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Human recombinant monoamine oxidase B as reliable and efficient enzyme source for inhibitor screening

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Abstract—Interest in inhibitors of monoamine oxidase type B (MAO B) has grown in recent years, due to their therapeutic potential in aging-related neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. This study is devoted to the use of human recombinant MAO B obtained from a *Baculovirus* expression system (Supersomes™ MAO B, BD Gentest, MA, USA) as reliable and efficient enzyme source for MAO B inhibitor screening. Comparison of inhibition potencies (pIC_{50} values) determined with human cloned and human platelet MAO B for the two series of MAO B inhibitors, coumarin and 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives, showed that the difference between pIC_{50} values obtained with the two enzyme sources was not significant ($P > 0.05$, Student's *t*-test). Hence, recombinant enzyme is validated as convenient enzyme source for MAO B inhibitor screening.

1. Introduction

Monoamine oxidase B (MAO B, EC 1.4.3.4) is one of two flavin-adenine dinucleotide-depending isozymes playing a key role in the regulation of endogenous monoamine neurotransmitters as dopamine and serotonin.

A number of studies have shown that MAO B is involved in aging-related neurodegenerative diseases,[1,2] resulting in an increased interest in this enzyme as a target in the search of neuroprotective agents. Ontogenetic studies have demonstrated that MAO B activity stays unchanged until the 60th year of life and then increases nonlinearly. This is in contrast to what has been observed for most enzymes, as generally their activities decrease with advancing years.[3,4]

MAO B plays a double role in the pathophysiology of Parkinson's disease (PD). It is the main enzyme implicated in the metabolism of dopamine and is also supposed to be involved in the formation of free radicals and other neurotoxic species. In order to restore a sufficient striatal dopamine level, MAO B inhibitors such as *l*-deprenyl are currently coadministrated with L-DOPA in the symptomatic treatment of PD.

Increased levels of MAO B have also been observed in plaque-associated astrocytes of brains of patients suffering from Alzheimer's disease (AD).[5] Studies have shown that MAO B activity can increase up to 3-fold in the temporal, parietal, and frontal cortex of AD patients compared with controls. The increased MAO B activity produces an elevation of hydroxyl radicals, which has been correlated with the formation of Aβ plaques. Currently, acetylcholinesterase inhibitors are the only drugs approved for the treatment of cognitive dysfunction in AD. Hence, MAO B inhibitors might offer an alternative in the therapy of AD.

These observations stress the need for new MAO B inhibitors with antiapoptotic and neuroprotective properties for the therapy of neurodegenerative diseases.[1] A condition for the screening of new MAO B inhibitors is a reliable screening system, that is easy to handle and allows to obtain representative trustworthy inhibition values of human MAO B.

Keywords: Human recombinant MAO B; Human platelet MAO B; Inhibitor screening; Neurodegenerative diseases.
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Since human platelets contain almost exclusively monoamine oxidase type B, they became, in recent years, one of the most used biological markers in MAO B-related psychiatric diseases.[6,7] Platelet MAO B has the same amino acid sequence as human brain MAO B.[8] Thus, human platelets offer an excellent peripheral model to indirectly assess MAO B activity in the CNS. Platelet MAO B activity can be measured in whole blood,[9] platelet rich plasma (PRP),[10] platelet homogenate,[11,12] or even purified platelet mitochondrial fraction.[13] The higher the purity of the enzyme preparation, the smaller the risk of erroneous results due to contaminants. Indeed, substrates of MAO B being generally also substrates of plasma amine oxidases, such as semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6), the screening of MAO B inhibitors on whole blood or PRP may lead to unfounded values.[14] On the other hand, since MAO B is located in the outer mitochondrial membrane of cells, its activity depends on the microenvironment where the protein is inserted. As a consequence, purification procedures risk to provide altered enzyme with a very low specific activity, if not done carefully. Hence, the platelet MAO B isolation method used in this work is based on the simple separation of platelets from plasma, avoiding further purification procedures.

Recently, expression systems permitting the production of large quantities of purified human MAO B have been developed. MAO B has been expressed from human MAO B-cDNA using different transfer vectors (e.g., plasmids[15] and *Baculovirus*[16]) and expression systems (e.g., *Pichia Pastoris*[15]). Up to now, the characterization of the catalytic behaviour of recombinant MAO B was mainly performed by studying its interaction with different substrates and by comparing the kinetic constants obtained with those found for MAO B extracted from tissue preparations.[15] Yan et al. also reported the sensitivity of recombinant enzyme toward a few known MAO B inhibitors such as deprenyl and tranylcypromine.[17] As MAO B is a membrane bound enzyme, the mitochondrial microenvironment is important for enzyme activity and inhibitor specificity. Hence, an evaluation of the influence on inhibitor specificities of any differences in the mitochondrial microenvironment of human platelets and insect cells with larger series of compounds is needed.

In this study, the use of human recombinant MAO B as a reliable and easily accessible enzyme source for inhibitor screening was confirmed with two larger series of compounds, namely coumarin ($n = 19$) and 5*H*-indeno[1,2-*c*]pyridazin-5-one ($n = 13$) derivatives.[18,19] The reliability of the recombinant enzyme was validated by comparison of inhibition potencies (pIC_{50} values) determined on human platelet and human recombinant MAO B obtained from *Baculovirus*-infected insect cells (Supersomes™ MAO B, BD Gentest, MA, USA) for both the series of compounds. Convenience for inhibitor screening and advantages of recombinant enzyme over more traditional enzyme sources, as human platelets, are discussed.

2. Results

2.1. Characterization of the enzymatic activity of Supersomes™ MAO B and human platelet MAO B

To characterize the enzymatic activity of the two MAO B sources used, K_m values of kynuramine and pIC_{50} values of three well-known MAO B inhibitors were determined and compared with literature data.

The K_m value of kynuramine, calculated by curve fitting according to the classical Michaelis–Menten equation, was 29 ± 2 μM for Supersomes™ MAO B and 30 ± 4 μM for human platelet MAO B. The results obtained for both the enzyme sources were in good agreement with the K_m values reported by Bembenek et al. (MAO B: 34 μM) on human liver mitochondria[20] and McEntire et al. (MAO B: 30 μM) on pooled platelet-rich plasma.[10]

Inhibition of Supersomes™ and blood platelet MAO B by three-well known MAO B inhibitors, namely deprenyl, pargyline, and iproniazid, was determined (Table 1). The pIC_{50} values obtained corresponded well with literature data.

2.2. Comparison of pIC_{50} values obtained with Supersomes™ MAO B and human platelet homogenate for two classes of inhibitors

To evaluate the reliability in using the cloned enzyme to screen MAO B inhibitors, pIC_{50} values of the two series of compounds, coumarin and 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives, were determined for human recombinant and human platelet MAO B.

pIC_{50} values determined for both the enzyme sources, as well as ΔpIC_{50} values (difference between pIC_{50} Supersomes™ and pIC_{50} human platelets), are reported in Tables 2 and 3 for coumarin 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives, respectively. As shown in Figures 1 and 2, for both the series of compounds, the correlation between the inhibitory potencies determined with the two MAO B sources, demonstrates a linear relationship. Eqs. 1 and 2 describe this linear dependence for coumarin and 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives, respectively:

$$pIC_{50\,platelets} = 1.023(\pm 0.054)pIC_{50\,Supersomes^{TM}} - 0.20(\pm 0.45)$$
$$n = 19;\ r^2 = 0.99;\ s = 0.10;\ F = 1550 \quad (1)$$

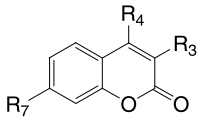
$$pIC_{50\,platelets} = 1.12(\pm 0.18)pIC_{50\,Supersomes^{TM}} - 0.92(\pm 1.35)$$
$$n = 13;\quad r^2 = 0.94;\quad s = 0.21;\quad F = 188 \quad (2)$$

Table 1. Comparison of pIC_{50} values obtained with literature data for three known MAO B inhibitors

	Literature data[a]	Supersomes™	Platelets
Deprenyl	7.22	7.25 ± 0.05	7.44 ± 0.05
Pargyline	6.22[b]	6.33 ± 0.08	6.56 ± 0.09
Iproniazide	4.80	4.28 ± 0.11	4.69 ± 0.18

[a] pIC_{50} values reported by BD Gentest using Supersomes™ as MAO B source (posted on BD Gentest website as poster, 2002).
[b] The value differs from the old published value ($pIC_{50} = 7.4$).[11]

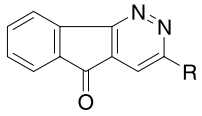
Table 2. MAO B inhibitory activities of coumarin derivatives towards Supersomes™ and human blood platelets



Compound	R_3	R_4	R_7	pIC_{50} human blood platelets	pIC_{50} Supersomes™	ΔpIC_{50}[a]
C-2	H	H	$OCH_2C_6H_5$	8.77 ± 0.10	8.77 ± 0.04	0.00
C-4	H	H	$CH_2NHC_6H_5$	7.20 ± 0.04	7.24 ± 0.06	0.04
C-10	H	CH_3	$OCH_2C_6H_5$	8.96 ± 0.05	8.80 ± 0.06	−0.16
C-12	H	C_6H_5	$OCH_2C_6H_5$	6.48 ± 0.14	6.59 ± 0.05	0.11
C-15	CH_3	CH_3	$OCH_2C_6H_5$	8.90 ± 0.20	8.97 ± 0.13	0.07
C-16	CH_3	CH_3	$NHCH_2C_6H_5$	8.69 ± 0.09	8.86 ± 0.08	0.17
C-21	(—CH=CH—)$_2$		$OCH_2C_6H_5$	9.03 ± 0.13	8.95 ± 0.09	−0.08
C-23	CH_3	CH_3	$NHCOC_6H_5$	8.18 ± 0.02	8.06 ± 0.03	−0.12
C-24	CH_3	CH_3	$OSO_2C_6H_5$	7.74 ± 0.11	7.80 ± 0.11	0.06
C-26	CH_3	CH_3	$OSO_2C_6H_4$—4′—OCH_3	6.60 ± 0.16	6.70 ± 0.05	0.10
C-30[b]	CH_3	CH_3	$OCH_2C_6H_5$	8.59 ± 0.08	8.41 ± 0.05	−0.18
C-50	CH_3	CH_3	$OCH_2C_6H_4$—2′—CN	8.62 ± 0.08	8.71 ± 0.06	0.09
C-54	CH_3	CH_3	$OCH_2C_6H_4$—3′—OCF_3	8.52 ± 0.10	8.44 ± 0.07	−0.08
C-56	CH_3	CH_3	$OCH_2C_6H_4$ 3′—$NHCOCH_3$	8.06 ± 0.17	8.10 ± 0.16	0.04
C-60	CH_3	CH_3	$OCH_2C_6H_4$—3′—CN	8.83 ± 0.07	8.85 ± 0.02	0.02
C-61	CH_3	CH_3	$OCH_2C_6H_4$—3′—NO_2	8.76 ± 0.05	8.96 ± 0.07	0.20
C-71	CH_3	CH_3	$OCH_2C_6F_5$	8.84 ± 0.03	8.79 ± 0.07	−0.05
C-75	H	H	$OSO_2C_6H_5$	5.60 ± 0.06	5.65 ± 0.06	0.05
C-85	CH_3	CH_3	$OCH_2C(=O)C_6H_5$	8.69 ± 0.06	8.63 ± 0.04	−0.06

[a] pIC_{50}Supersomes™-pIC_{50}human blood platelets.
[b] 6-Hydroxycoumarin derivative.

Table 3. MAO B inhibition data for 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives towards Supersomes™ and human blood platelets



Compound	R	pIC_{50} human blood platelets	pIC_{50} Supersomes™	$\Delta p\ IC_{50}$[a]
IP-12	$CH_2C_6H_5$	5.81 ± 0.15	6.12 ± 0.12	0.31
IP-15	*t*-CH=CHC_6H_5	7.57 ± 0.12	7.58 ± 0.06	0.01
IP-17	2′-Naphthyl	7.89 ± 0.22	8.20 ± 0.03	0.31
IP-33	C_6H_4—3′—OCH_3	7.68 ± 0.11	7.37 ± 0.04	−0.31
IP-34	C_6H_4—4′—OCH_3	7.02 ± 0.14	7.13 ± 0.13	0.11
IP-40	C_6H_4—3′—F	7.31 ± 0.17	7.37 ± 0.07	0.06
IP-43	C_6H_4—2′—Cl	6.75 ± 0.13	6.72 ± 0.13	−0.03
IP-47	C_6H_4—3′—Br	8.64 ± 0.21	8.25 ± 0.16	−0.39
IP-48	C_6H_4—4′—Br	8.35 ± 0.29	8.09 ± 0.04	−0.26
IP-51	C_6H_4—4′—CF_3	8.26 ± 0.10	8.27 ± 0.13	0.01
IP-53	C_6H_4—3′—CN	7.57 ± 0.12	7.48 ± 0.11	−0.09
IP-55	C_6H_4—2′—NO_2	6.09 ± 0.13	6.28 ± 0.22	0.19
IP-57	C_6H_4—4′—NO_2	7.98 ± 0.14	8.08 ± 0.05	0.10

[a] pIC_{50} Supersomes™-pIC_{50} human blood platelets.

where n is the number of compounds investigated, r^2 the squared correlation coefficient, s the standard deviation of the residuals, and F the Fischer test for significance of the equation. 95% confidence limits are given in parentheses.

A closer look at Eqs. 1 and 2 shows that identical inhibitor specificity of the two MAO B sources can be assumed. In fact, for both the series of compounds, the slope is one and y-intercepts are zero within the limits of experimental error.

The mean of the differences between the pIC_{50} values obtained for Supersomes™ and those determined for human platelets (ΔpIC_{50}) is 0.01 (±0.11) for the coumarin series and 0.002 (±0.220) for 5*H*-indeno[1,2-*c*]-pyridazin-5-one derivatives; 95% confidence limits are given in parentheses. Statistical analysis (paired two-tailed Student's *t*-test) corroborates the assumption of an identical inhibitor specificity of human recombinant and human platelet MAO B. Indeed, the difference between the pIC_{50} values obtained for the two enzyme sources resulted to be not significant ($P > 0.05$).

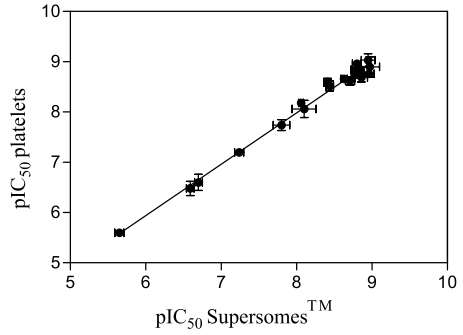


Figure 1. Linear relationship between inhibition potencies (pIC_{50}) of coumarin derivatives determined with human recombinant and human platelet MAO B.

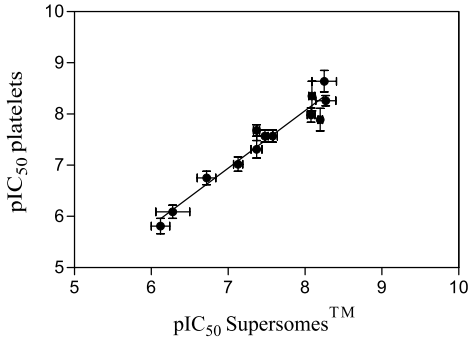


Figure 2. Linear relationship between inhibition potencies (pIC_{50}) of 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives determined with human recombinant and human platelet MAO B.

3. Discussion

The present results provide unequivocal evidence that human recombinant and human platelet MAO B show the same inhibitor specificity. Within experimental errors, the same inhibition potencies (pIC_{50} values) were obtained with human cloned and human platelet MAO B for the two series of MAO B inhibitors covering IC_{50} values of more than three orders of magnitude (pIC_{50} values of 5.6–9). Hence, any differences in the mitochondrial microenvironment of human platelets and insect cells have an insignificant influence on the catalytic properties and inhibitor specificities of recombinant and natural MAO B.

Human recombinant MAO B is not only a reliable but also an efficient human enzyme source for medium- and high-throughput inhibitor screening, presenting notable advantages over blood platelets and mitochondrial fractions of tissue homogenates. Principal demands on an enzyme source for medium- and high-throughput inhibitor screening are (i) prompt availability of sufficient quantities for several hundreds of enzyme assays per day, and (ii) well-defined, pure enzyme systems with reproducible substrate and inhibitor specificities. Indeed, the analyses of inhibition data from well-defined systems such as recombinant enzymes, rather than crude tissue homogenates, allow for a more detailed description of inhibitor binding.[21] Human recombinant MAO B satisfies both of these requirements. Subsequent to the isolation and publication of its gene sequence, expression systems permitting the production of large quantities of purified human MAO B have been developed.[22] Human recombinant MAO B is now also commercially available. On the other hand, the use of human blood platelets or mitochondrial fractions of human tissue homogenates as an enzyme source for medium- and high-throughput inhibitor screening involves some drawbacks; above all, the limited accessibility to outdated human platelet-rich plasma or human tissue.

Since MAO B is an integral protein of the outer mitochondrial membrane, tedious experimental procedures are required to obtain purified enzyme with an intact mitochondrial microenvironment. Therefore, the platelet MAO B isolation method used in this study was based on a careful separation of platelets from plasma, avoiding further purification procedures. An enzyme fraction with unaffected specific activity was obtained, the K_m value of kynuramine being identical with literature data.[10] However, the platelet homogenate obtained was about 33 times less pure than the human recombinant enzyme used, as can be illustrated by the final protein concentration required in the assay mixture, that was set to 0.5 mg/mL for human platelet homogenate and to 0.015 mg/mL for human recombinant MAO B.

In summary, human recombinant MAO B was shown to be a reliable enzyme source, easy to handle, with reproducible substrate and inhibitor specificities, suitable for medium- and high-throughput inhibitor screening.

4. Experimental

4.1. Materials

Kynuramine, deprenyl, and iproniazide were obtained from Sigma–Aldrich Chemical (St. Louis, MA, USA). DMSO (microselect for molecular biology), 4-hydroxyquinolin, pargyline, potassium phosphate salts, potassium chloride, and sodium hydroxide were obtained from Fluka AG (Buchs, CH, Switzerland). The synthesis of all compounds not commercially available was performed in the Dipartimento Farmaco-Chimico (Università di Bari, Bari, Italy). The details of the synthesis of coumarin derivatives have been reported in Gnerre et al.,[18] except for compounds **75** and **85** whose preparation is described below. The synthesis of 5*H*-indeno[1,2-*c*]pyridazin-5-one derivatives has been described by Carotti et al.[23] for compounds **17**, **53** and **55**, and by Kneubühler and co-workers[19,24] for compounds **12**, **15**, **33**, **34**, **40**, **43**, **47**, **48**, **51** and **57**.

4.2. Synthesis of coumarin derivatives 75 and 85

Chemicals and reagents were obtained from Sigma–Aldrich Chemical. The purity of compounds was checked by microanalysis (±0.40% of theoretical values for C, H and N) and ^{1}H NMR. IR and ^{1}H NMR data are listed below. For the former, only the most signifi-

cant absorption bands (per cm) are reported, and for the latter, chemical shifts are expressed in parts per millions (δ) and the coupling constants (J) in Hertz. Classical abbreviations are used for the multiplicities.

4.2.1. 7-Benzensulfonyloxy-2*H*-chromen-2-one (75). 7-Hydroxycoumarin (1.0 mmol) was dissolved in 5 mL of anhydrous pyridine and 3.0 mmol of benzenesulfonyl chloride were added. The mixture was refluxed for 30 min, then cooled, poured onto ice and acidified with diluted HCl. A solid precipitate was formed. It was collected by filtration and recrystallized (98% yield). Mp 134–135 °C from ethanol. IR (per cm) 1734, 1378, 1193. 7.85 (d, 2H, H(2′), H(6′), $J_o = 7.4$), 7.70 (t, 1H, H(4′), $J_o = 7.4$), 7.64 (d, 1H, H(4), $J_o = 9.5$), 7.55 (t, 2H, H(3′), H(5′), $J_o = 7.8$), 7.42 (d, 1H, H(5), $J_o = 8.4$), 7.03 (dd, 1H, H(6), $J_o = 8.4$, $J_m = 2.2$), 6.86 (d, 1H, H(8), $J_m = 2.2$), 6.39 (d, 1H, H(3), $J_o = 9.5$).

4.2.2. 7-Benzoylmethyloxy-3,4-dimethyl-2*H*-chromen-2-one (85). Ten millimoles of 7-hydroxy-3,4-dimethylcoumarin, α-bromoacetophenone, and potassium carbonate were heated in 10 mL of anhydrous dimethylformamide at 120 °C for 3 h. After cooling, the mixture was poured onto ice, and the formed precipitate was collected by filtration and recrystallized (51% yield). Mp 188–189 °C from ethanol. IR (per cm) 1695, 1615, 1235. ^{1}H NMR 7.99–7.95 (m, 2H, H(2′), H(6′)), 7.63 (tt, 1H, H(4′), $J_o = 7.4$, $J_m = 0.9$), 7.53–7.48 (m, 3H, H(5) + H(3′) + H(5′)), 6.92 (dd, 1H, H(6), $J_o = 8.9$, $J_m = 2.6$), 6.76 (d, 1H, H(8), $J_m = 2.6$), 5.34 (s, 2H, CH_2), 2.34 (s, 3H, CH_3(4)), 2.16 (s, 3H, CH_3(3)).

4.3. Biological assay

4.3.1. Supersomes™. Human MAO B Supersomes™, purchased from BD Gentest (Woburn, MA, USA), are membrane fractions of insect cells containing human recombinant MAO B. Supersomes™ were stored at −80 °C. After initial thawing, small aliquots were refrozen.

To determine pIC_{50} values, a fluorescence-based screening method (end point lecture) was adapted from a standard BD Gentest protocol. The substrate used for the assay was kynuramine, which is nonfluorescent until undergoing oxidative deamination by MAO B resulting in the fluorescent metabolite 4-hydroxyquinolin ($\lambda_{Ex} = 310$ nm, $\lambda_{Em} = 400$ nm). Product formation was quantified by comparing the fluorescence emission of the samples to that of known amounts of authentic metabolite 4-hydroxyquinolin.

Reactions were performed in black, flat-bottomed polystyrene 96-well microtiter plates with enhanced assay surface (FluoroNunc/LumiNunc, MaxiSorp™ Surface, NUNC™, Roskild, Denmark) using a final volume of 200 μL. The wells, containing 140 μL of potassium phosphate buffer (0.1 M, pH 7.4, made isotonic with KCl), 8 μL of an aqueous stock solution of kynuramine (0.75 mM to get a final concentration corresponding to its K_m value), and 2 μL of a DMSO inhibitor solution (final DMSO concentration of 1% v/v), were preincubated at pH 7.4, 37 °C for 10 min. As positive control, 2 μL of pure DMSO were used at the place of the inhibitor solution. Diluted human recombinant enzyme (50 μL) was then delivered to obtain a final protein concentration of 0.015 mg/mL in the assay mixture. Incubation was carried out at 37 °C and the reaction was stopped after 20 min by addition of 75 μL of NaOH (2 N). Fluorescence emission at 400 nm was measured with a 96-well microplate fluorescent reader (FLx 800, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Data analysis was performed with Prism V4.0 (GraphPad Software Inc., CA, USA). The kinetic parameters K_m and V_{max} were obtained by curve fitting according to the classical Michaelis–Menten equation and the degree of inhibition pIC_{50} ($-\log IC_{50}$) was assessed by a sigmoidal dose–response curve. ΔpIC_{50} is the difference between the pIC_{50} values found for the same compound using two MAO B sources.

4.3.2. Human platelets. Different batches of outdated human platelet-rich plasma (PRP) preparations were kindly donated by the Laboratoire Central d'Hématologie, Centre Hopitalier Universitaire Vaudois, Lausanne, Switzerland. Each batch had an approximate volume of 200 mL. Platelets were separated from PRP by centrifugation at 6000*g* for 5 min at 5–15 °C. Plasma was discarded and the platelets were suspended in 0.1 M potassium phosphate buffer, pH 7.4, made isotonic with KCl. This platelet suspension was kept frozen at −20 °C overnight. Samples were thawed by addition of a volume of distilled water equal to half of the original volume of PRP. Afterward, the platelets were homogenized in a glass homogenizer with a Teflon pestle for 1 min at 600 rpm. The homogenate was separated in aliquots (1 mL) and kept frozen until use.

The protein concentration of each batch was determined according to Lowry et al., with bovine serum albumin as standard.[25] In the assay mixture, the final protein concentration of the platelet homogenate was set to 0.5 mg/mL. To determine pIC_{50} values, the same fluorescent inhibitor-screening assay developed for human recombinant enzyme was employed.

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