Identification of a Slow Tight-Binding Inhibitor of 3-Deoxy-D-*manno*-octulosonic Acid 8-Phosphate Synthase

Matthew R. Birck,[†] Tod P. Holler,[‡] and Ronald W. Woodard*,[†]

College of Pharmacy, University of Michigan Ann Arbor, Michigan 48109-1065 Parke-Davis Pharmaceutical Research Division of Warner-Lambert, 2800 Plymouth Road Ann Arbor, Michigan 48105

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To overcome the recent emergence of drug-resistant bacteria, it is necessary to discover new and innovative antibiotics with novel targets so as to prevent cross-resistance with present agents. The reaction catalyzed by 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) synthase (EC 4.1.2.16), the condensation of phosphoenolpyruvate (PEP) and arabinose 5-phosphate (A5P) (Figure 1),[1] is the first committed step in the synthesis of KDO,

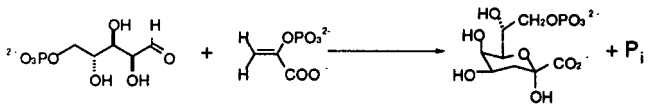

Figure 1. Reaction catalyzed by KDO 8-P synthase.

an integral part of the lipopolysaccharide (LPS) layer in Gram negative bacteria. Once incorporated into the LPS, between one and three KDO molecules act as a linker between lipid A and the inner core region. At least a single KDO moiety has been observed in all Gram negative bacteria, including those lacking the O-antigen.[2] The activity of KDO 8-P synthase is a necessary part of the cell cycle; disruption of KDO 8-P synthase leads to a halt in protein, RNA, and DNA synthesis after a single generation.[3] Although several attempts have been made to design a potent inhibitor of KDO 8-P synthase, most based on purported transition state structures, none have been sufficiently potent to warrant further investigation;[4−7] the most potent to date, an acyclic bisubstrate analogue (**2**), has a K_i of 3.3 μM.[4] Our search for novel inhibitors of KDO 8-P synthase began by screening a library of approximately 150 000 compounds using the continuous coupled phosphate assay described by Webb[8] with modifications for the 96-well plate format.[9] Compounds demonstrating >80% inhibition compared to control were reanalyzed individually to confirm inhibition. To eliminate inhibitors of the coupling enzyme, PNPase, a second screening assay was used[10] based on the method of Aminoff[11] as modified by Ray.[12] Seventy-two compounds were screened using this method and the IC_{50} was determined for compounds appearing to inhibit KDO 8-P synthase >50% at 100 μM. Of these, 22 compounds have an $IC_{50} \leq 10$ μM. The minimum inhibitory concentration of these compounds was determined using a wild-type *Escherichia coli* strain, a membrane permeable *E. coli* strain (*tolC*$^-$), and a strain of *Staphylococcus aureus*. Of the tested compounds, only one was able to selectively inhibit Gram negative bacteria (32 μg/mL against the Gram negative *E. coli* strains and >64 μg/mL against the Gram positive *S. aureus*). This compound, PD 404182 (Figure 2), was taken on for further analysis.

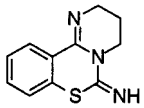

Figure 2. PD 404182 [6*H*-6-imino-(2,3,4,5-tetrahydropyrimido)[1,2-*c*]-[1,3]benzothiazine].

Initial characterization shows PD 404182 to inhibit KDO 8-P synthase competitively with respect to PEP and noncompetitively with respect to A5P. These experiments also show inhibition at very low concentrations, suggesting the possibility of a covalent adduct. To determine which kinetic model should be used for a detailed kinetic analysis, the possibility that PD 404182 was covalently modifying the enzyme was investigated. Modification[13] was examined by both electrospray ionization[14] (ESI) and matrix-assisted laser desorption ionization[15] (MALDI) mass spectrometry (MS). MALDI-MS was chosen to determine the mass of the monomeric species in the presence of inhibitor while ESI-MS was selected to observe the quaternary form of the enzyme. Monomer molecular mass as determined by MALDI-MS is 30842 ± 15 Da (calcd: 30842 Da). Mass of the quaternary complex as determined by ESI-MS is 123 381 ± 56 Da, consistent with the tetrameric structure proposed by Gatti et al.[16] These results clearly show that PD 404182 is not covalently bound to the enzyme.

To determine the kinetic mechanism and individual equilibrium constants, a continuous assay monitoring the disappearance of the PEP double bond was used.[17] Initial experiments demonstrated that PD 404182 is a time-dependent inhibitor, requiring several minutes to achieve full inhibition at low concentrations. It was also observed that PD 404182 inhibits KDO 8-P synthase at concentrations similar to those of the enzyme. Therefore, PD 404182 was treated as a slow tight-binding inhibitor. Techniques

[†] University of Michigan.
[‡] Parke-Davis Pharmaceutical Research.

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(9) A typical reaction contained, in a final volume of 200 μL, 100 μM Tris-OAc (pH 7.4), 30 μM PEP, 30 μM A5P, 100 μM 7-methylthioguanosine [MESG, Berry & Associates, Inc. (Ann Arbor, MI)], 50 nM KDO 8-P synthase, 50 nM purine nucleoside phosphorylase (PNPase), 1% DMSO, and approximately 100 μg/mL inhibitor. The reaction mixtures were assembled in a polystyrene 96-well plate (Rainin) without A5P and kept at room temperature for 5 min before initiation with A5P. The conversion of MESG to 7-methylthioguanine was monitored for 2 min at 350 nm in a Molecular Devices SpectraMAX 340 microplate reader.

(10) In a total of 50 μL, individual compounds (100 μM) were incubated under conditions similar to those above[9] (100 μM Tris-OAc, pH 7.4, 30 μM PEP, 50 nM KDO 8-P synthase). The reaction was initiated by addition of A5P to 30 μM. The total amount of KDO 8-P produced in 5 min was determined and the resulting chromophore quantitated at $\lambda = 549$ nm ($\epsilon_{549} = 103\,000\ M^{-1}\ cm^{-1}$). IC_{50} values were also determined using this assay.
(11) Aminoff, D. *Biochem. J.* **1961**, *81*, 384−393.
(12) Ray, P. H. *J. Bacteriol.* **1980**, *2*, 635−644.
(13) 0.5 mg/mL KDO 8-P synthase was incubated with [I] ≅ 10 × [E] for approximately 1 h.
(14) ESI-MS (KDO 8-P synthase ± PD 404182) was accomplished using a double focusing hybrid mass spectrometer (EBqQ geometry, Finnigan MAT 900Q, Bremen, Germany) with a mass-to-charge (*m*/*z*) range of 10 000 at 5 kV full acceleration potential. A position and time-resolved ion counting (PATRIC) scanning array detector with an 8% *m*/*z* range of the *m*/*z* centered on the detector was used.
(15) KDO 8-P synthase solutions (±PD 404182) were mixed with a sinapinic acid matrix for analysis by MALDI-MS on a PerSeptive Voyager Elite operated in linear mode.
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(17) All assays were done in 1 mL total with 100 mM Tris-OAc (pH 7.4), 150 μM PEP, 150 μM A5P, 200−1200 nM PD 404182, initiated with 20 nM KDO 8-P synthase, and were monitored continuously for PEP disappearance at $\lambda = 232$ nm ($\epsilon_{232} = 2840\ M^{-1}\ cm^{-1}$) at 37 °C.

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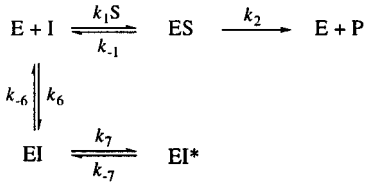
were employed which allow for observation of both the fast and slow phases of inhibition (for additional details, see Supporting Information).

The slow-binding kinetic properties of PD 404182 were analyzed assuming the kinetic mechanism shown in Scheme 1

Scheme 1. Proposed Mechanism of Inhibition[21]



where EI is a rapidly formed complex and EI* is the product of a slow, unimolecular rearrangement or conformational change from EI. This kinetic mechanism was chosen over a single slow step inhibition because the data clearly show inhibition at the initiation of the experiment.[18,19] The initial inhibition of the enzyme by PD 404182 is described by the inhibition constant K_i, and the overall inhibition by K_i* (eq 1). Data were analyzed using progress curve analysis as described by Reczkowski and Markham.[20]

$$K_i^* = \frac{[E][I]}{[EI] + [EI^*]} = K_i\left(\frac{k_{-7}}{k_7 + k_{-7}}\right) \qquad (1)$$

Rates were easily measurable between 200 nM and 1.2 μM PD 404182. Due to the increased rapid equilibrium concentration of EI above [I] = 1.2 μM, the rate of transition from the weak complex to the strong became too rapid for analysis with the employed techniques. The reversible nature of the inhibition is demonstrated by the fact that a measurable equilibrium rate of catalysis was routinely achieved. Observed inhibition constants are corrected for [PEP] to afford the true K_i and K_i*. Analysis of the progress curve data shows that PD 404182 inhibits KDO 8-P synthase with $K_i = 26 \pm 1$ nM and $K_i^* = 240 \pm 60$ pM. This is a 4 orders of magnitude decrease in inhibition constant as compared with compounds published previously.[5−7]

Many of the compounds designed as bisubstrate (**2**[6]) or transition state analogues (**1**,[7] **3**[5]) of KDO 8-P synthase, in addition to being poor inhibitors, lack many of the qualities associated with effective small molecule drug compounds. Specifically, these compounds are extremely hydrophilic, susceptible to hydrolysis, and require multistep synthesis, including many protection/deprotection steps. Figure 3 shows three of the most potent KDO 8-P synthase inhibitors reported to date. Compound **2**, a bisubstrate analogue, and **3**, a transition state analogue, were designed as inhibitors of the proposed linear reaction mechanism which is initiated by attack of an activated water molecule on $C2^{PEP}$.

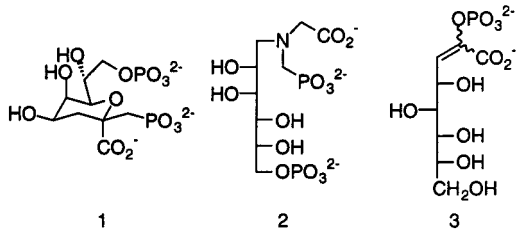


Figure 3. KDO 8-P synthase inhibitors.

Compound **1** was intended to be a transition state analogue for the proposed cyclic reaction mechanism where $C2\text{-}OH^{A5P}$ began the reaction by attack upon $C2^{PEP}$. The acyclic mechanism is the more likely based on crystallographic studies from our laboratory.[16] Compounds **1** and **2** have in vitro K_i values of 4.9 and 3.3 μM, respectively. Compound **3** showed a small in vivo activity.[22] In contrast, PD 404182 is a relatively hydrophobic, stable compound that is very amenable to synthesis and modification by both standard and combinatorial synthetic techniques.

That PD 404182 inhibits KDO 8-P synthase very effectively in vitro while being a poor inhibitor of bacterial growth suggests that PD 404182 has less than optimal bioavailability or that the compound is rapidly exported from the cell or metabolized. Future iterations of this compound will provide structure−activity relationship data that can be used to probe the reasons for reduced in vivo activity and to improve the ability of this novel class of compounds to inhibit KDO 8-P synthase and by extension, cellular growth. To begin this process, efforts to cocrystallize PD 404182 with KDO 8-P synthase are currently underway and preliminary modeling studies have been performed. Using the docking algorithm implemented in Molecular Operating Environment (Chemical Computing Group), PD 404182 consistently docks within the active site of the enzyme, engaging in several interactions with residues normally involved in binding PEP. This is, of course, consistent with the competitive kinetic mechanism described herein. The very simplicity of the synthesis of PD 404182[23] and the availability of substituted starting materials logically lends this compound to a comprehensive study of the structure−activity relationship using standard medicinal chemistry techniques. More work in this area is clearly warranted and has been initiated in our laboratory.

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Supporting Information Available: Full set of equations for inhibition constant determination (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) Scheme 1 represents the simpliest model system that adequately accounts for the experimental data.

(22) No in vitro testing was reported on **3** and bacterial growth was not inhibited at 500 μg/mL although bacterial morphology was altered slightly in the Gram negative bacteria tested.[5]

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