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Binding kinetics and duration of in vivo action of novel prolyl oligopeptidase inhibitors

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ABSTRACT

Prolyl oligopeptidase (POP) is a serine protease that specifically hydrolyses small peptides at the carboxyl end of the proline residue. POP has gained pharmaceutical interest, since its inhibitors have been shown to have antiamnesic properties in rat. We examined the effect of the 2(*S*)-substituents CN and $COCH_2OH$ at the P1 site of the parent inhibitors isophthalic acid 2(*S*)-(cyclopentanecarbonyl)pyrrolidine-L-prolyl-pyrrolidine amide and 4-phenylbutanoyl-L-prolyl-pyrrolidine and bulky 5-*t*-butyl group at the P2 site L-prolyl residue of the parent inhibitor 4-phenylbutanoyl-L-prolyl-pyrrolidine on the binding kinetics to the enzyme. In addition, we studied the duration of POP inhibition in the rat tissues in vivo after i.p. administration. CN and $COCH_2OH$ substituents at the P1 site pyrrolidine group were found to greatly increase the affinity of the inhibitor and the enzyme–inhibitor complex half-life. In addition, 5-*t*-butyl group at the P2 site L-prolyl residue increased the dissociation half-life of the enzyme–inhibitor complex, without much affecting the inhibitory potency. The duration of the inhibition in the rat tissues followed the inhibition kinetic properties in that the compounds with fast dissociation produced shorter inhibition in the rat tissues than the compounds with slow dissociation. The duration of POP inhibition of compounds was evidently not governed by their serum clearance. The fact that the in vivo pharmacodynamic behaviour of POP inhibitors can be predicted by their in vitro-properties may be of importance when designing therapeutically useful POP inhibitors.

1. Introduction

Prolyl oligopeptidase (POP) (EC 3.4.21.26) is an 80 kDa enzyme that belongs to a POP family of serine proteases. The POP family includes POP, dipeptidyl peptidase IV (EC 3.4.14.5), oligopeptidase B (EC 3.4.21.83) and acylaminoacyl peptidase (EC 3.4.19.1). This enzyme family is of ancient origin and it is different from the classical trypsin- and subtilisin-like serine proteases in their selectivity for small peptide subtrates and in the order of catalytic triad residues [1–3].

POP preferentially hydrolyses small (<30 aa) peptides at the carboxyl side of a proline residue. POP degrades several proline

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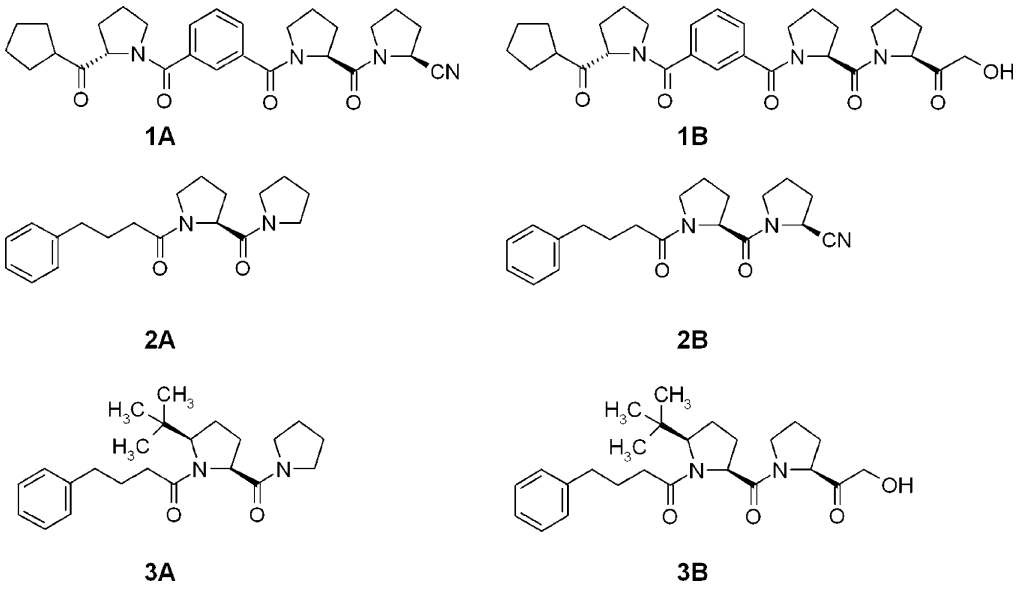


Fig. 1 – The prolyl oligopeptidase inhibitors used in this study: (1A) isophthalic acid 2(*S*)-(cyclopentanecarbonyl)pyrrolidine-L-prolyl-2(*S*)-cyanopyrrolidine amide; (1B) isophthalic acid 2(*S*)-(cyclopentanecarbonyl)pyrrolidine-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine amide; (2A) 4-phenylbutanoyl-L-prolyl-pyrrolidine (SUAM-1221); (2B) 4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine; (3A) 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine; (3B) 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine.

containing neuropeptides in the central nervous system and several of its substrates, such as substance P, vasopressin, neurotensin and thyroliberin, are involved in learning and memory [4]. In addition, low levels of substance P are commonly found in the brains of Alzheimer's patients and administration of substance P has been reported to block β-amyloid-induced neurotoxicity [5,6]. Confusingly, both increased and decreased POP activity levels have been reported from post mortem brain tissues of Alzheimer patients [7–9]. It was recently reported that POP gene transcription was decreased when mice were exposed to an enriched environment and the transcription was increased many-fold in hypothalamus and cortex from aged mice [10,11]. As a summary, centrally acting POP inhibitors might theoretically be beneficial in patients with cognitive disturbances. Indeed, POP inhibitors have been shown to increase the brain levels of several neuropeptides, to reverse scopolamine-induced amnesia in rats and to improve cognition in old rats and MPTP-treated monkeys [12–15]. POP inhibitors have also been reported to prevent β-amyloid deposition in a neuroblastoma cell line and in senescence-accelerated mouse [16,17]. The possible cognition enhancing properties of POP inhibitors has gained pharmaceutical interest and several potent compounds have been synthesized. To date, however, none of the compounds has entered into the market.

Recently we have studied the effect of different 2(*S*)-substituents at pyrrolidine ring at the P1 site of the parent inhibitor dicarboxylic acid bis(L-prolyl-pyrrolidine) amide on the binding kinetics to the active site of POP [18]. We found that the addition of 2-(*S*)-substituents CN, $COCH_2OH$ or CHO at the inhibitor P1 site pyrrolidine ring results in slow binding inhibition kinetics and slow dissociation from the enzyme active site. It has been shown that Z-prolylprolinal with 2(*S*)-CHO substituent at the P1 site pyrrolidine ring forms a hemiacetal adduct with the Ser554 residue at the active site of POP [19]. It is probable that the CN and $COCH_2OH$ groups act similarly and form imino ether and hemiketal adducts with the active site Ser554 residue and these covalent adducts cause the slow binding and dissociation. Slow binding inhibition is a phenomenon in which inhibition of enzyme activity occurs relatively slowly and not at diffusion controlled rates. Analysis of slow binding inhibition allows the calculation of enzyme–inhibitor association and dissociation rates [20]. In this study, we determined the slow binding inhibition kinetic parameters and ex vivo inhibition profiles of six potent POP inhibitors with 2-(*S*)-substituents CN or $COCH_2OH$ at the P1 site pyrrolidine ring and 5-*t*-butyl substituent at the P2 site prolyl residue (see Fig. 1). K_i, the association and dissociation rate constants and the dissociation half-lives of the enzyme–inhibitor complex were measured using purified pig POP. In animal experiments, rats were given single i.p. injections of the inhibitors and the liver and brain POP activities were measured ex vivo at different time points. The correlation between the duration of POP inhibition and the in vitro dissociation half-life of the inhibitor was examined. In some cases also serum clearance of the compounds was measured.

2. Materials and methods

2.1. Compounds and reagents

Compounds **1A**, **1B**, **2B**, **3A** and **3B** were synthesized in the Department of Pharmaceutical Chemistry, University of Kuopio as described earlier [21–24]. The reference compound SUAM-1221 [25] (compound **2A**) was synthesized using slightly

modified reported method [26]. For in vitro studies, 0.01 M stock solutions were made in DMSO, further dilutions were made in 0.1 M Na-K-phosphate buffer, pH 7.0. For ex vivo studies, the compounds were dissolved and diluted in sterile water (Aqua Sterilisata, Orion Pharma, Espoo, Finland). None of these experimental compounds have been approved for human use.

N-Benzyloxycarbonyl-glycyl-prolyl-7-amino-4-methylcoumarin (Z-Gly-Pro-AMC) and *N*-succinyl-glycyl-prolyl-7-amino-4-methylcoumarin (Suc-Gly-Pro-AMC) were obtained from Bachem AG (Bubendorf, Switzerland). Na_2HPO_4 and KH_2PO_4 were obtained from Merck (Darmstadt, Germany), methanol from Labscan Ltd. (Dublin, Ireland) and dimethyl sulfoxide (DMSO) from Riedel-de Haën (Seelze, Germany).

2.2. Expression and purification of porcine and human POP

Expression and purification of porcine POP were performed as reported previously and the active enzyme concentration was determined using the tight binding POP inhibitor, JTP-4819 [27,28]. Purified pig POP was used as the enzyme in all in vitro measurements.

Human POP cDNA was synthesized by reverse transcriptase reaction (AMV reverse transcriptase, Promega, USA) on a human brain poly-A+ RNA preparation (Clonotech, USA) using 5′-TAAAATCCCACGGCAGTTC-3′ as primer. cDNA was amplified by PCR with 5′-TTCGATTATCCATGGTGTCC-TTCC-3′ (*Nco*I site added) and 5′-TTAAGCTTATGGAATCC-3′ (*Hin*dIII site added) as 5′ and 3′ primers, respectively. PCR product was A-tailed (Taq-DNA polymerase, Promega, USA) and ligated to pGEM-T-Easy (Promega, USA) for sequencing. Correct sequence harbouring clones were grown, the plasmid prepared and digested with *Nco*I and *Hin*dIII and the resulting fragment was sub-cloned into the same restriction sites in pBAD/*myc* His A (Life Technologies, USA). The final construct, pBADHumPOP, contained the stop codon before the 6× His sequence of the original plasmid pBAD7*myc* His A, such that no addition of the histidine tail was obtained in the expressed protein. pBADHumPOP was used to transform *E. coli* TOP10 strain for expression. The expression and purification of human POP was performed as for pig POP.

2.3. Generation of antibody against human POP

Purified *E. coli* expressed human POP was used to generate antibodies against POP. Two 50 μg protein dosages (in 50% Freund's complete adjuvant), with two weeks interval, were injected to hens and egg yolk *anti*-POP titer was followed thereafter. POP-specific IgY was then purified by affinity chromatography (HiTrap NHS column coupled with purified POP) using the high titer yolk preparations.

3. In vitro studies

3.1. Progress curve experiments

Slow binding inhibition measurements were made at 23 °C in 0.1 M Na-K-phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol at an enzyme concentration of 0.14 nM. Z-Gly-Pro-AMC was used as a substrate at 10 μM, which is $0.5K_m$. Due to its poor solubility, Z-Gly-Pro-AMC was dissolved and diluted in methanol. The final methanol concentration in the reaction mixture was 1% which inhibited POP by less than 5% [28]. The reaction was initiated by the addition of the enzyme and the progress curves of AMC liberation were monitored every 1 min over 60 min using a Victor2 fluorescence plate reader at excitation and emission wavelengths of 360 and 460 nm, respectively. Five to six inhibitor concentrations were used for each inhibitor. Blank values (substrate without enzyme) were subtracted from data and the progress curves were fitted to the rate equation of slow binding inhibition [20] (Eq. (1)) using GraphPad Prism version 4.00 software (GraphPad Software, San Diego, CA, USA):

$$[P] = v_s t + \frac{v_0 - v_s}{k_{obs}}(1 - e^{-k_{obs}t}) \quad (1)$$

where [P] is the product concentration, v_0 and v_s the initial and final steady state reaction rates, t the time and k_{obs} is the apparent first-order rate constant for the establishment of the final steady-state velocity.

3.2. Calculation of association and dissociation constants

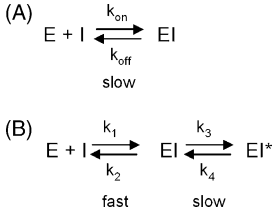
The association and dissociation constants of a slow-binding inhibitor can be determined by plotting the apparent first-order rate constant k_{obs} against inhibitor concentration. Depending on the inhibition mechanism, the plot is a straight line (for the direct binding model, Scheme 1A) or a hyperbola (for the isomerization model, Scheme 1B). All the inhibitors in this study obeyed the direct binding model, as detected by the straight k_{obs} versus inhibitor concentration plots and the data could be fitted to Eq. (2) to solve the association rate constants:

$$k_{obs} = k_{off} + k'_{on}[I] \quad (2)$$

where k_{off} and k'_{on} are the dissociation and association constants, respectively. k'_{on} was determined from the slope of the plot and then corrected for substrate competition using Eq. (3):

$$k_{on} = k'_{on}\left(\frac{1+S}{K_m}\right) \quad (3)$$

Due to the low k_{off} values, the interceptions of the y-axis. Fig. 3 (see Eq. (5)) did not provide accurate estimates of the dissociation constants for most of the studied inhibitors.



Scheme 1 – Kinetic schemes for slow binding inhibition. (A) The binding of the inhibitor to the enzyme active site is a single step process, while in the two-step (B), the inhibitor first binds rapidly to the enzyme (complex EI formation), which then undergoes a slow conformational change to form the inactive complex EI*.

Instead, the dissociation rate constants were calculated from Eq. (4):

$$k_{off} = k_{on}K_i \quad (4)$$

This kind of analysis has been shown to give reliable estimates for the dissociation rate constants [18].

3.3. Calculation of K_i values

The K_i values of compounds **2A** and **3A** were calculated using Eq. (5) and the results from the progress curve experiments:

$$\frac{v_s}{v_0} = \frac{1}{[I]/K_i^{app} + 1} \quad (5)$$

K_i^{app} is an apparent inhibition constant that was converted to a real K_i value by Eq. (6):

$$K_i = \frac{K_i^{app}}{1 + [S]/K_m} \quad (6)$$

in which [S] is the substrate concentration (10 μM) and K_m is the Henri–Michaelis–Menten constant of Z-Gly-Pro-AMC (20 μM). The K_m value was determined as previously reported [28].

K_i values of compounds **1A**, **1B**, **2B** and **3B** could not be determined by the same method as for the compounds **2B** and **3A**, because they were shown to be tight binding inhibitors of POP (their K_i values are of the same order of magnitude as the enzyme concentration in the reaction mixture). Instead, their slow dissociation from the enzyme allowed the direct determination of the real K_i value. The enzyme (0.57 nM) was incubated for 2 h in the presence of various concentrations of inhibitors at room temperature. The reaction was started by addition of Z-Gly-Pro-AMC (final concentration 10 μM) and the reaction was monitored every 1 min for 10–15 min. Over that time range, the product formation was linear, indicating that the inhibitor did not dissociate markedly from the enzyme. K_i values were calculated using the Morrison equation that takes the tight binding inhibition into account (Eq. (4)) [29]:

$$\frac{v_i}{v_0} = 1 - \frac{(E + I + K_i) - \sqrt{(E + I + K_i)^2 - 4EI}}{2E} \quad (7)$$

where v_0 and v_i are the reaction velocities in the absence and presence of the inhibitor (I), respectively, K_i the inhibition constant of the inhibitor and E is the active enzyme concentration in the reaction medium (0.57 nM). Since the inhibitor did not dissociate from the enzyme during the measurement, competition of binding between substrate and inhibitor did not occur, and hence, the calculated K_i value is the real dissociation constant of the inhibitor [30].

4. Ex vivo experiments

4.1. Ex vivo inhibition of POP activity after i.p. administration

Male Wistar rats weighing from 250 to 350 g were treated i.p. with 9 μmol/kg of compounds **1A**, **1B**, **3A** and **3B** or 3 mg/kg of compounds **2A** and **2B**. The doses of compounds **2A** (9.5 μmol/kg) and **2B** (8.8 μmol/kg) differed less than 6% from the standard 9 μmol/kg dose. Rats were decapitated at 10 min–168 h after administration of a single dose. Blood samples were collected from studies with compounds **1A**, **1B** and **2A**. Blood was allowed to clot after which serum was prepared by centrifugation and stored at −70 °C until analyzed. The brains and liver samples were dissected and frozen in liquid nitrogen. The brain and liver samples were stored at −70 °C until homogenized in three volumes of assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0). The homogenate was centrifuged at 10,000 × g, +4 °C, for 20 min. Aliquots of supernatant were frozen and stored at −70 °C until assayed. All procedures with animals were reviewed by the Animal Ethics Committee of the University of Kuopio and approved by the local Provincial Government.

The POP activity was determined in 48 well plates. Ten microlitres of supernatant was preincubated with 465 μl of assay buffer at 30 °C for 30 min. The reaction was initiated by addition of 25 μl of 4 mM Suc-Gly-Pro-AMC and the plates were incubated for 60 min at 30 °C. The reaction was terminated by the addition of 500 μl of 1 M sodium acetate buffer (pH 4.0). The formation of AMC was measured fluorometrically using Victor2 fluorescence plate reader at the excitation and emission wavelengths of 360 and 460 nm.

The protein concentration of the supernatants were determined with Bio-Rad protein assay kit. POP activities were calculated as pmol AMC/(min mg protein) and expressed as percent of vehicle-treated control animals.

4.2. Bioassay of serum concentrations of compounds 1A, 1B and 2A

Since it was not feasible to develop analytical MS chromatography assays to all novel compounds, the concentrations of compounds **1A**, **1B** and **2A** in the serum were estimated by measuring the degree of POP inhibition the serum samples from different time points after compound administration cause. 0.57 nM of purified pig POP was preincubated with appropriately diluted serum sample in 0.1 M Na-K-phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol at 30 °C for 30 min. The reaction was initiated by adding Z-Gly-Pro-AMC (final concentration 10 μM) and the samples collected at different time points were incubated for 30 min at 30 °C. The reaction was terminated by the addition of 500 μl of 1 M sodium acetate buffer (pH 4.0). The formation of AMC was measured fluorometrically using Victor2 fluorescence plate reader at the excitation and emission wavelengths of 360 and 460 nm, respectively. Standards were treated equally to the serum samples except that different concentrations of studied compounds were used instead of serum samples. The concentrations of compounds in serum samples were calculated using this standard curve. The POP activity in serum samples was negligible compared to that of the purified POP protein used in these studies. The detection limit of this biological assay depends on the affinity of the studied compound, being about 2 nM for compounds with a K_i value of less than 0.1 nM and 10 nM for a compound with a K_i value of 1 nM. This bioassay was adequate for the determination of elimination half-lives of the compounds but not for exact pharmacokinetic analysis.

4.3. *Western blot*

The rat brain samples of ex vivo study with compound **3B** were subjected to Western blot analysis to study whether the POP protein level changed due to the reduced enzyme activity. Replicate samples of each time point were pooled (same amount of protein was taken from each replicant). Twenty micrograms protein samples were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes. POP was detected by the primary antibody (IgY against human POP) and secondary antibody (rabbit anti-chicken IgY-HRP, Pierce, 1:20,000) and visualized by chemiluminescence with Super Signal West Femto (Pierce). Analysis of Western blot films was performed using Bio-Rad GS710 densitometer and Quantity One software.

5. Results

5.1. *Slow binding inhibition*

Some representative progress curves of the inhibition of POP by compounds **1A** and **1B** are shown in Fig. 2. In the absence of inhibitor, the steady-state rate of the substrate hydrolysis is reached instantaneously whereas in the presence of the inhibitors, the rate decreases in a time- and concentration-dependent manner. Similar slow binding inhibition was observed also with other inhibitors used in this study (data not shown). The linear plot of k_{obs} versus [I] in Fig. 3 indicates that the inhibition process involves only one step for compounds **1A** and **1B**. The inhibition mechanism was same for all compounds studied.

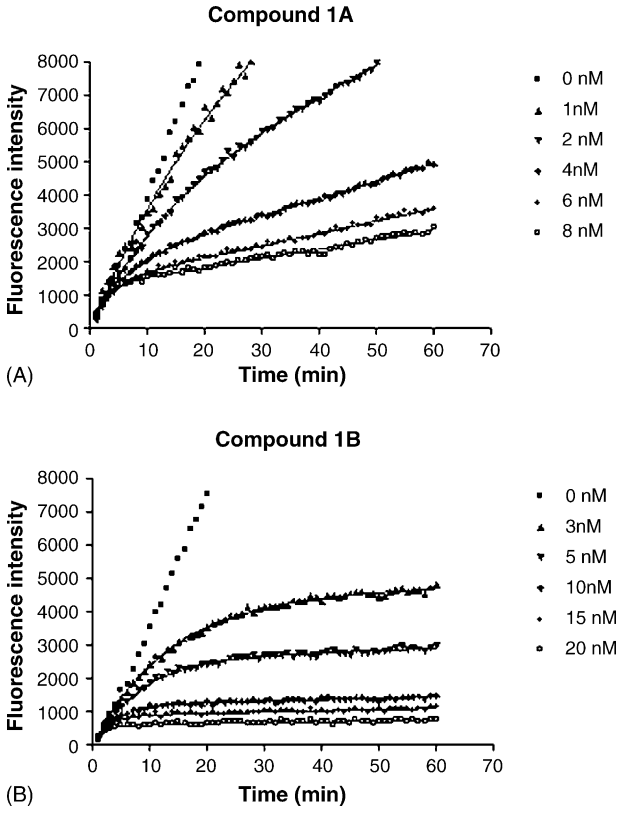


Fig. 2 – Slow binding inhibition of POP. Progress curves for the generation of AMC in the presence of various concentrations of compound 1A (A) and 1B (B) using 10 μM Z-Gly-Pro-AMC as a substrate. The reaction was monitored in 0.1 M Na-K-phosphate buffer, pH 7.0, containing 0.1 mM DTT at 23 °C. The solid curves represent the best fit of the data to Eq. (1) for slow binding inhibition.

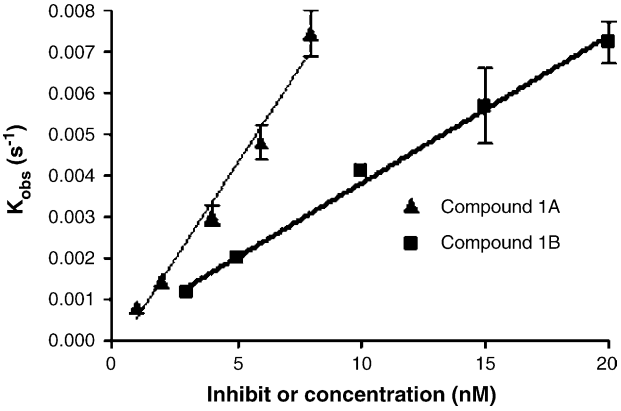


Fig. 3 – Determination of association constant k'_{on} from a plot of k_{obs} (calculated from slow binding inhibition experiments in Fig. 2) vs. inhibitor concentration for compounds 1A and 1B. The lines represent the best fit of the data to Eq. (2). Values are shown as mean ± S.E.M. of three to five separate experiments.

The high potencies and relatively slow onset of inhibition of the studied compounds allowed us to determine the kinetic parameters K_i, k_{on}, k_{off} and the half-life of dissociation (Table 1). The K_i values were at subnanomolar range for all compounds, from 0.022 to 0.97 nM. The CN and $COCH_2OH$ groups at the P1 site (compounds **2B** and **3B**) increased the affinity significantly compared to the inhibitors without functional group (compounds **2A** and **3A**), decreasing the K_i value below 0.03 nM. In compound group 1, a compound with a $COCH_2OH$ substituent had higher affinity than compound CN group. However, also the compound with CN group had 10-fold lower K_i value than the parent compound without functional group at the P1 site (data not shown).

The 2(S)-CN and 2(S)-$COCH_2OH$ substituents at the P1 site pyrrolidine ring also decreased the association rates of the inhibitors when compared to the parent inibitors without the functional groups, as shown in compound groups 2 and 3. The compound pair 1 shows that the addition of $COCH_2OH$ group at the P1 site resulted in slower association than CN group. Interestingly, also the 5-*t*-butyl group at the S2 site proline ring slowed down the inhibitor binding to the enzyme, as shown by the five-fold decrease in k_{on} values between the compounds **2A** and **3A**.

The dissociation half-lives of the enzyme–inhibitor complexes varied from 1.8 min (compound **2A**) to 14.0 h (compound **3B**). CN and $COCH_2OH$ substituents at the P1 site pyrrolidine group increased the half-lives of enzyme–inhibitor complex. In case of compound groups 2 and 3, CN and $COCH_2OH$ substituents increased the half-life of dissociation from 1.8 min to 4.8 h and from 20 min to 14.0 h, respectively.

Table 1 – Kinetic paramaters for POP inhibitors

Compound	K_i (nM)	k_{on} ($\times 10^{-5} M^{-1} s^{-1}$)	k_{off} ($\times 10^{-5} s^{-1}$)	EI half-life	log P
1A	0.36 ± 0.06	12.1 ± 0.9	43.5 ± 8.0	27 ± 5 min	0.8[a]
1B	0.048 ± 0.009	4.92 ± 0.30	2.36 ± 0.46	8.2 ± 1.6 h	0.2[a]
2A	0.97 ± 0.04	67.8 ± 7.5	658 ± 77	1.8 ± 0.2 min	1.8[b]
2B	0.023 ± 0.005	17.5 ± 1.4	4.03 ± 0.87	4.8 ± 1.0 h	1.6[c]
3A	0.39 ± 0.02	14.5 ± 1.6	56.5 ± 6.8	20 ± 2 min	3.3[b]
3B	0.022 ± 0.007	6.29 ± 0.47	1.38 ± 0.45	14.0 ± 4.6 h	2.3[b]

The inhibition kinetic assays were conducted with 10 μM Z-Gly-Pro-AMC as a substrate in 0.1 M Na-K-phosphate buffer, pH 7.0, containing 0.1 mM DTT at +23 °C. Values are mean ± S.E.M. of three to five measurements.

[a] The log P values were adapted from Ref. [22].

[b] The log P values were adapted from Ref. [23].

[c] The log P values were adapted from Ref. [21].

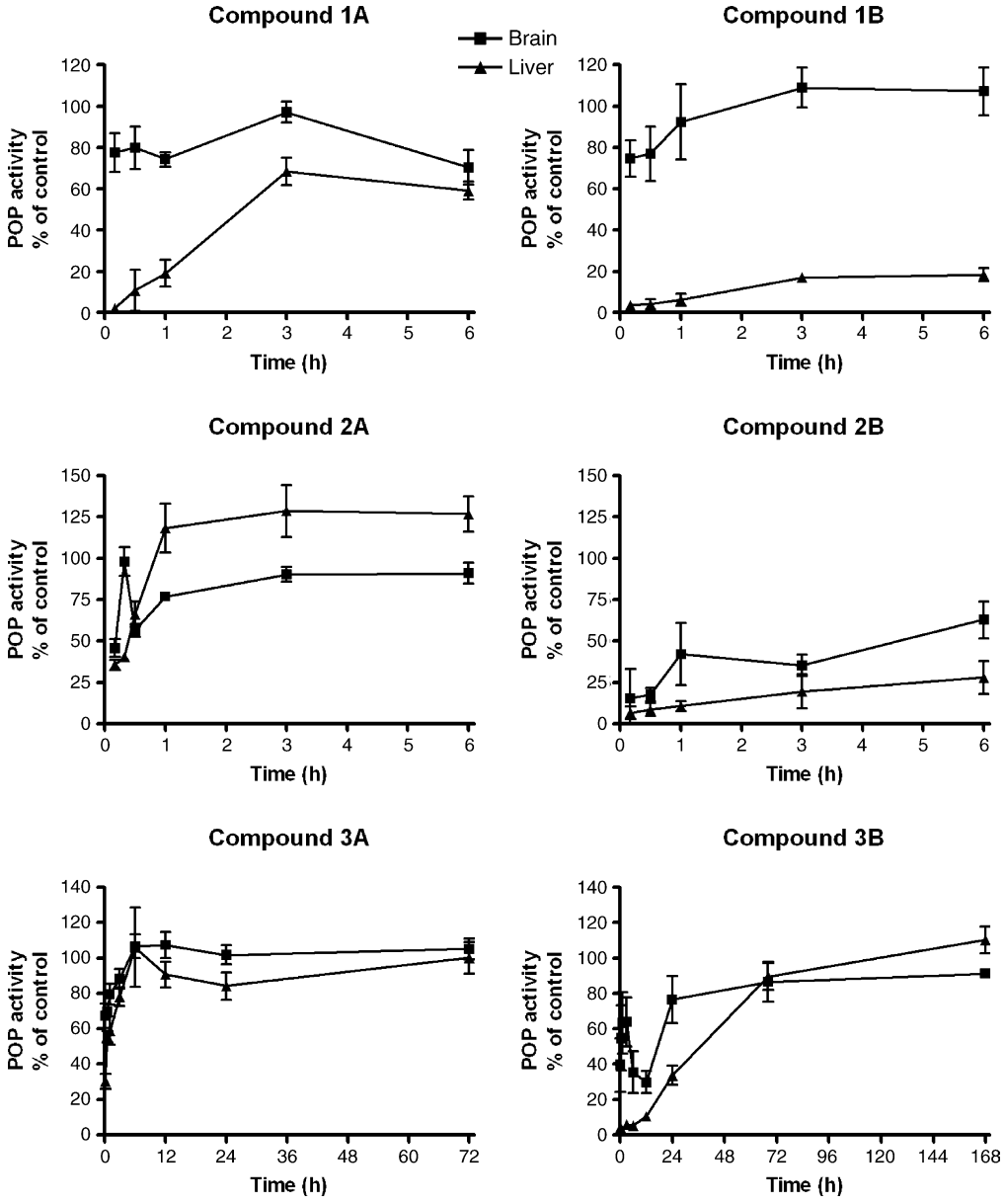


Fig. 4 – Time courses of POP inhibition in rat brain and liver after i.p. injections of studied compounds. Activity is expressed as a percentages of control (vehicle-treated) activities. Values are shown as mean ± S.E.M. of four to six animals for each time point. POP activities were measured using Suc-Gly-Pro-AMC as a substrate.

The $COCH_2OH$ group promoted formation of longer living complex than CN group, as can be seen with compound group 1. Also the 5-t-butyl group at the S2 site proline ring increased the dissociation half-life by one order of magnitude, from 1.8 min of compound **2A** to 20 min of compound **3A**.

5.2. *Ex vivo inhibition studies*

The rat brain and liver POP activities after administration of each compound are shown as a function of time in Fig. 4.

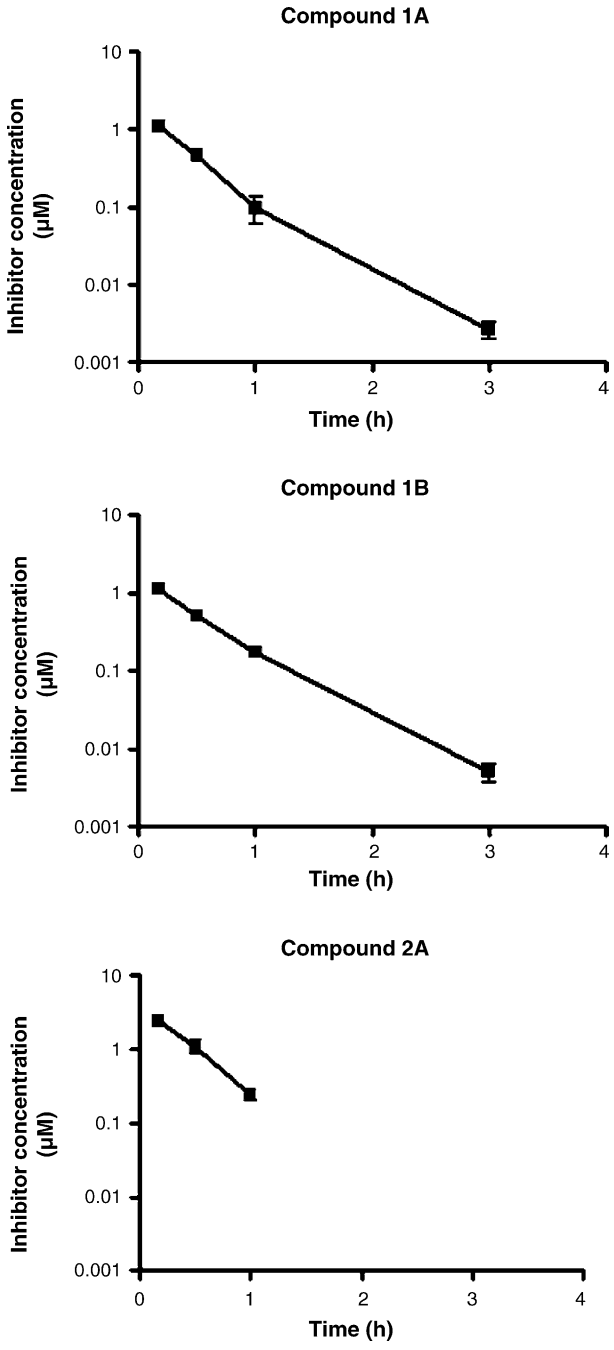


Fig. 5 – The serum concentration vs. time profiles of compounds 1A, 1B and 2A following i.p. administration (9 μmol/kg) in rat. The data represents mean ± S.E.M. of four animals for each time point.

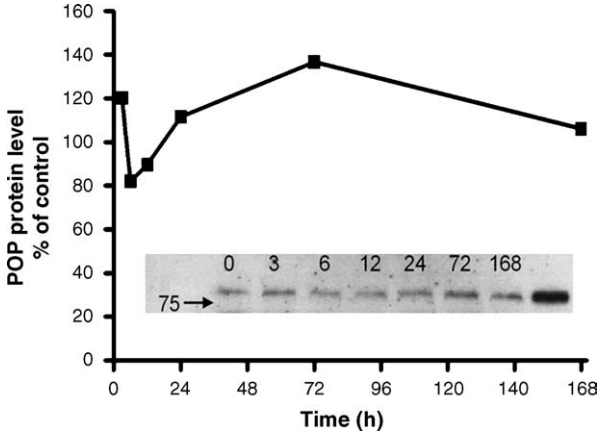


Fig. 6 – Rat brain POP protein levels after i.p. injection of compound 3B analysed from Western blot films. The inset indicates western blot analysis of control brain sample (0) and brain samples of 3, 6, 12, 24, 72 and 168 h after the injection of compound 3A. The outermost lane on the right is a purified pig POP sample.

Both compounds **1A** and **1B** inhibited POP activity only marginally (20–25% inhibition) in the brain but blocked the liver POP activity completely (nearly 100% inhibition). Administration of compound **1B** having 2(*S*)-$COCH_2OH$ at the P1 site pyrrolidine ring resulted in longer-lasting POP inhibition in liver than compound **1A** with CN group; there was still a marked 80% inhibition in the rat liver at 6 h but only 40% inhibition after administration of compound **1A**.

The compound group 2 produced much higher brain POP inhibition than compound group 1; compounds **2A** and **2B** inhibited POP activity by 55% and 85% at 10 min, respectively. The degree of inhibition of liver POP activity was slightly higher than in the brain. In compound **2A** treated animals, the POP activity recovered to the control level within 1–3 h, whereas compound **2B** having CN substituent at the P1 site produced longer-lasting POP inhibition. At 6 h after the administration of compound **2B**, the degree of inhibition of brain and liver POP activity were still 40% and 80%, respectively.

Compound **3B** having the $COCH_2OH$-substituent at the P1 pyrrolidine ring and 5-t-butyl group at the P2 site proline residue had the longest duration of action, there was still a marked inhibition 24 h after the administration. In contrast, the POP activity had fully recovered already at 6 h after the administration of compound **3A**, which does not contain the P1 site substituent group.

5.3. *Serum concentration versus time profiles of compounds 1A, 1B and 2A*

The serum concentrations of compounds **1A**, **1B** and **2A** following single administration were measured to study if the different functional groups would have remarkably altered the serum pharmacokinetics of the compounds. The serum concentration–time profiles of all three compounds were practically identical (Fig. 5). Their absorption was rapid and they had equal and very short terminal half-lives of about

0.3 h. At 1 h after the injection, the serum concentrations of all the compounds were about 10% of the concentration at 10 min and at 3 h, the serum concentrations of compounds **1A** and **2B** were further diminished to less than 1%. The serum concentration of compound **2A** could not be reliably detected at 3 h after the injection because of its lower affinity to POP. The identical serum concentration–time profiles of compounds **1A**, **1B** and **2A** are in sharp contrast to their very different POP inhibition profiles in ex vivo studies (Fig. 4).

5.4. *Western blot analysis of ex vivo samples*

POP protein levels in the rat brain after the administration of compound **3B** are shown in Fig. 6. The ex vivo study with compound **3B** was selected for western blot analysis since it produced longest lasting POP inhibition. The POP protein level in the rat brain remained constant over the 7 days. Thus, it seems that there is no increase in POP synthesis as a response to reduced enzyme activity, at least after administration of a single dose.

6. Discussion

The observed affinity improving effects of CN and $COCH_2OH$ substituents at the P1 site pyrrolidine ring against POP are in line with our earlier studies [18,22]. These functional groups also reduced association and dissociation rates of the inhibitors. The high affinity and slow binding inhibition of the compounds having CN and $COCH_2OH$ substituents are most probable due to the formation of an imino ether and a hemiketal adducts with the Ser554 residue in the active site of POP. The formation of the adduct is a slow process causing the slow association rates. The high affinity of the core structures of compound groups 2 and 3 also allowed us to determine the kinetic constants of inhibitors without the functional groups, contrary to earlier studies [18]. These compound groups showed that the 2(*S*)-CN and 2(*S*)-$COCH_2OH$ substituents decrease the association rates by several folds. By improving the affinity, the substituents also decrease the dissociation rate of the enzyme–inhibitor complex. $COCH_2OH$ substituent resulted in higher affinity and lower dissociation rates than CN.

In this study, also the 5-*t*-butyl group at the P2 site prolyl residue affected the kinetic parameters. The decrease in K_i value was only marginal (from 0.97 to 0.39 nM), but the *t*-butyl group caused a 4-fold decrease in the association rate of the inhibitor. The increased affinity and decreased association rate can be also seen in the dissociation rate of the inhibitor—the addition of the *t*-butyl group at the P2 site increased the dissociation half-life of the enzyme–inhibitor complex by one order of magnitude.

The association rate lowering effects of both P1 site 2(*S*)-$COCH_2OH$ substituents and P2 site 5-*t*-butyl group were seen in compound **3B**. The association rate of this compound was 10 times lower than that of its counterpart without the $COCH_2OH$ and *t*-butyl substituents (compound **2A**). High affinity and slow association rate ensure a very long half-life of 14 h for the enzyme–inhibitor complex, which is 500 times longer than for the parent compound **2A**.

Ex vivo studies showed that there are striking differences in the brain activity and duration of POP inhibition between the compounds studied. Compound pair 1 inhibited POP activity only marginally (25%) in the brain whereas the compound pairs 2 and 3 inhibited brain POP much more effectively. This data suggests that the compounds **1A** and **1B** have poor brain penetration which could result from their low log *P* values (0.8 and 0.2, respectively) and possibly also from their relatively high molecular weights (around 500). The durations of POP inhibitory actions in vivo of the studied compounds were in line with the dissociation half-lives of enzyme–inhibitor complex. In group 1, the $COCH_2OH$-derivative **1B** produced much longer inhibition than its CN-containing counterpart **1A**. Compound **2A**, which had the dissociation half-life of 1.8 min, caused a very short POP inhibition in the rat tissues. In contrast, CN- and *t*-butyl-derivatives of this parent compound produced much longer-lasting POP inhibition both in vitro and in vivo. Compound **2B**, which had an EI half-life of 4.8 h, inhibited liver POP activity by 80% after 6 h of the administration. After administration of the compound **3A** with the EI half-life of 20 min, the POP activity recovered within 6 h. Compound **3B** contains both the P1 site $COCH_2OH$ and P2 site *t*-butyl groups and it produced very long EI half-life of 14 h. As predicted by its EI-half-life, this compound also caused very long-lasting POP inhibition in the rat brain and liver, producing still extensive POP inhibition at 24 h after administration.

The differences in the duration of POP inhibition did evidently not result from the different serum pharmacokinetic profiles of the compounds. Pharmacokinetics of the compounds **1A**, **1B** and **2A**, which had striking differences in the duration of POP inhibition were studied as examples using serum samples collected from the same rats that were used for ex vivo studies. These compounds had remarkably similar serum concentration versus time profiles. Even though compounds **1A** and **1B** produced long-lasting POP inhibition in the liver, they had very fast terminal half-lives of about 0.3 h in serum which was comparable to that of very short-acting inhibitor **2A**. Hence, the long-lasting inhibition of the studied compounds resulted from slow dissociation from the target enzyme and not from the slow clearance from serum. The slow dissociation of the compounds from POP cannot be seen in the serum concentration profiles, since the amount of POP enzyme in the rat is less than 1% of the amount of administrated compound (calculation based on the rat brain POP concentration [28]).

The brain penetrating and long-acting POP inhibitors may have clinical potential in patients with cognitive disturbances and mood disorders, partly owing to their potential to increase the brain levels of several neuropeptides [14,31,32] and partly by their ability to interfere in phosphoinositol pathways connected to depression and mania [33–35]. An interesting extra benefit may come from saving the β-amyloid peptide reinternalization motif, a substrate of POP, that is important in the secretion of β-amyloid elimination [36,37]. A very long half-life of the POP enzyme–inhibitor complex would allow once daily administration or even longer dosing interval.

In conclusion, we demonstrated in the present study that in addition to 2(*S*)-$COCH_2OH$ and 2(*S*)-CN groups at the P1 site pyrrolidine ring of the inhibitor, also bulky 5-*t*-butyl group at

the P2 site prolyl residue can be used to increase the dissociation half-life of the POP-inhibitor complex. In vivo studies showed that the duration of POP inhibition in the rat tissues can be predicted to certain degree by the dissociation constants of the inhibitors. These findings are valuable when designing new POP inhibitors for therapeutic use.

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