Brief Articles

Analysis of 6-(2,2-Dichloroacetamido)chrysene Interaction with the Hypoxanthine Phosphoribosyltransferase from *Trypanosoma cruzi*

Francisco J. Medrano,[†] Mary A. Wenck,[‡] Juan C. Engel,[§] and Sydney P. Craig, III*

Laboratory of Molecular Parasitology & Drug Design, Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3044 and San Francisco VA Medical Center, San Francisco, California 94121

Selective inhibition is needed for drugs targeting the hypoxanthine phosphoribosyltransferase of *Trypanosoma cruzi*, etiologic agent of Chagas' disease. 6-(2,2-Dichloroacetamido)chrysene, was shown herein to be a selective inhibitor of the trypanosomal enzyme. SAR analysis revealed that the 6-amido moiety was essential, but the dichloroaceto moiety was not essential for achieving the low K_i for this inhibitor. Understanding the molecular basis for these interactions could facilitate the design of selective inhibitors without a chrysene moiety.

Introduction

Chagas's disease results from infection by the protozoan parasite, *Trypanosoma cruzi*. More than 16 million inhabitants of Central and South America and 300 000 immigrants to the United States are infected with this disease.[1] Chagas' disease is a major cause of death due to heart failure and continues to have a negative impact on the economic growth of several countries in Latin America.[2] The drug Benznidazole (Rochagan, Roche 7−1051, Radamil) has been demonstrated to have efficacy approaching 56% in the treatment of acute and indeterminate cases of the disease. However, there is presently no available drug recommended for patients with evidence of cardiomyopathy or one of the megasyndromes (mega colon or mega esophagus).[2] Thus, new drugs with improved efficacy are needed for the treatment of this disease.

The hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8; inosine monophosphate:pyrophosphate phosphoribosyltransferase) of *T. cruzi* has been identified as a potential target for drugs in the treatment of Chagas' disease.[3] Inhibitors of the enzyme have been shown to kill insect stage parasites (epimastigotes) in axenic cell cultures and to block the replication of amastigotes in infected mammalian cells. Partial reversal of both the killing of epimastigotes and the replication of amastigotes, by the addition of excess substrate (hypoxanthine) to the media, provides evidence for HPRT being the target of the lead inhibitors.[3]

Defects in the human HPRT can result in gouty arthritis[4,5] or Lesch−Nyhan syndrome.[6,7] Thus, for FDA approval, drugs targeted to the HPRT of a human parasite are likely to be required to be selective inhibitors of the enzymes from the parasite. In the present study, 6-(2,2-dichloroacetamido)chrysene was shown to be a selective inhibitor of the HPRT from *T. cruzi*. Furthermore, studies of structure−activity relationships (SAR) revealed that the dichloroaceto moiety of this inhibitor was not essential for potent inhibition of the trypanosomal HPRT, but the formation of a hydrogen bond involving the 6-amino group potentially was essential for interactions contributing to the sub-micromolar inhibition constants of chrysene-containing compounds.

Although chrysene-containing compounds are likely to be tumorogenic, understanding the molecular basis for the selective inhibition of the trypanosomal HPRT could help in guiding organic synthetic strategies intended to improve target specificity among non-chrysene-containing drug leads.

Table 1. Kinetic Inhibition Constants for Lead Inhibitors with HPRTs

lead inhibitor[a]	K_i vs *T. cruzi* HPRT, μM	K_i vs human HPRT, μM	K_i vs bacterial HPRT, μM
1159	2.2 ± 0.2 (C)	5.4 ± 1.1 (C)	-
32980[b]	0.5 ± 0.06 (C)	16.0 ± 9.2 (M)	≫25
141430	11.8 ± 0.8 (U−C)	28.8 ± 4.7 (M)	48.1 ± 1.8 (M)
156385	2.0 ± 0.2 (C)	10.9 ± 1.6 (C)	13.9 ± 0.7 (C)

[a] MFCD no.'s and K_i's vs the HPRT of *T. cruzi* are from Freymann et al.[3] The K_i's shown were determined with PRPP as the variable substrate: (C) = competitive, (U−C) = uncompetitive, and (M) = mixed inhibition. [b] 6-(2,2-dichloroacetamido)-chrysene.

Materials and Methods

Chrysene (98% pure), 6-nitrochrysene (95% pure), and 6-aminochrysene (97% pure) were from Aldrich. 6-Fluorochrysene, from Sigma Aldrich rare chemicals, formerly has been purified by crystallization and purity checked by NMR.[8] For the present study, the commercial product was reexamined by NMR and found to be approximately 90% pure with the major contaminant appearing to be nonfluorinated chrysene.

Compounds previously reported to be inhibitors of the HPRT from *T. cruzi*[3] are identified in Table 1 by their MFCD numbers

* Corresponding author. Telephone: (860) 486-1831, facsimile: (860) 486-1784, e-mail: scraig@uconnvm.uconn.edu.

[†] Present address: Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Sincrotron, Caixa Postal 6192, Campinas − SP, CEP 13084-971, Brasil.

[‡] Present address: Department of Epidemiology, School of Public Health, University of North Carolina, Chapel Hill, NC 27599-7453.

[§] San Francisco VA Medical Center and Department of Pathology at UCSF, 4150 Clement St., San Francisco, CA 94121.

10.1021/jm030061i CCC: $25.00
Published on Web 05/10/2003

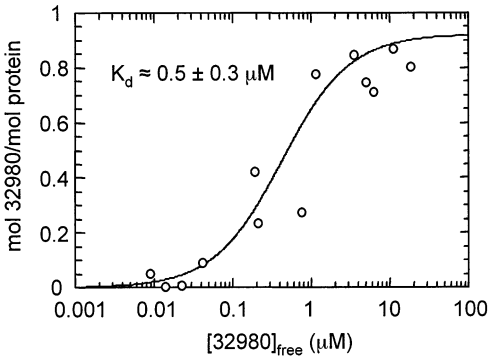

Figure 1. Binding isotherm of 6-(2,2-dichloroacetamido)-chrysene (MFCD no. 32980) to the trypanosomal HPRT.

provided by MDL Information Systems. These include the 2,4,7-trinitro-9-fluorenylidenemalononitrile from ACROS (MFCD no. 1159), 6-(2,2-dichloroacetamido)chrysene from Aldrich (MFCD no. 32980), 3-(2-fluorophenyl)-5-(2,4-dichlorophenoxy)-1,2,4-triazolo[4,3-*c*]quinazoline from Bionet (MFCD no. 141430), and 3,5-diphenyl-4′-methyl-2-nitrobiphenyl from Aldrich (MFCD no. 156385). The purities of compounds 32980, 141430, and 156385 were formerly confirmed by reverse phase HPLC.[3] For the present study, 6-(2,2-dichloroacetamido)chrysene was examined by NMR and again confirmed to be pure.

Recombinant HPRTs were purified by affinity chromatography using GMP-agarose as described.[9] Enzyme concentrations were determined by the Bradford method using immunoglobulin G (IgG) as the standard protein (Protein Assay Kit, Bio-Rad, Hercules, CA).

Fluorescence Spectroscopy. Fluorescence measurements were made with an Aminco SLM 8100 spectrofluorometer. Excitation and emission bandwidths were 4 and 8 nm, respectively. The fluorescence cell (0.5 × 1.0 cm) was mounted on a thermostated holder. The inner filter effect was minimized by using sample absorbances of less than 0.05.

Binding Measurements. Binding measurements were carried out by fluorometric titration and were conducted as follows. The fluorescence of the free ligand was measured, and its value (almost negligible) was subtracted from all further measurements. The fluorescence intensity of bound ligand was determined by titrating a fixed concentration of ligand (one that gave no appreciable inner filter effect) with HPRT until the fluorescence signal due to bound ligand reached saturation, thereby yielding the fluorescence intensity per micromolar concentration of ligand. All measurements were made at 25 °C in 20 mM bis-Tris buffer at pH 7.5 containing 6 mM $MgCl_2$ using an excitation wavelength of 325 nm and an emission wavelength of 395 nm. The ligand excitation wavelength was selected to avoid excitation of intrinsic protein fluorescence. Subsequently, fluorescence was monitored with the concentration of enzyme fixed and at various increasing concentrations of ligand (Figure 1). Bound ligand was estimated from its corrected fluorescence (subtracting fluorescence due to ligand alone), and the free ligand concentration was calculated as the difference between the known total concentration and the determined bound concentration of ligand. The values of the binding equilibrium constant and the number of sites were obtained from Scatchard plots of the data.

Determinations of K_i Values. A continuous spectrophotometric assay[3,10] was used for HPRT activity studies and the determination of inhibition constants. All measurements were carried out in 100 mM Tris-HCl, pH 7.5, and 12 mM $MgCl_2$ at 37 °C. Enzymes were briefly preequilibrated with PRPP and inhibitor at 37 °C before initiating reactions by the addition of hypoxanthine as described.[3] Production of IMP was monitored continuously at a wavelength of 245 nm. First-order reaction kinetics was approximated for the multisubstrate reaction by holding one substrate at a fixed concentration and varying the other. In this manner Lineweaver−Burk plots were generated for inhibitor effects versus the variable substrate and then the experiments were repeated varying the second substrate.[11] For a two substrate reaction, the ideal concentration for the fixed substrate should be at or near its K_m.[11] The rationale for this is that if a bound inhibitor overlaps the binding domains of both substrates, then high to saturating concentrations of the second substrate could interfere with inhibitor binding. In practice, the actual concentration for the fixed substrate needed to be above its K_m value in order to obtain significant rates for the reactions at low concentrations of the variable substrate. For the studies reported here, the concentration of hypoxanthine was fixed at 60 μM, while PRPP was varied between 13 and 215 μM. The concentration of the enzyme for these assays was at 300 nM, which was well below the lowest concentration tested for the inhibitors. For each K_i determination, more that 40 assays were conducted and no fewer than 16 individual data points were used in the final calculations. Results were analyzed using the program "EnzymeKinetics" (copyright 1990, 1991 by Jacek Stanislawski, from Trinity Software, Campton, NH). Lineweaver−Burke plots were used to classify the type of inhibition as competitive, noncompetitive, mixed, or uncompetitive based on the point of intersection for the lines.[3] All K_i values were from nonlinear regression analysis of the data.

Results and Discussion

Previously, four unrelated compounds targeted to the HPRT from *T. cruzi* were identified as leads for drug design.[3] Three of these compounds were shown to be effective against the pathogen, but 6-(2,2-dichloroacetamido)chrysene was ineffective as an inhibitor of parasite growth. Initially, this compound was included as a lead for drug design because it yielded a sub-micromolar K_i versus the trypanosomal HPRT.

For the present study, fluorescence spectroscopy was used to estimate the kinetic binding constant (K_d) of 6-(2,2-dichloroacetamido)chrysene for the trypanosomal HPRT (Figure 1). The data show that the K_d of 0.5 μM for 6-(2,2-dichloroacetamido)chrysene with the trypanosomal HPRT is consistent with the K_i of 0.5 μM formerly reported for this compound.[3]

Many of the amino acids surrounding the active site of the trypanosomal HPRT are identical to those in the active site of the homologous human enzyme. For this reason, it is widely believed that the design or discovery of a selective inhibitor targeted to the active site of the trypanosomal HPRT could be difficult, if not an impossible task. With this in mind, each of the previously reported drug design leads was tested kinetically as an inhibitor of the human HPRT catalyzed reaction, and three of the four compounds also were tested as inhibitors the *Salmonella* HPRT.[12] Of the three inhibitors that showed bioactivity toward live trypanosomes, all yielded K_i's values versus the human, bacterial, and trypanosomal enzymes that were within a 5-fold range of concentration of each of the inhibitors (Table 1). However, 6-(2,2-dichloroacetamido)chrysene (MFCD no. 32980) yielded a K_i versus the human enzyme that was 32-fold higher than that obtained with the trypanosomal HPRT. Furthermore, this compound did not significantly inhibit the HPRT from *S. typhimurium* at concentrations approaching 50-fold above its K_i value with the trypanosomal enzyme.

These results demonstrate that selective inhibition of the trypanosomal HPRT is possible and provide further justification for the inclusion of 6-(2,2-dichloroacetamido)chrysene among the leads for drug design. Furthermore, if the molecular basis for the selective inhibition of the trypanosomal enzyme were elucidated, this

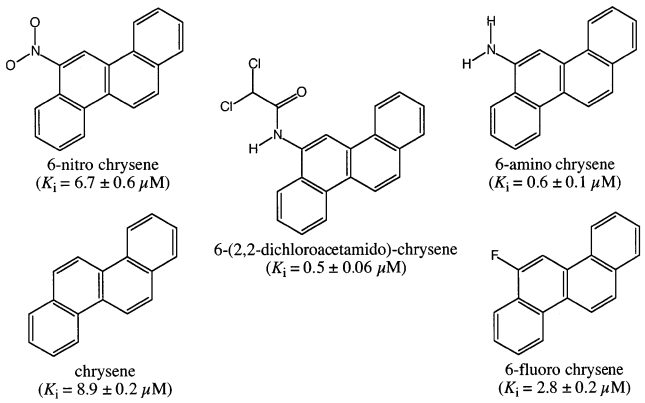

Figure 2. Analogues of 6-(2,2-dichloroacetamido)chrysene were acquired and tested as inhibitors of the HPRT from *T. cruzi*. Kinetic inhibition constants (K_i's) were determined for HPRT-catalyzed reactions with variable concentrations of PRPP and fixed concentrations of hypoxanthine. All of these compounds showed competitive inhibition versus PRPP as the variable substrate.

information could be used to guide synthetic strategies for compounds designed to be selective inhibitors of the target enzyme, but which would not possess a potentially toxic moiety such as chrysene.

The commercial availability of a several chrysene-containing compounds enabled the initiation of an analysis of structure−activity relationships (SAR) for compounds related to 6-(2,2-dichloroacetamido)chrysene without the need for synthesis of novel compounds. The results of these studies (Figure 2) show that 6-amino-chrysene yielded a K_i of 0.6 μM versus the trypanosomal HPRT, which was very close to the K_i of 0.5 μM for 6-(2,2-dichloroacetamido)chrysene. Since both of these compounds were ≥97% pure, the results suggest that the dichloroaceto moiety of the lead inhibitor does not contribute significantly to favorable interactions with the active site of the trypanosomal HPRT. Also, since the K_i's for chrysene (8.9 μM), 6-fluoro-chrysene (2.8 μM), and 6-nitro-chrysene (6.7 μM) were significantly higher than for 6-amino chrysene (at 0.6 μM), a hydrogen bond involving the 6-amino moiety is likely essential for achieving the low kinetic inhibition constants for the chrysene containing inhibitors.

Like 6-(2,2-dichloroacetamido)chrysene, 6-amino-chrysene at 10 μM was ineffective as an inhibitor of parasite growth in infected mouse macrophage cells (results not shown). Possibly the absence of in vivo activity was due to the inability of chrysene-containing compounds to diffuse or be transported through the plasma membranes of host cells. Nevertheless, these results provide a foundation for the initiation of synthetic strategies intended to enhance binding interactions with the trypanosomal HPRT and to improve the pharmacological properties of both chrysene and non-chrysene-containing lead inhibitors.

Although interactions between 6-(2,2-dichloroacetamido)chrysene and the trypanosomal HPRT have been predicted computationally,[3] efforts are underway to solve crystal structures of both the trypanosomal and human HPRTs with this compound or 6-aminochrysene bound in the active sites. These crystal structures should provide details of interactions associated with the selective inhibition of the trypanosomal enzyme, and this information might be used in the design of selective inhibitors without chrysene.

Acknowledgment. This research was supported by NIH Grant Numbers RO1-AI45021 and PO1-AI35707. We thank Martha Morton, Director of the NMR Laboratory at the University of Connecticut, for analysis of compound purities.

Appendix

Abbreviations: HPRT, EC 2.4.2.8, hypoxanthine phosphoribosyltransferase; PRPP, 5-phospho-α-D-ribosyl-1-pyrophosphate; PPi, inorganic pyrophosphate; IMP, inosine monophosphate; GMP, guanosine monophosphate; Hx, hypoxanthine; DTT, dithiothrietol.

References

(1) Panosian, C. The parasite of the poor. *Discover* **1998**, *19*, 38−41.
(2) Magill, A. J.; Reed, S. G. American Trypanosomiasis. *Hunter's Tropical Medicine and Emerging Infectious Diseases*, 8th ed.; W. B. Saunders Company: Philadelphia, 2000; pp 653−664.
(3) Freymann, D. M.; Wenck, M. A.; Engel, J. C.; Feng, J.; Focia, P., J.; Eakin, A. E.; Craig, S. P., III. Efficient identification of inhibitors targeting the closed active site conformation of the HPRT from *Trypanosoma cruzi*. *Chem. Biol.* **2000**, *7*, 957−968.
(4) Kelley, W. N.; Greene, M. L.; Rosenbloom, F. M.; Henderson, J. F.; Seegmiller, J. E. Hypoxanthine-guanine phosphoribosyltransferase deficiency in Gout. *Ann. Intern. Med.* **1969**, *70*, 155−206.
(5) Sculley, D. G.; Dawson, P. A.; Emmerson, B. T.; Gordon, R. B. A review of the molecular basis of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. *Human Genet.* **1992**, *90*, 195−207.
(6) Seegmiller, J. E.; Rosenbloom, F. M.; Kelley, W. N. Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science* **1967**, *155*, 1682−1684.
(7) Nyhan, W. L. The recognition of Lesch-Nyhan syndrome as an inborn error of purine metabolism. *J. Inherited Metab. Dis.* **1997**, *20*, 171−178.
(8) Laali, K. K.; Hollenstein, S.; Harvey, R. G.; Hansen, P. E. Stable ion studies of the chrysene skeleton. Protonation of chrysene, 6-halochrysenes, 6-acetylchrysene, and 4*H*-cyclopental[*def*]chrysene: NMR studies of charge distribution in chrysenium cations and AM1 calculations. *J. Org. Chem.* **1997**, *62*, 4023−4028.
(9) Eakin, A. E.; Guerra, A.; Focia, P. J.; Torres-Martinez, J.; Craig, S. P. 3rd. Hypoxanthine Phosphoribosyltransferase from *Trypanosoma cruzi* as a Target for Structure-Based Inhibitor Design: Crystallization and Inhibition Studies with Purine Analogues. *Antimicrob. Agents Chemother.* **1997**, *41*, 1686−1692.
(10) Yuan, L.; Craig, S. P., III; McKerrow, J. H.; Wang, C. C. Steady-State Kinetics of the Schistosomal Hypoxanthine-Guanine Phosphoribosyltransferase. *Biochemistry* **1992**, *31*, 806−810.
(11) Segel, I. H. Simple inhibition systems. *Enzyme Kinetics; behavior and analysis of rapid equilibrium and steady-state enzyme systems*; John Wiley & Sons: New York, 1975; pp 100−160.
(12) Lee, C. C.; Craig, S. P., III; Eakin, A. E. A single amino acid substitution in the human and a bacterial hypoxanthine phosphoribosyltransferase modulates specificity for the binding of guanine. *Biochemistry* **1998**, *37*, 3491−3498.

JM030061I