 10 Hz), 8.0 (1H, d, *J* = 2 Hz), 8.10 (1H, d, *J* = 8 Hz), 8.80 (1H, s). Anal. Calcd For $C_{14}H_{13}ClN_2O$: C, 64.49; H, 5.03; N, 10.74. Found: C, 64.45; H, 4.75; N, 10.60. Yield: 84 % of the thioamide **7**, mp 108-109°. IR (KBr) 3204, 1529, 1452, 1390, 1130, 1111; ^{1}H NMR ($CDCl_3$): δ 3.10 (4H, s), 7.15 (5H, s), 7.55 (1H, dd, *J* = 2, 9 Hz), 8.10 (1H, d, *J* = 2 Hz), 9.0 (1H, d, *J* = 8 Hz), 10.0 (1H, s). Anal. Calcd for $C_{14}H_{13}ClN_2S$: C, 60.75; H, 4.73; N, 10.12. Found: C, 61.04; H, 4.63; N, 9.88.

N-(5-chloropyridin-2-yl)-4-phenylthiobutanamide (8)

Yield: 67 % of the amide intermediate, mp 103-104° IR (KBr) ν 3250, 2932, 1692, 1582, 1528, 1377; ^{1}H NMR ($CDCl_3$): δ 1.95-2.45 (4H, m), 2.65 (2H, t, *J* = 12 Hz), 7.15 (5H, s), 7.5 (1H, dd, *J* = 2, 8 Hz), 8.10 (1H, d, *J* = 2 Hz), 8.20 (1H, d, *J* = 9 Hz), 8.75 (1H, s). Anal. Calcd $C_{15}H_{15}ClN_2O$: C, 65.57; H, 5.50; N, 10.20. Found: C, 65.31; H, 5.44; N, 10.41. Yield: 68 % of the thioamide **8**, IR (KBr) ν 3211, 1570, 1522, 1492, 1298, 1114; ^{1}H NMR ($CDCl_3$): $CDCl_3$; δ 2.05 (2H, p), 2.25-3.0 (4H, t), 7.0 (5H, s), 7.60 (1H, dd, *J* = 2, 8 Hz), 8.15 (1H, d, *J* = 2 Hz), 8.80 (1H, d, *J* = 9 Hz). Anal. Calcd for $C_{15}H_{15}ClN_2S$: C, 61.95; H, 5.20; N, 9.63. Found: C, 61.91; H, 5.22; N, 9.46.

The Thioamides 1,2 and 3 were Prepared According to the Scheme 2 with the Analytical Data Listed Below

N-(4-chlorophenyl)-2-pyridin-2-ylthioethanamide (1)

Yield: 23 % mp 120-121°. IR (KBr) ν 3163, 1593, 1550, 1476, 1434, 1133, 746; ^{1}H NMR ($CDCl_3$): δ 4.25 (2H, s),

7.20 (2H, d, *J* = 8), 7.70 (2H, d, *J* = 9 Hz), 7.0-7.60 (3H, m), 8.50 (1H, dd, *J* = 1, 6 Hz). Anal. Calcd for $C_{13}H_{11}ClN_2S$: C, 59.42; H, 4.22; N, 10.66. Found: C, 59.83; H, 4.04; N, 10.51.

N-(2-chlorobenzyl)-2-pyridin-2-ylthioacetamide (2)

Yield: 16 % mp 123-124°C IR (KBr) 3164, 1595, 1550, 1434, 1125, 746; ^{1}H NMR ($CDCl_3$): δ 4.20 (2H, s), 4.95 (2H, d, *J* = 5 Hz), 6.95 (6H, m), 8.30 (1H, dd, *J* = 1, 6 Hz), 10.25 (1H, s). Anal. Calcd for $C_{14}H_{13}ClN_2S$: C, 60.75; H, 4.73; N, 10.12. Found: C, 61.05; H, 4.59; N, 10.00.

N-(2-phenethyl)-2-pyridin-2-ylethioethanamide (4)

Yield: 10 % mp 80-81° IR (KBr) ν 3177, 1565, 1475, 1448, 1128, 753; ^{1}H NMR ($CDCl_3$): δ 2.95 (2H, t, *J* = 16 Hz), 3.80 (2H, q, *J* = 18 Hz), 4.20 (2H, s), 7.10 (5H, broad s), 7.50 (1H, dt, *J* = 2, 16 Hz), 8.10 (1H, dd, *J* = 1, 6 Hz), 8.90 (1H, s). Anal. Calcd for $C_{15}H_{16}N_2S$: C, 70.27; H, 6.29; N, 10.93. Found: C, 70.41; H, 5.99; N, 10.91.

BIOLOGICAL EVALUATION

HIV-1 Reverse Transcriptase Assay

The compounds were submitted to Southern Research Institute (SRI)-Fredrick for the biological testing. The assay utilized recombinant, purified HIV-1 reverse transcriptase (RT) enzymes containing the following defines amino acid substitutions within the RT gene: K103N, L100I and Y181I. Additionally, wild type HIV-1 RT was also used as part of the evaluation panel. Characterization of the RT inhibitory properties of the test compounds was performed utilizing an RT assay described by Boyer *et al.* [21]; with minor modifications as described below. Briefly, recombinant RT enzymes were assayed in microtiter plates in a 100 µl reaction mixture containing 25 mM Tris-HCl, pH 8.0, 75 mM $MgCl_2$, 2 mM DTT, 10 µM dGTP, 0.01 U rC:dG template (Pharmacia), 10 µCi [^{32}P]-α-dGTP (800 Ci/mmol), and the test compound at the following concentrations: 0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM. the RT enzyme concentration employed in these assays ranged from 0.4-0.9 µg/ml for different recombinant proteins; all the RT preparations utilized in these studies displayed linear enzyme kinetics for at least 45 min at 37°C under these reaction conditions. For each assay, the reverse transcription enzyme reactions were allowed to proceed for 30 min at 37°C before termination of the enzyme reaction by addition of 10 µL of trichloroacetic acid (TCA); 10 µg of heat-denatured, sonicated salmon sperm DNA was also added to aid DNA precipitation and recovery. Upon termination of the enzyme reactions, the TCA precipitated DNAS was harvested onto glass-fiber filters (GF/C), washed twice with ice cold 10 % TCA and subjected to liquid scintillation counting. To increase sample throughput and minimize sample handling for this assay, they employed a 96-well glass-fiber plate and vacuum manifold (Millipore) to harvest and wash the DNA. The labeled DNA samples are subsequently counted directly in the multiwell plate by addition of 20 µl scintillation fluid (OptiPhase SuperMix, Wallac) to each well and using a MicroBeta 96-well scintillation counter (Wallac). Serial dilutions of each compound were included in individual reaction mixtures to determine if the compounds were inhibitory toward wild type HIV-1 RT enzyme as well as against the panel of RT enzymes that contained the defined amino acid substitutions. In conjunction with the biochemical assay described above, the test compounds which showed IC_{50} values lower than 100 µM were evaluated to determine their efficacy in preventing HIV-induced cell killing of CEM-SS cell as monitored with the XTT assay. The XTT assay provides information regarding the antiviral properties and toxicity of each test compound.

Anti-HIV-1 Cytoprotection Assay

Cell Preparation

CEM-SS cells were passaged in T-75 flasks prior to use in the anti-viral assay. On the day preceding the assay, the cells were split 1:2 to assure they were in an exponential growth phase at the time of infection. Total cell and viability quantification was performed using a hemacytometer and tryptan blue exclusion. The cell viability was greater than 95 % for the cells to be utilized in the assay. The cells were resuspended at 5 x 10^4 cells/ml in tissue culture medium and added to the drug-containing microtiter plates in a volume of 50 µl.

Virus Preparation

The virus for the assay was the lymphocytotropic virus strain HIV-1_{RF}. This virus was obtained from the NIH AIDS Research and Reference Reagent Program and was grown in CEM-SS cells for the production of stock virus pools. A pretittered aliquot of virus was removed from the freezer (-80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus was re-suspended and diluted into tissue culture medium such that the amount of the virus added to each well in a volume of 50 µl was the amount determined to give between 85 to 95 % cell killing at 6 days post infection. $TCID_{50}$ calculations by end point titration in CEM-SS cell indicated that the multiplicity of infection of these assays were approximately 0.01.

Plate Format

The format of the test plate has been standardized by SRI. Each plate contained cell control wells (cells only), virus control wells (cells plus virus), drug cytotoxicity wells (cells plus drug only), drug colorimetric control well (drug only) as well as experimental wells (drug plus cells plus virus). Samples were evaluated with triplicate measurement using 6 concentrations at half-log dilutions in order to determine IC_{50} and to measure cellular cytotoxicity, if detectable.

MTS staining for cell viability: At assay termination the assay plates stained with the soluble terazolium-based dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-caroxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to determine cell viability and quantify compound toxicity. MTS is metabolized in mitochondrial enzymes of metabolically active cells to yield a soluble formazan product, allowing the rapid quantitative analysis cell viability and compound toxicity. The MTS is a stable solution that does not require preparation before use. At termination of the assay, 20 µl of MTS was added per well. The wells were incubated overnight for the HIV cytoprotection assay at 37°C. the incubation intervals were chosen based on empirically determined times for optimal dye reduction in each cell type. Adhesive plate sealers

were used in place of the lids, the sealed plate was inverted several times to mix the soluble formazan product and the plate was read spectrophotometrically at 490 nm with a Molecular Devices Vmax plate reader.

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