Bioorganic & Medicinal Chemistry Letters 19 (2009) 6379–6381

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Identification of the amino acid-AZT-phosphoramidase by affinity T7 phage display selection

Jilin Cheng [a,b], Xin Zhou [a], Tsui-Fen Chou [a], Brahma Ghosh [a], Baoling Liu [a], Carston R. Wagner [a,*]

[a] Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA
[b] Shanghai Public Health Clinical Center affiliated to Fudan University, Shanghai, China

ARTICLE INFO

Article history:
Received 25 July 2009
Revised 17 September 2009
Accepted 17 September 2009
Available online 22 September 2009

Keywords:
Pronucleotide
AZT
Prodrug
Hint1
Phosphoramidate

ABSTRACT

A CEM cell cDNA T7 phage display library was prepared and used to screen for activating enzymes of phosphoramidate prodrugs of AZT monophosphate. Although, inefficient compared to ribonucleotide based phosphoramidates, hHint 1 was identified as the likely intracellular pronucleotide activating enzyme.

Nucleosides are an important class of agents for both viral and cancer chemotherapy, as well as immunosuppressive, anti-ischemic and antibacterial agents. Unfortunately, the therapeutic usefulness of nucleosides has been limited by their short half-life in vivo and the requirement for intracellular phosphorylation.

In theory, the requirement for phosphorylation could be overcome by the use of nucleotides. Nucleotides, however, are too polar to cross most cell membranes and are rapidly dephosphorylated by plasma and membrane bound phosphatases.[1] The development of a prodrug strategy (i.e., pronucleotide) for the in vivo delivery of nucleotides has been proposed.[1]

Previous work from our laboratory has demonstrated that amino acid phosphoramidate monoesters of antiviral and antitumor nucleosides are water soluble, non-toxic, highly stable and potent antiviral and antitumor agents.[2–5] Mechanistic studies of phosphoramidates of nucleosides, such as 3′-azido-3′-deoxythymidine (AZT) and 5′-fluoro-2′-deoxyuridine (FudR), have indicated that the antiviral and antitumor activity of these compounds is dependent on enzymatic P–N bond hydrolysis.[2–4] In addition, direct evidence of intracellular P–N bond hydrolysis has been demonstrated by monitoring the release of O^{18} labeled AZT-MP from O^{18} AZT tryptophan methyl ester phosphoramidate by capillary LC-ESI-MS/MS.[6,7] Subsequent, subfractionation experiments revealed that 80% of the AZT-phosphoramidase activity was associated with the cytoplasm. (S. L. Chang and C. R. Wagner, unpublished data) Taken together, these results suggest that a nucleoside phosphoramidate hydrolase(s) is likely responsible for AZT phosphoramidate hydrolysis.

Recently, human histidine triad nucleotide binding protein 1 (hHint1) has been found to be a nucleoside phosphoramidase and acyl-AMP hydrolase.[8–11] Hints are ubiquitously expressed in both prokaryotes and eukaryotes, containing a highly conserved His-X-His-X-His-XX motif in the active site, where X is a hydrophobic amino acid.[12] Structure–activity relationship studies have revealed that hHint1 prefers purines over pyrimidines and D-amino acids over L-amino acids.[9] In addition, kinetic and crystallographic studies demonstrated that the 2′- and 3′-hydroxyl groups of the ribose ring are required for maximal efficiency.[9] In particular, although AZT-L-phenylalanine methyl ester, compound **1** (Fig. 1), was found to have both antiviral and antitumor activity[3,4,13] and to undergo intracellular conversion to AZT-MP[6,7], a ^{31}P NMR based assay was unable to demonstrate that it was a substrate for hHint1, thus casting doubt on the hypothesis that it is responsible for the intracellular conversion of AZT phosphoramidates to AZT-MP.[9]

Consequently, we have chosen to prepare a T7 phage CEM cell cDNA library and identify potential AZT-phosphoramidases by selection with an affinity column prepared from an inhibitor of AZT phosphoramidate hydrolysis. Phage can be used to display peptides and proteins as fusion proteins to capsid proteins.[14,15] T7 phage display was preferred over conventional M13 phage display, because secretion and extrusion through the periplasmic space can be deleterious to mammalian cytoplasmic proteins.[16,17]

* Corresponding author.
E-mail address: wagne003@umn.edu (C.R. Wagner).

0960-894X/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2009.09.067

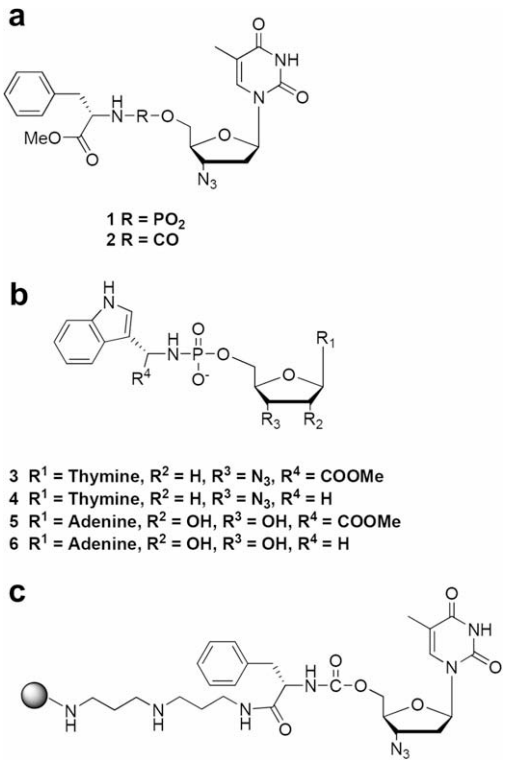

Figure 1. Structures of (a) & (b): Phosphoramidate substrates and Ara-C prodrugs, (c) affinity column. The free acid of compound **2** was coupled to the diaminodipropylamine resin.

In order to carry out our strategy for identifying the putative AZT-phosphoramidase, we first designed and synthesized a potential stable analog of **1**, the carbamate analog, compound **2** (Fig. 1).[3] Compound **2** (100 μM) was shown to inhibit greater than 99% of the conversion of **1** by CEM cell lysates.[3] An affinity column was then prepared by coupling the corresponding free acid to diaminodipropylamine resin (Pierce, IL).

A T7 phage library displaying CEM cDNA was screened for proteins binding specifically to the carbamate (compound **2**) affinity column and eluted with compound **1**. The phage titer of the eluted solutions increased from 10^3 pfu/ml after the first round to 10^5 pfu/ml at the third round of binding and elution. To confirm the enrichment of specific phage particles cDNAs inserted into the eluted phages were analyzed by PCR with primers flanking the library construction site of the T7 phage DNA. The phage PCR products from the first round of elution appeared as smear bands (data not shown). The PCR products of the eluted phages from the second-, and third-round amplifications (Fig. 2, lanes 2, 3, 7 and 10),

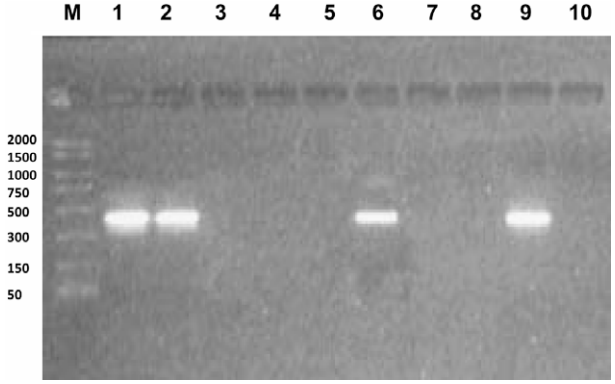

Figure 2. PCR of ten individual clones after third round of biopanning.

Table 1
Steady-state kinetic parameters for hydrolysis of phosphoramidates by hHint1 in HEPES buffer (pH 7.2) at 25 °C using continuous fluorescence assay

Compds	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/M^{-1})
3	460 (±252)	0.0033 (±0.0008)	7 (±6)
4	981 (±634)	0.0247 (±0.008)	25 (±24)
5	41 (±4)[a]	0.012 (±0.001)[a]	230 (±40)[a]
6	0.13 (±0.02)[a]	2.1 (±0.01)[a]	15,000,000 (±3,000,000)[a]

[a] Values are taken from Ref. 9.

however, revealed bands around 400–600 bp. These results indicated that specific phages were selectively enriched by the biopanning procedure. PCR and DNA sequences were determined for 10 phages eluted and selected from the third round of biopanning. Four of the isolated clones had a PCR product of 381 bp (Fig. 2, lanes 2, 3, 7 and 10) that produced an open reading frame of 126 amino acids. The BLAST search with the nucleotide sequences matched with human Hint1 gene in GeneBank (gi: 4885412).

Previously, direct intracellular P–N bond cleavage of the AZT phosphoramidates, **1** and **3** has been observed by ESI-MS.[3] Since earlier ^{31}P NMR based assays were unable to observe the conversion of **1** to AZT-MP[18], we chose to use a more sensitive fluorogenic assay that relies on the enhanced fluorescence of tryptophan or 3-indolyl upon P–N bond cleavage, which allows the detection of the fluorogenic substrate conversion at low micromolar concentrations.[9] The k_{cat} value for hHint1 hydrolysis of **3** was determined to be 0.0033 ± 0.0008 s^{-1}, and the K_m was 460 ± 252 μM, yielding a second order rate constant of k_{cat}/K_m (7 ± 6) M^{-1} s^{-1} (Table 1). Substrate specificity studies have shown that hHint1 favors the configuration at the α carbon of the amino acid, exhibiting preferences for D- over L-tryptophan by about 70–120-fold.[9] Since removal of the carboxymethyl moiety has been shown to greatly enhance phosphoramidate hydrolysis by hHint1, we prepared and evaluated the ability of the 3-indolyl phosphoramidate analog, **4**, to serve as a substrate for hHint1. As expected, the k_{cat} increased by greater than sevenfold, while the K_m value was largely unaffected, resulting in a modest threefold enhancement in substrate specificity for the non-carboxymethyl ester containing substrate.

Since hHint1 has been found to prefer phosphoramidates containing purines and a ribose ring with 2′- and 3′-hydroxyl groups, we compared the ability the enzyme to hydrolyze the AZT phosphoramidates, **3** and **4**, with the corresponding adenosine phosphoramidates, **5** and **6**.[9] The k_{cat} values for the L-tryptophan methyl ester phosphoramidates were found to differ by a modest fourfold and the K_m by greater than 10-fold, resulting in a 32-fold preference for the adenosine phosphoramidate, **5**, over the AZT phosphoramidate, **3**. In contrast, the k_{cat} values for the 3-indolyl containing phosphoramidates were found to differ by 85-fold and the K_m by greater than 7500-fold, resulting in a nearly million-fold preference for the adenosine over the AZT phosphoramidate. Thus, similar to the results for **3** and **4**, removal of the carboxy methyl ester moiety of the amino acid enhances hHint1 selectivity.

Previously, we have demonstrated by both intact and cell free metabolism studies that antiviral and antitumor AZT phosphoramidates are converted to the corresponding AZT 5′-monophosphates.[4,6,7,19] The identity of this enzyme(s) has remained unknown. Based on our results from T7 phage display, affinity chromatography and steady-state kinetics carried out with a sensitive fluorogenic assay, hHint1 appears likely to be the enzyme responsible for AZT phosphoramidate bioactivation by PBMCs and CEM cells.

Acknowledgments

This work was partially supported by Grant CA-61909 and the University of Minnesota Graduate School (C.R.W).

References and notes

1. Wagner, C. R.; Iyer, V. V.; McIntee, E. J. *Med. Res. Rev.* **2000**, *20*, 417.
2. Abraham, T. W.; Kalman, T. I.; McIntee, E. J.; Wagner, C. R. *J. Med. Chem.* **1996**, *39*, 4569.
3. Chang, S.; Griesgraber, G. W.; Southern, P. J.; Wagner, C. R. *J. Med. Chem.* **2001**, *44*, 223.
4. McIntee, E. J.; Remmel, R. P.; Schinazi, R. F.; Abraham, T. W.; Wagner, C. R. *J. Med. Chem.* **1997**, *40*, 3323.
5. Song, H.; Griesgraber, G. W.; Wagner, C. R.; Zimmerman, C. L. *Antimicrob. Agents Chemother.* **2002**, *46*, 1357.
6. Kim, J.; Chou, T. F.; Griesgraber, G. W.; Wagner, C. R. *Mol. Pharm.* **2004**, *1*, 102.
7. Kim, J.; Park, S.; Tretyakova, N. Y.; Wagner, C. R. *Mol. Pharm.* **2005**, *2*, 233.
8. Bieganowski, P.; Garrison, P. N.; Hodawadekar, S. C.; Faye, G.; Barnes, L. D.; Brenner, C. *J. Biol. Chem.* **2002**, *277*, 10852.
9. Chou, T. F.; Baraniak, J.; Kaczmarek, R.; Zhou, X.; Cheng, J.; Ghosh, B.; Wagner, C. R. *Mol. Pharm.* **2007**, *4*, 208.
10. Chou, T. F.; Tikh, I. B.; Horta, B. A.; Ghosh, B.; De Alencastro, R. B.; Wagner, C. R. *J. Biol. Chem.* **2007**, *282*, 15137.
11. Chou, T. F.; Wagner, C. R. *J. Biol. Chem.* **2007**, *282*, 4719.
12. Brenner, C.; Bieganowski, P.; Pace, H. C.; Huebner, K. *J. Cell. Physiol.* **1999**, *181*, 179.
13. Iyer, V. V.; Griesgraber, G. W.; Radmer, M. R.; McIntee, E. J.; Wagner, C. R. *J. Med. Chem.* **2000**, *43*, 2266.
14. Hoess, R. H. *Chem. Rev.* **2001**, *101*, 3205.
15. Smith, G. P.; Petrenko, V. A. *Chem. Rev.* **1997**, *97*, 391.
16. Sche, P. P.; McKenzie, K. M.; White, J. D.; Austin, D. J. *Chem. Biol.* **1999**, *6*, 707.
17. Takakusagi, Y.; Takakusagi, K.; Kuramochi, K.; Kobayashi, S.; Sugawara, F.; Sakaguchi, K. *Bioorg. Med. Chem.* **2007**, *15*, 7590.
18. Chou, T. F.; Bieganowski, P.; Shilinski, K.; Cheng, J.; Brenner, C.; Wagner, C. R. *J. Biol. Chem.* **2005**, *280*, 15356.
19. Kim, J.; Drontle, D. P.; Wagner, C. R. *Nucleosides Nucleotides Nucleic Acids* **2004**, *23*, 483.