Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 87–90

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Antioxidant Function of Phenethyl-5-bromo-pyridyl Thiourea Compounds with Potent Anti-HIV Activity

Yanhong Dong, T. K. Venkatachalam, Rama Krishna Narla, Vuong N. Trieu, Elise A. Sudbeck and Fatih M. Uckun*

Drug Discovery Program, Departments of Chemistry, Virology, Radiation Biology and Structural Biology, Hughes Institute, St Paul, MN 55113, USA

Received 1 June 1999; accepted 31 August 1999

Abstract—In a systematic search for novel dual function antioxidants with potent anti-HIV activity, we evaluated 9 rationally designed non-nucleoside inhibitors (NNI) of HIV-1 RT for antioxidant and anti-HIV activities. Our lead phenethyl-5-bromopyridyl thiourea (PEPT) compounds, *N*-[2-(2-methoxyphenylethyl)]-*N*′-[2-(5-bromopyridyl)]-thiourea (**2**) and *N*-[2-(2-chlorophenylethyl)]-*N*′-[2-(5-bromopyridyl)]-thiourea (**9**), inhibited the oxidation of ABTS to $ABTS^{\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide with EC_{50} values of 79 and 75 μM, respectively. Both compounds effectively inhibited the oxidation-induced green fluorescence emission from the free radical-sensitive indicator dye 2′,7′-dichlorodihydrofluorescein diacetate in CEM human T-cells and Nalm-6 human B-cells exposed to hydrogen peroxide. To our knowledge, compounds **2** and **9** are the first NNI of HIV-1 RT with potent anti-oxidant activity. Furthermore, the activity center was defined as the sulfhydryl group since alkylated PEPT derivatives were inactive. The presence of a free thiourea group was also essential for the anti-HIV activity of the PEPT compounds.

Introduction

Free radicals and other reactive oxygen species (ROS) are constantly generated in the human body and are involved in various physiologically important biologic reactions. However, high levels of free radicals (i.e. oxidative stress) can cause oxidative damage to biomolecules such as lipids, proteins and DNA within cells.[1] The oxidation of these biomolecules may play an important role in the pathogenesis of inflammatory diseases, atherosclerosis, aging, Alzheimer's disease, Parkinson's disease, stroke, cancer and AIDS.[2–4] Living organisms have antioxidant defense systems to remove excess damaging free radicals. Superoxide dismutases, catalases, glutathione peroxidases and glutathione reductase are such enzymatic defense systems. In addition, there are a variety of small molecules distributed widely in biological systems capable of scavenging free radicals non-enzymatically. These small molecules include glutathione, vitamin C, α-tocopherol (vitamin E), β-carotene, uric acid, taurine and hypotaurine. Synthetic or natural antioxidants are important in the management of severe oxidative stress conditions where oxidative stress cannot be adequately managed by the various components of the endogenous antioxidative defense system. Synthetic antioxidants that are currently being developed as therapeutic agents against oxidative stress include derivatives of natural antioxidants (e.g. α-tocopherol analogues), phenolic antioxidants (such as Probucol and Nitecapone), sulfhydryl-containing compounds (thiazolidine, ebselen, dithiolethiones) and inorganic coordination complexes as superoxide dismutase mimics.[5] Recent evidence suggests that oxidative stress-induced apoptosis of T-lymphocytes may also play an important role in the pathophysiology of AIDS. HIV-infected patients have subnormal levels of natural antioxidants in their plasma.[6–8] Therefore, oxidative stress-induced apoptosis may contribute to T-lymphocyte depletion in HIV-infected patients. Oxidative stress also contributes to the rising viral load in HIV-infected patients by activating NF kappa B, a cellular factor necessary for HIV transcription and replication.[9,10] A known antioxidant, *N*-acetylcysteine (NAC), exhibits potent antiviral activity in vitro by inhibiting these processes.[9,11–13] Consequently, dietary supplementation with vitamins E and C produces a trend toward a reduction in viral load.[14] In this study we evaluated the antioxidant activity of a series of phenethyl-5-bromopyridyl thiourea (PEPT) compounds with potent anti-HIV activity in order to identify new anti-HIV agents with antioxidant characteristics. All of our PEPT

*Corresponding author.

0960-894X/00/$ - see front matter
PII: S0960-894X(99)00581-8

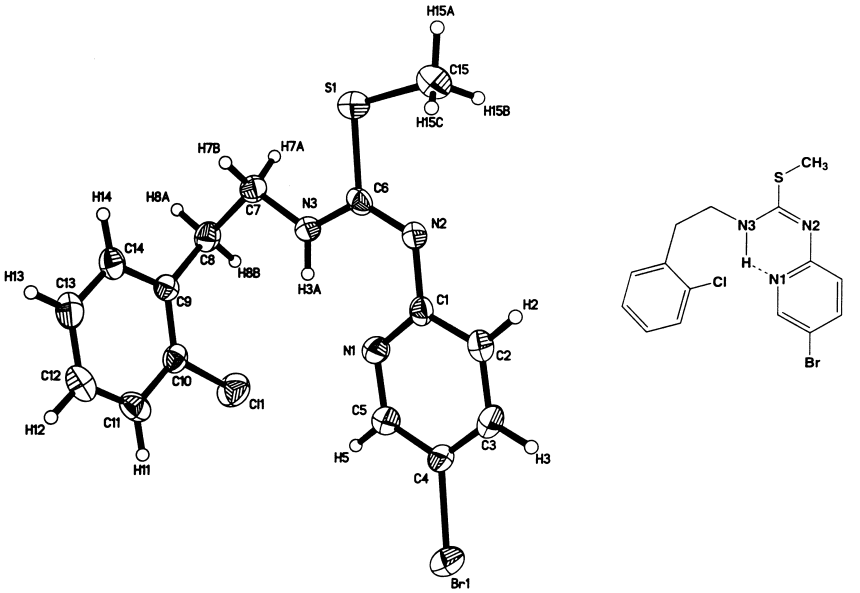


Figure 1. ORTEP drawing of compound 13 with atom-labeling scheme (30% probability ellipsoids, temp. = 296(2) K). Selected bond lengths (Å) are as follows: C(6)–S(1), 1.776(4); C(6)–N(2), 1301(5); C(6)–N(3), 1.329(4); N(1)···N(3), 2.656(4). $R = 0.052$, 3565 indep. Reflections, 191 parameters, goodness of fit on $F^2 = 0.908$, $\lambda = 0.71073$ Å.

compounds exhibited significant antioxidant activity. These agents may be particularly useful in the treatment of AIDS due to their dual function.

Cell-free assays of antioxidant activity

The antioxidant activity of the test compounds was measured using the total antioxidant status kit from Calbiochem, San Diego, CA, USA. This spectrophotometric assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide.[15] The amount of $ABTS^{\bullet+}$ produced was monitored at 620 nm using an ELISA plate reader every 5 min for 90 min. The rate of $ABTS^{\bullet+}$ production as ΔOD_{620}/min was plotted against drug concentrations (170, 80, 40 and 20 μM) for the determination of the EC_{50}, the concentration of the drug necessary for 50% inhibition of $ABTS^{\bullet+}$ production. Experiments were performed in triplicate.

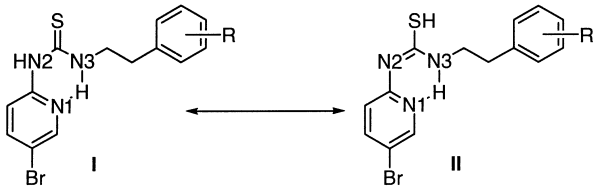
Cellular assays of antioxidant activity

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR, USA) is a free radical-sensitive indicator dye, which has been extensively used for evaluation of oxidative stress in cells.[16–19] This non-fluorescent dye emits green fluorescence upon oxidation by reactive oxygen species. CEM human T-cells (CCRF-CEM; ATCC, Rockville, MD, cat# CCL-119) and Nalm-6 human B-cells were incubated with the test compounds **1**–**9** (250 μM, 2 h incubation at 37°C in the dark), washed with fresh culture medium and then loaded with 20 μM H_2DCFDA for 30 min in the dark. Cells were washed to remove excess H_2DCFDA and exposed to 1 mM hydrogen peroxide for 15 min. Green fluorescence was measured by quantitative flow cytometry, as previously reported.[16–19] The percent reduction of H_2O_2-induced H_2DCFDA fluorescence was calculated using the formula: % Reduction = $100-[100\,(\mathbf{I}_{\text{compound}+H_2O_2}-\mathbf{I}_{\text{control}})/(\mathbf{I}_{H_2O_2}-\mathbf{I}_{\text{control}})]$, where $\mathbf{I}_{\text{compound}+H_2O_2}$, $\mathbf{I}_{H_2O_2}$, $\mathbf{I}_{\text{control}}$ are the mean H_2DCFDA fluorescence in cells treated with compound + H_2O_2, with H_2O_2 alone, or untreated, respectively.

Results and Discussion

Thiourea and 1,3-dimethyl-2-thiourea (DMTU) are effective scavengers of reactive oxygen intermediates (ROI).[20–24] DMTU was reported to be capable of preventing ROI-induced lung injury in vitro and in vivo.[21,25] In this study, we determined the antioxidant activities of a series of novel PEPT compounds with potent anti-HIV activity.[26–28]

The PEPT compounds that have been recently designed and synthesized in our laboratory are composed of a 5-bromopyridyl moiety and a substituted phenylethyl linked by a thiourea group.[26,27] The pyridylthiourea group in PCPT as well as in *S*-alkylated derivatives forms an intramolecular hydrogen-bonded heterocyclic ring observed by X-ray crystallography (Fig. 1), which would facilitate the formation of the sulfhydryl form (**II**) of the compounds (Scheme 1). Furthermore, in tautomer **I** the proton on N2 is expected to be more labile (relative to N3) due to the 5-bromopyridyl group and thus would be more involved in the formation of tautomer **II**. The existence of the sulfhydryl tautomeric form (**II**) was supported by the successful synthesis of *S*-alkylated PEPT derivatives (Table 1, compounds **10**–**13**) and consistent with the crystal structure of **13** (Fig. 1) which shows the deprotonated N2 and the protonated N3 (involved in a hydrogen bond with N1).

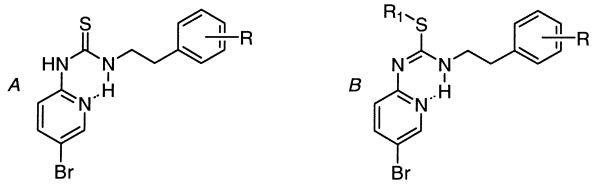


Scheme 1.

As shown in Table 1, all phenethyl-5-bromopyridyl thiourea compounds (**1**–**9**), exhibited antioxidant activity against the metmyoglobin/hydrogen peroxide-induced oxidation of ABTS at micromolar concentrations and also showed potent anti-HIV activity. The most active antioxidants, *N*-[2-(2-methoxyphenylethyl)]-*N*′-[2-(5-bromopyridyl)]-thiourea (**2**, $EC_{50} = 79$ μM) and *N*-[2-(2-chlorophenylethyl)]-*N*′-[2-(5-bromopyridyl)]-thiourea (**9**, $EC_{50} = 75$ μM), were more active than the unsubstituted parent compound (**1**, $EC_{50} = 98$ μM). *S*-Alkylation eliminated the antioxidant activity and the anti-HIV activity of the compounds (**10**–**13**), which indicated that the unalkylated thiourea group is critical to both antioxidant activity and anti-HIV activity. We also compared side by side the antioxidant activity of vitamin E and propyl gallate to estimate the potency of our sufhydryl-containing PEPT derivatives. As shown in Table 1, PEPT derivatives showed potent antioxidant activity of similar magnitude as vitamin E and propyl gallate. We examined the effects of the compounds on H_2O_2-induced oxidative stress in human lymphocyte cell lines, because oxidative stress-induced apoptosis may contribute to lymphocyte depletion. As shown in Figure 2 and Table 1, compounds **2** and **9** effectively inhibited the oxidation-induced green fluorescence emission from the free radical-sensitive indicator dye 2′,7′-dichlorodihydrofluorescein diacetate in CEM human T-lymphocytes exposed to hydrogen peroxide. The antioxidant activity of these compounds was not limited to T cells since similar effects were also observed in NALM-6 human pre-B-cells (Table 1). The values for the percent reduction of H_2DCFDA fluorescence intensity (as measured by the mean channel of green fluorescence) by compound **2** were 72.3% in CEM cells and 94.2% in NALM-6 cells. The values for the percent reduction of H_2DCFDA fluorescence intensity (as measured by the mean channel of fluorescence) by compound **9** were 56.3% in CEM cells and 32.0% in NALM-6 cells.

We have previously reported that compounds **2** and **9** inhibit HIV replication in peripheral blood mononuclear cells with average IC_{50} values of 0.01 μM and < 0.001 μM, respectively.[26] To our knowledge, these phenethyl-5-bromopyridyl thiourea compounds are the first dual-function NNI of HIV-1 RT discovered to have antioxidant activity. We postulate that the sulfhydryl group (**II**) (Scheme 1) is responsible for the antioxidant activity due to its favorable electron-donating characteristics.[21,29] In PEPT, the phenyl group does not directly connect to the thiourea group, therefore, the electron donating (or withdrawing) ability of the substituents on the phenyl ring should not significantly

Table 1. Antioxidant activity of phenethyl-5-bromopyridyl thiourea compounds



Compound	R (*A*) or R, R_1 (*B*)	EC_{50} anti-ox.[a] (μM)	% Reduction of H_2O_2-induced H_2DCFDA fluorescence[b]		IC_{50} rRT[c] (μM)
			CEM T-cells	NALM-6 B-cell	
1	H	98	32.1	37.1	1.3[d]
2	2-OMe	79	72.3	94.2	1.0[e]
3	3-OMe	90	19.7	30.4	0.4[e]
4	4-OMe	176	21.4	0.0	0.9[e]
5	2,5-di-OMe	89	29.4	11.8	0.1[d]
6	2-F	109	47.0	—	0.6[e]
7	3-F	100	45.0	—	0.7[e]
8	4-F	89	13.0	2.0	6.4[e]
9	2-Cl	75	56.3	32.0	0.7[e]
10	2,5-di-OMe,$C_6H_5CH_2$-	> 1000	—	—	> 100[d]
11	2-F,$C_6H_5CH_2$-	> 1000	—	—	> 100[d]
12	2-Cl,$C_6H_5CH_2$-	> 1000	—	—	> 100[d]
13	2-Cl,CH_3-	> 1000	—	—	> 100[d]
AZT		> 1000	—	—	> 100[e]
Vitamin E		377	—	—	—
Propyl gallate		84	—	—	—

[a]EC_{50} anti-ox. values represent the inhibition of the oxidation of ABTS to $ABTS^{\bullet+}$ by metmyoglobin in the presence of H_2O_2.
[b]Percent reduction of H_2O_2-induced H_2DCFDA fluorescence.
[c]IC_{50} rRT values represent the inhibition of recombinant HIV reverse transcriptase.
[d]This work.
[e]ref 26.

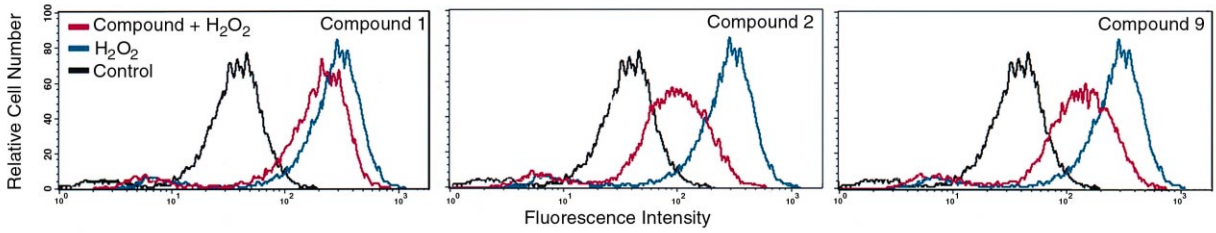


Figure 2. Antioxidant activity of phenethyl-5-bromopyridyl thiourea compounds against H_2O_2-induced oxidative stress in CEM T-cells. The cells were preincubated with the compounds for 2 h, loaded with H_2DCFDA and then challenged with H_2O_2. H_2DCFDA fluorescence was measured by quantitative flow cytometry. The fluorescence intensity of untreated control cells, H_2O_2-treated cells, and H_2O_2 + compound treated cells are shown in black, blue and red, respectively.

affect their antioxidant activity; however, it may be responsible for the subtle differences in activity among the various sulfhydryl-containing PEPT derivatives. To better understand the role played by the sulfhydryl group in antioxidant activity, we synthesized *S*-alkylated derivatives of compound **10** and others (Table 1) which lack a sulfhydryl group. Alkylation was achieved by using methyl as well as benzyl groups. None of the alkylated derivatives were active as an antioxidant (Table 1), which is consistent with the hypothesis that the sulfhydryl group of tautomer **II** (Scheme 1) is responsible for the antioxidant activity. Furthermore, alkylated derivatives did not show any anti-HIV activity, implicating for the first time that this unalkylated thiourea group is also critical for anti-HIV activity. The lack of anti-HIV activity of the alkylated derivatives is probably due to impaired binding to the NNI pocket because of steric hindrance caused by the substituents.

In conclusion, a series of PEPT compounds with anti-HIV activity were found to have potent antioxidant activity as well. These dual-function compounds may be particularly useful in the treatment of AIDS, as antioxidants have been shown to suppress viral replication and lymphocyte depletion. Further studies are in progress to determine the actual antioxidant mechanism of these novel PEPT derivatives.

References and Notes

1. Halliwell, B.; Cross, C. E. *Environ. Health Perspect* **1994**, *102*, 5–12.
2. Martinez-Cayuela, M. *Biochimie* **1995**, *77*, 147–262.
3. Lee, R.; Beauparlant, P.; Elford, H.; Ponka, P.; Hiscott, J. *Virology* **1997**, *234*, 277–290.
4. Chanarat, N.; Chanarat, P.; Suttajit, M.; Chiewsilp, D. *J. Med. Assoc. Thai.* **1997**, *80*, S116–S119.
5. Packer, L.; Cadenas, E., Eds.; *Handbook of Synthetic Antioxidants*; Marcel Decker: New York, 1996.
6. Pace, G. W.; Leaf, C. D. *Free Rad. Biol. Med.* **1995**, *19*, 523–528.
7. Israel, N.; Gougerot-Pocidalo, M. A. *Cell Mol. Life Sci.* **1997**, *53*, 864–870.
8. Allard, J. P.; Aghdassi, E.; Chau, J.; Salit, I.; Walmsley, S. *Am. J. Clin. Nutr* **1998**, *67*, 143–147.
9. Malorni, W.; Rivabene, R.; Lucia, B. M.; Ferrara, R.; Mazzone, A. M.; Cauda, R.; Paganelli, R. *AIDS Res. Hum. Retroviruses* **1998**, *14*, 1589–1596.
10. Dobmeyer, T. S.; Findhammer, S.; Dobmeyer, J. M.; Klein, S. A.; Raffel, B.; Hoelzer, D.; Helm, E. B.; Kabelitz, D.; Rossol, R. *Free Rad. Bio. Med.* **1997**, *22*, 775–785.
11. Raju, P. A.; Herzenberg, L. A.; Herzenberg, L. A.; Roederer, M. *AIDS Res. Hum. Retroviruses* **1994**, *10*, 961–967.
12. Aillet, F.; Gougerot-Pocidalo, M. A.; Virelizier, J. L.; Israel, N. *AIDS Res. Hum. Retroviruses* **1994**, *10*, 405–411.
13. Staal, F. J.; Roederer, M.; Raju, P. A.; Anderson, M. T.; Ela, S. W.; Herzenberg, L. A.; Herzenberg, L. A. *AIDS Res. Hum. Retroviruses* **1993**, *9*, 299–306.
14. Allard, J. P.; Aghdassi, E.; Chau, J.; Tam, C.; Kovacs, C. M.; Salit, I. E.; Walmsley, S. L. *AIDS* **1998**, *12*, 1653–1659.
15. Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. *Clinical Science* **1993**, *84*, 407–412.
16. Yuan, L.; Inoue, S.; Saito, Y.; Nakajima, O. *Exp. Cell Res* **1993**, *209*, 375–381.
17. Casado, J. A.; Merino, J.; Cid, J.; Subira, M. L.; Sanchez-Ibarrola, A. *J. Immunol. Methods* **1993**, *159*, 173–176.
18. Model, M. A.; Kuruga, M. A. J. *Immunol. Methods* **1997**, *202*, 105–111.
19. Kalinich, J. F.; Ramakrishnan, N.; McClain, D. E. *Free Rad. Res.* **1997**, *26*, 37–47.
20. Wasil, M.; Halliwell, B.; Grootveld, M.; Moorhouse, C. P.; Hutchison, D. C. S.; Baum, H. *Biochem. J.* **1987**, *243*, 867–870.
21. Fox, R. B. *J. Clin. Invest.* **1984**, *74*, 1456–1464.
22. Curtis, W. E.; Muldrow, M. E.; Parker, N. B.; Barkley, R.; Linas, S. L.; Repine, J. E. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 3422–3425.
23. Beehler, C. J.; Simchuk, M. L.; McCord, J. M.; Repine, J. E. *J. Lab. Clin. Med.* **1992**, *119*, 508–513.
24. Kelner, M. J.; Bagnell, R.; Welch, K. J. *J. Biol. Chem.* **1990**, *265*, 1306–1311.
25. Lai, Y.-L.; Wu, H.-D.; Chen, C.-F. *J. Cardiovascular Pharmacology* **1998**, *32*, 714–720.
26. Vig, R.; Mao, C.; Venkatachalam, T. K.; Tuel-Ahlgren, L.; Sudbeck, E. A.; Uckun, F. M. *Bioorg. Med. Chem.* **1998**, *6*, 1789–1797.
27. Mao, C.; Vig, R.; Venkatachalam, T. K.; Sudbeck, E. A.; Uckun, F. M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2213–2218.
28. All new compounds gave satisfactory NMR and MS results. The crystal structure of compound **13** was solved and its NMR, IR and MS data are as follows: ^{1}H NMR ($CDCl_3$) δ 10.52 (bs, 1H), 8.11–8.10 (dd, 1H), 7.62–7.59 (dd, 1H), 7.39–7.36 (m, 1H), 7.29–7.19 (m, 3H), 6.92–6.89 (dd, 1H), 3.69–3.62 (m, 2H), 3.10–3.06 (t, 2H), 2.49 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 159.8, 145.8, 142.1, 139.7, 136.0, 134.1, 131.1, 129.6, 128.1, 126.7, 122.5, 111.5, 43.1, 34.2, 13.9. IR (KBr) ν 3067, 2927, 2871, 2845, 2360, 2339, 1600, 1561, 1452, 1416, 1365, 1230, 1091, 1055, 998, 967, 916, 838, 751, 679, 642, 586, 514 cm^{-1}. MALDI-MS: calcd for $[M+H]^+$ ($M=C_{15}H_{15}BrClN_3S$): 385.4; found: 385.9.
29. Hicks, M.; Wong, L. S.; Day, R. O. *Biochem. Pharmacol* **1992**, *43*, 439–444.