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Structure–Activity Relationship on Human Serum Paraoxonase (PON1) Using Substrate Analogues and Inhibitors

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Abstract—Substrate analogues based on the parent compounds paraoxon and phenyl acetate were tested on human serum paraoxonase (PON1) to explore the active site of the enzyme. Replacement of the nitro group of paraoxon with an amine or hydrogen, as well as electronic changes to the parent compound, converted these analogues into inhibitors. Introduction of either electron-withdrawing or donating groups onto phenyl acetate resulted in reduction in their rate of hydrolysis by PON1.

Human serum paraoxonase (PON1, EC 3.1.8.1) is a calcium-dependent glycoprotein with hydrolase activity.[1] The enzyme derives its name from its ability to hydrolyse paraoxon **1a** into *p*-nitrophenol and diethyl phosphate, a reaction that was first demonstrated by Aldridge in 1953 (Scheme 1).[2] Paraoxon, a potent acetylcholinesterase inhibitor, is metabolically generated in vivo from the insecticide parathion by mitochondrial oxidation involving the cytochrome-P450 pathway.[3] PON1 is synthesised in the liver and then secreted into the bloodstream where it becomes tightly bound to high-density lipoprotein (HDL).[4] The enzyme catalyses the hydrolysis of a broad range of substrates including arylesters **2a**,[5] carbamates,[6] as well as cyclic carbonates and lactones **3** (Scheme 1).[7] It has been shown that the incorporation of the latter two groups as hydrolysable moieties onto a glucocorticosteroid nucleus generates an ideal antedrug strategy that could offer safer treatment of asthma.[7] This antedrug strategy is possible because of the tissue-specific distribution of PON1. Its presence in the blood and liver renders the drug inactive. However, PON1 activity is absent in the lungs, where stability of the compound is required for maximal efficacy. PON1 can also activate an unsaturated cyclic carbonate prodrug of quinolone antibiotic in the plasma[8] and the enzyme can detoxify nerve agents, such as sarin **4**[9] (Scheme 1).

PON1 is a 354 amino acid polymorphic protein of 43–45k Da with amino acid substitutions at positions 54 (L or M) and 191 (Q or R) giving rise to several isozymes in the human blood which display marked differences in activities towards some substrates.[10] For example, the differential rates of detoxification of warfare agents by Q, R and QR phenotypes has been linked to susceptibility of soldiers to developing the Gulf War Syndrome.[11]

Over the last decade it has become apparent that PON1 also performs an important physiological function by retarding the oxidative modification of low-density lipoprotein (LDL) in the arterial wall. This oxidation is thought to be the initial step in the cascade of events which eventually lead to atherosclerosis.[12] The R/L phenotype has been associated with a risk of developing cardiovascular disease, while in populations with the Q/M phenotype, a lower risk of atherosclerosis has been observed,[13] although this is still controversial.[14]

As yet, the natural substrate of PON1 remains in doubt, but a recent report suggests that it might be platelet-activating factor (PAF).[15] This phospholipid is a good substrate for PON1, with a catalytic efficiency 750-fold greater than that for paraoxon. Interestingly, PAF seems to be hydrolysed in the absence of Ca^{2+} ions in vitro, possibly suggesting the presence of two different active sites on the enzyme. At present, little is known about how PON1 processes any of its substrates.

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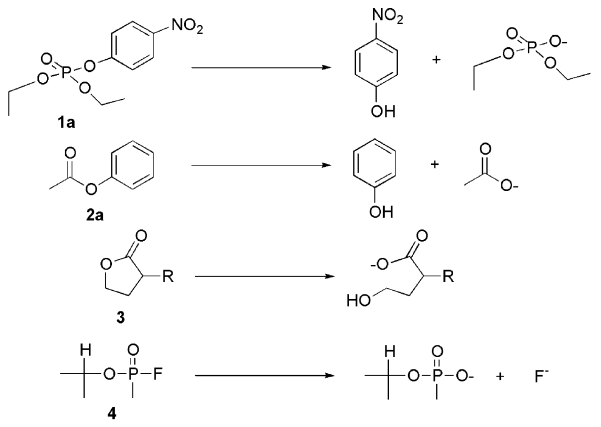
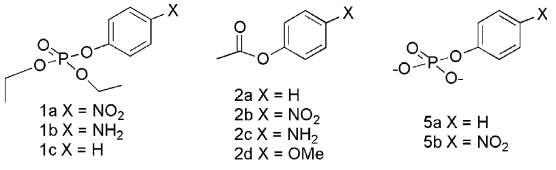


Scheme 1. Some known enzymatic reactions of PON1 with synthetic substrates.

Reports concerning the relationship between structure and activity of PON1 using synthetic substrates are scarce, but recently lactone substrate specificity and inhibition by lactams on PON1 has been investigated.[16] Previously, plasma arylesterase activity was quantitatively related to structural effects of various aryl and aliphatic esters.[17] Studies that offer a mechanistic and catalytic insight into the action of PON1 could provide important clues to the in vivo function of the enzyme, thereby leading the way to new antedrug and prodrug strategies.

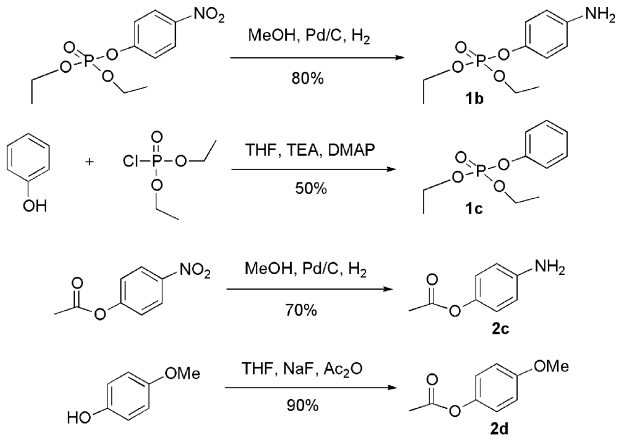
We report here results from the use of substrate analogues based on the parent compounds paraoxon **1a** and phenyl acetate **2a** (Scheme 2) to define the relation between the structure and the function of human serum PON1. Analogues **1b**, **1c**, **2c**, and **2d** were synthesised in our laboratory (Scheme 3) whilst others were commercially available.

Our initial approach was to examine the time course of hydrolysis of compounds **1a**–**1c**, **2a**–**2d**, **5a** and **5b** using whole serum, which had been pre-treated with phenylmethylsulfonyl fluoride (PMSF) to inactivate other esterases within it, particularly acetylcholinesterase which is known to have arylesterase activity.[1] We have demonstrated that 4 mM PMSF causes a 20% reduction in arylesterase activity, using **2a** as substrate, which is primarily due to inhibition of acetylcholinesterase (unpublished data). We further confirmed this by showing that in the presence of serum treated with PMSF, no significant hydrolysis of acetylcholine occurred (data not shown). All substrates and analogues were tested under identical assay conditions with fixed amounts of compound and serum. Although crude serum has previously been used to measure PON1 activities spectrophotometrically,[18] this technique is limited to substrates and products exhibiting chromophoric groups with different spectral properties. We have now developed a novel PON1 assay system that employs NMR spectroscopy to monitor continuous enzyme-catalysed reactions.[20] This system has none of the limitations of spectrophotometric techniques and it can be used to generate qualitative and quantitative data on novel compounds.



Scheme 2. Substrate analogues of PON1.

Using this assay protocol, we tested the hydrolysis of our compounds with serum PON1 by measuring the relative changes of reactants and products over time. From this data, the time taken for 50% of the test compound to be consumed by serum PON1 was determined, which is reported as $t_{1/2}$ (Table 1). A wide range of activities was observed. Substrate **2a** was hydrolysed most rapidly ($t_{1/2}$ 1.5 min), while the rate of hydrolysis of the phosphate ester **1a** was very low ($t_{1/2}$ 1460 min). No hydrolysis was detected with compounds **1b**, **1c**, **5a** and **5b** even after 24 h incubation with serum. Control experiments indicated that diluted serum [10% (v/v)] was stable at 30 °C for up to 21 h without loss of PON1 activity. Introduction of the electron-withdrawing nitro-group to the *para*-position of the parent ester **2a** slowed down the hydrolysis of the resultant molecule **2b** more than 2-fold. Conversely, the presence of NO_2 group was essential for the hydrolysis of **1a** because its removal from the phenyl ring rendered the analogue **1c** stable. Attachment of electron-donating groups (NH_2/OMe) to



Scheme 3. Reagents and conditions for the synthesis of substrate analogues of PON1. All final compounds exhibited satisfactory analytical data in accordance with the structures proposed.[19]

Table 1. Effect of substrates and their analogues on PON1

Compd	$t_{1/2}$ (min)	IC_{50} (mM)
2a	1.5	na
2b	3.5	na
2c	20	na
2d	20	na
1a	1460	na
1b	nh	1.28
1c	nh	3.27
5b	nh	3.25
5a	nh	18.5

IC_{50} values were determined from plots of enzyme activity versus inhibitor concentration using GraFit (Erithacus Software, Staines, UK). na = not active, nh = not hydrolysed.

the *para* position of **2a** caused over 13-fold reduction in the rate of hydrolysis (**2c** and **2d**, respectively). Substitution of the NO_2-group of paraoxon **1a** with a NH_2-group rendered the substrate analogue **1b** non-hydrolysable by serum PON1. Because compounds **1b**, **1c**, **5a** and **5b** did not serve as substrates for serum PON1, we tested whether these acted as inhibitors of the enzyme.[21] All four derivatives caused varying degrees of inhibition with **1b**, **1c**, and **5b** displaying IC_{50} values between 1.28 and 3.27 mM (Table 1), whilst **5a** was the weakest inhibitor in the series (IC_{50} of 18.5 mM).

These studies provide curious observations associated with substituent effects for carboxylic ester substrates of PON1. Coupled with this study, we have previously demonstrated that phenyl acetate **2a** is a better substrate than the 4-nitro analogue **2b** for PON1 in that **2a** shows a 50-fold greater V_{max}/K_m value, despite both exhibiting similar K_m values of around 0.7 mM (unpublished results). These findings suggest that the Lewis basicity of the carbonyl oxygen is more important in stabilizing the rate-limiting transition state than a low pK_a for the leaving group of structures **2a**–**2d**. The enzyme could stabilize the negative charge on oxygen through interaction with a metal ion such as Ca^{2+} or by protonation.

The reduction in the rate of hydrolysis caused by the presence of amine or methoxy groups on phenyl acetate was most likely due to steric hindrance. Substrate analogues of paraoxon in the phosphate ester series (**1b**, **1c**, **5a** and **5b**) were all inhibitors suggesting that they bind to PON1, but the enzyme does not catalyze their hydrolysis at a significant rate. These inhibitors either had an overall negative charge or were hydrophobic, both of which characteristics were detrimental to hydrolysis by PON1. There is a free cysteine residue at position 283 in PON1 which is not involved in arylesteraser/paraoxon catalysis. Evidence for this came from site-directed mutagenesis (SDM) experiments in which cysteine 283 was replaced with either alanine or serine and the resulting mutants retained both paraoxonase and arylesterase activities.[22] Other SDM studies have revealed that several histidine and tryptophan residues are essential for PON1 arylesterase and paraoxonase activities.[23] The aromatic nature of these amino acids suggests that the active site of the enzyme is hydrophobic, which could also explain why PON1 favours lipophilic substrates, such as **2a** and PAF. But, paraoxon **1a**, which itself has a polar NO_2 group, is also hydrolyzed by PON1, albeit at a much reduced rate, suggesting that there may be a different mode of interaction with phosphate and carboxylic acid esters.

In summary, we have demonstrated that both electron-withdrawing and -donating groups in the *para*-position of the parent phenyl acetate esters lead to significant reductions in the rate of hydrolysis. Using the NMR assay described here, it should be possible to determine K_m and V_{max} for these and other substrate analogues of PON1 using purified single isomorphs. The structure–activity data generated could then be used to extrapolate whether the analogues have an effect on the enzyme through binding or catalysis. In addition, the mode of inhibition of the paraoxonase derivatives also needs to be evaluated. Data from these concerted efforts will further our knowledge of the active site of PON1 and pave the way towards understanding the catalytic mechanism of this physiologically important enzyme.

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19. **1b** HRMS calcd for $C_{10}H_{16}NO_4NaP$ (M + Na^+) 268.0715, found 268.0725. ^1H NMR (300 MHz, $CDCl_3$): δ 1.32 (t, $J=6.99$ Hz, 6H), 4.17 (m, 4H), 6.61 (d, $J=8.81$ Hz, 2H), 6.99 (d, $J=8.81$ Hz, 2H). ^{13}C NMR (75.5 Hz, $CDCl_3$): δ 143.5, 143.2, 120.8 and 115.8 (Ar_C), 64.4 and 64.4 (2×CH_2), 16.1 and 16.0 (2×CH_3). ^{31}P NMR (121.5 Hz, $CDCl_3$): δ −5.68. IR (KBr, cm^{-1}): 3380 br, 3025, 1504, 1210, 1028, 967, 757. **1c** HRMS calcd for $C_{10}H_{15}O_4NaP$ (M + Na^+) 253.0606, found 253.0599. ^1H NMR (300 MHz, CD_3OD): δ 1.34 (t, $J=7.06$ Hz, 6H), 4.22 (m, 4H), 7.22 (m, 3H), 7.38 (m, 2H). ^{13}C NMR (75.5 Hz, $CDCl_3$): δ 148.9, 129.6, 124.8, 112.0 and 119.8 (Ar_C), 64.5 and 64.4 (2×CH_2), 16.0 and 15.9 (2×CH_3). ^{31}P NMR (121.5 Hz, $CDCl_3$): δ_P −5.62. IR (KBr, cm^{-1}): 3013, 1601, 1488, 1273, 1221, 1041, 970, 749. **2c** HRMS calcd for $C_8H_9NO_2Na$ (M + Na^+) 174.0531, found 174.0532. ^1H NMR (300 MHz, $CDCl_3$): δ 2.26 (s, 3H), 6.66 (m, 2H), 6.86 (m, 2H). ^{13}C NMR (75.5 Hz, $CDCl_3$): δ 170.3 (C=O), 144.2, 142.9, 122.2 and 115.6 (Ar_C), 21.0 (CH_3). IR (KBr, cm^{-1}): 3361, 3338, 3016, 1751, 1616, 1508, 1219, 1186, 1014, 752. **2d** HRMS calcd for $C_9H_{10}O_3Na$ (M + Na^+) 189.0528, found 189.0522. ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H), 3.79 (s, 3H), 6.88 (m, 2H), 7.00 (m, 2H). ^{13}C NMR (75.5 Hz, $CDCl_3$): δ 169.9 (C=O), 157.2, 144.2, 122.3 and 114.4 (Ar_C), 55.6 (OCH_3), 21.0 (CH_3). IR (KBr, cm^{-1}): 3015, 2940, 2907, 2832, 1753, 1594, 1509, 1462, 1298, 1097, 909.

20. PON1 enzyme activity assays were conducted in a total volume of 2.5 mL containing 20 mM Tris–HCl buffer in D_2O (pH 7.4), 2 mM $CaCl_2$ and 5 μm of the test compound. The reaction was initiated by adding 100 μL crude serum, which had been pre-treated for 1 h with PMSF, and an aliquot of the reaction mixture was quickly transferred to an NMR tube for time course analysis, which was done at 30 °C. From the ^1H NMR spectra, integration assignment was performed to determine the amount of substrate remaining over time, which was plotted to obtain $t_{1/2}$ of the compound. A control tube was also prepared for each compound which did not contain serum.

21. Inhibition of serum PON1 activity was tested with 1 mM paraoxon in the presence of various concentrations of inhibitor with crude serum, which had been pre-treated for 1 h with PMSF. This mixture was incubated at 37 °C for 10 min prior to assay. Assays were preformed as described,[1] except that 20 mM Tris–HCl (pH 7.4) buffer containing 2 mM $CaCl_2$ was used and that 200 μL treated serum were assayed in a total volume of 3 mL at 30 °C.

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