Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 8066–8072
Bioorganic & Medicinal Chemistry

New biscoumarin derivatives-cytotoxicity and enzyme inhibitory activities

Muhammad Iqbal Choudhary,[a,b,*] Naheed Fatima,[b] Khalid M. Khan,[a,b] Saima Jalil,[a] Sajjid Iqbal[b] and Atta-ur-Rahman[a,b]

[a]*Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi 75270, Pakistan*
[b]*H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi 75270, Pakistan*

Received 3 May 2006; revised 19 July 2006; accepted 20 July 2006
Available online 17 August 2006

Abstract—Biscoumarin derivatives **1**–**27** were tested for their inhibition of snake venom and human nucleotide pyrophosphatase phosphodiesterase-1 enzymes. Lineweaver–Burk and Dixon plots and their secondary replots showed that these compounds are pure non-competitive inhibitors of both the enzymes. K_i and IC_{50} values of biscoumarins were found to be in the range of 50 to 1000 and 164 to >1000 μM, respectively, against human recombinant phosphodiesterase 1 enzyme and 8.0 to 1150 and 9.44 to >1000 μM, respectively, against snake venom phosphodiesterase. Compounds **1**, **3**, **4**, **6**, **7**, **17**, **26**, and **30** were found to be non-competitive and non-cytotoxic upto a concentration of 200 μg/mL as evident by less than 10% cell death after 3 h of incubation.

1. Introduction

Biscoumarins [3,3-methylene *bis*-(4-hydroxycoumarin)] are reported to have anticoagulant activity and also identified as hemorrhagic agents in the spoiled clover disease of cattles.[1] They are generally synthesized by condensing formaldehyde with two equivalents of 4-hydroxycoumarin. We have previously reported the urease inhibitory activity of biscoumarins,[2] while Hecht et al. reported the DNA polymerase beta lyase inhibition activity of some naturally occurring biscoumarins.[3] During the current study, a series of biscoumarin derivatives (Tables 1 and 2) were screened for their inhibitory potency against the NPP1 enzymes of two different origins. Detail kinetic studies of these compounds against nucleotide pyrophosphatase phosphodiesterase-1 from snake venom and pure recombinant human source were carried out. This is the first report of the inhibition of phosphodiesterases by biscoumarin compounds (see Schemes 1, 2).

Nucleotide pyrophosphatases/phosphodiesterases (NPPs) are hydrolases that act on diesters of phosphoric acid. They catalyze the release of nucleoside-5′-monophosphates from various pyrophosphate bonds (in, e.g., nucleoside diphosphates and triphosphates, NAD, FAD, and UDP-glucose) and phosphodiester bonds (in oligonucleotides and exogenous substrates like di-*p*-nitrophenyl phosphate, and *p*-nitrophenyl ester of TMP).[4] The NPP family comprises of three members known as NPP-1, NPP-2, and NPP-3. They are widely distributed in mammalian intestinal mucosa, liver cells, and serum, snake venom, and in various plants. They have distinct patterns of distribution in different cell types and even within the same types of cells.[5] NPP-1 has been localized on cells of the distal convoluted tubule of the kidney, chondrocytes, osteoblasts, epididymic, and hepatocytes. NPP1 or PC1 (plasma cell membrane glycoprotein) is a key regulator of calcification of bone and other tissues. Overexpression of NPP1 has been associated with chondrocalcirosis,[6] while underexpression causes severe periarticular calcification in mice[7] and the syndrome of idiopathic infantile arterial calcification in humans.[8]

In continuation of our ongoing studies on the discovery of new phosphodiesterase 1 inhibitors, we now describe the snake venom phosphodiesterase (SVPDE) and human NPP1 (PC-1) inhibitory activities, inhibition

Keywords: Biscoumarins; Enzyme inhibition; Human recombinant phosphodiesterase; PC-1; Kinetics; Cytotoxicity; Nucleotide pyrophosphatase phosphodiesterase-1.
* Corresponding author. Tel.: +92 21 4819010; fax: +92 21 4819018 19; e-mail: hej@cyber.net.pk

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.07.037

Table 1. In vitro snake venom phosphodiesterase 1 activity of biscoumarins **1**–**27**

Compound	IC_{50} (μM)[a] ± SEM[b]	K_i[c] (μM) ± SEM[b]
1	9.44 ± 0.5	8.0 ± 2.1
2	13.34 ± 0.24	12.5 ± 0.82
3	61.3 ± 1.2	150 ± 3.0
4	68.05 ± 1.2	54.5 ± 1.3
5	73.1 ± 0.5	54.5 ± 1.8
6	109.5 ± 3.21	75 ± 0.8
7	147.4 ± 0.4	150 ± 1.2
8	175.2 ± 0.67	125 ± 2.2
9	201.6 ± 0.09	525 ± 2.8
10	257.9 ± 0.76	350 ± 1.8
11	300.8 ± 0.48	350 ± 2.4
12	301 ± 2.8	375 ± 2.0
13	304.8 ± 1.3	225 ± 4.0
14	382 ± 1.55	450 ± 1.2
15	406.7 ± 1.8	500 ± 4.0
16	412.9 ± 2.0	1050 ± 2.4
17	413.1 ± 7.4	225 ± 3.1
18	416.6 ± 3.2	475 ± 0.67
19	487.1 ± 0.78	900 ± 0.8
20	600 ± 4.5	1150 ± 1.0
21	652.2 ± 2.7	1100 ± 2.9
22	654 ± 1.34	350 ± 0.74
23	678 ± 3.41	900 ± 1.3
24	735 ± 3.7	450 ± 0.8
25	>1000	
26	>1000	
27	>1000	

[a] $IC_{50}$is the % inhibitory concentration.
[b] SEM, Standard mean error of 3–5 experiments.
[c] K_i, is the mean of five values calculated from Lineweave–Burk plot, its secondary replots and Dixon plot.

Table 2. In vitro human phosphodiesterase 1 activity of biscoumarins **1**–**27**

Compound	IC_{50} (μM)[a] ± SEM[b]	K_i[c] (μM) ± SEM[b]
1	>1000	—
2	>1000	—
3	261 ± 1.5	250 ± 1.5
4	250 ± 2.5	311 ± 4.0
5	>1000	—
6	688 ± 0.8	850 ± 0.9
7	322 ± 1.2	250 ± 1.7
8	686 ± 0.8	850 ± 4.2
9	761 ± 2.4	850 ± 4.5
10	424 ± 0.87	360 ± 2.1
11	251 ± 0.6	150 ± 0.8
12	995 ± 2.4	990 ± 3.2
13	684 ± 1.4	1000 ± 1.9
14	164 ± 0.4	50 ± 3.5
15	516 ± 2.1	150 ± 4.8
16	850 ± 3.4	250 ± 2.6
17	>1000	—
18	461 ± 2.5	360 ± 2.4
19	563 ± 1.8	600 ± 3.8
20	441 ± 0.95	675 ± 2.4
21	471 ± 2.0	320 ± 2.2
22	870 ± 4.8	250 ± 2.5
23	920 ± 1.2	1000 ± 3.1
24	840 ± 3.8	1000 ± 2.0
25	>1000	—
26	491 ± 1.5	220 ± 3.7
27	>1000	—

—, not tested due to weak inhibitory activity.
[a] IC_{50} is the % inhibitory concentration.
[b] SEM, Standard mean error of 3–5 experiments.
[c] K_i, is the mean of five values calculated from lineweave–Burk plot, its secondary replots and Dixon plot.

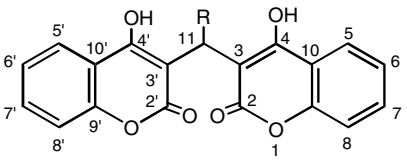
kinetics, and cytotoxicity of a series of biscoumarin derivatives **1**–**27**. In the present study, inhibitory activity was measured spectrophotometrically, while cytotoxicity was assessed on human neutrophils.[9,10]

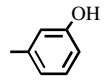
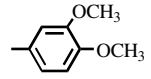
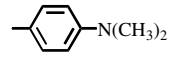
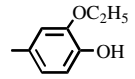
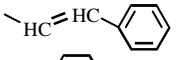
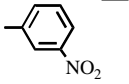
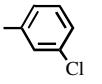
2. Results and discussion

Twenty-seven biscoumarins **1**–**27** were initially evaluated for in vitro snake venom NPP1 inhibitory activity. Determination of IC_{50} values indicated that the compounds **1**–**24** have exhibited significant inhibitory activity against the snake venom phosphodiesterase 1. 4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) methyl]-2*H*-chromen-2-one (**1**) was found to be the most active member of this series with an IC_{50} value of 9.44 ± 0.5 μM against snake venom phosphodiesterase1 (Table 1). Same compounds were then checked for inhibitory effect against the pure human recombinant NPP1. 4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(2-chlorophenyl) methyl]-2*H*-chromen-2-one (**14**) was found to be the most active member of this series with an IC_{50} value of 164 ± 0.4 μM (Table 2). All the active compounds were subjected to kinetic studies and found to be non-competitive inhibitors of both the enzymes. Results are given in Tables 1 and 2. Graphical representations of studies on 4-hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-pyridinyl)methyl]-2*H*-chromen-2-one (**3**), as an example of non-competitive inhibition against the snake venom and pure human recombinant phosphodiesterase 1 enzymes, are presented as Figures 1–4.

2.1. SAR studies on biscoumarins against SVPDE

Twenty-seven biscoumarin derivatives were screened for their snake venom NPP1 inhibitory potential and it was deduced that a substitution at C- 11 plays a pivotal role in increasing or decreasing the activity against the snake venom NPP1. When one of these positions is substituted by any substituent, except for H, a decline in activity was observed. 4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) methyl]-2*H*-chromen-2-one (**1**), having a hydrogen at position C-11, was found to be the most active compound of the series (IC_{50} = 9.44 ± 0.5 μM), even far better than both standards (cystein IC_{50} = 748 μM and EDTA IC_{50} = 274 μM). The second most active compound 4-hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-hydroxyphenyl)methyl]-2*H*-chromen-2-one (**2**) having a 3-hydroxy phenyl at position 11 showed a slightly lower activity (IC_{50} = 13.34 ± 0.24 μM), whereas analogous **15** (IC_{50} = 406.7 ± 1.8 μM) and **19** (IC_{50} = 487.1 ± 0.78 μM) having 3-aminophenyl and 3-methoxy phenyl, respectively, demonstrated a drastic decrease in activity, as compared to compound **2**. The other active compounds **3** (IC_{50} = 61.3 ± 1.2 μM), **4** (IC_{50} =



Compounds	R
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) methyl]-2*H*-chromen-2-one (**1**)	H
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-hydroxyphenyl) methyl]- 2*H*-chromen-2-one (**2**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-pyridinyl) methyl]-2*H*-chromen-2-one (**3**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (1*H*-pyrol-2-yl) methyl]-2*H*-chromen-2-one (**4**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3,4-dimethoxyphenyl) methyl]-2*H*-chromen-2-one (**5**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-*N,N*-dimethyl aminophenyl) methyl]-2*H*-chromen-2-one (**6**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-pyridinyl) methyl]-2*H*-chromen-2-one (**7**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-hydroxy-3-ethoxyphenyl) methyl]-2*H*-chromen-2-one (**8**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) ((*E*)-2-phenylethenyl) methyl]-2*H*-chromen-2-one (**9**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-nitrophenyl) methyl]-2*H*-chromen-2-one (**10**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-chlorophenyl) methyl]-2*H*-chromen-2-one (**11**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) ethyl]-2*H*-chromen-2-one (**12**)	CH_3

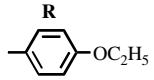
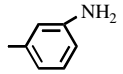
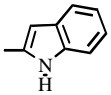
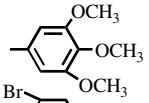
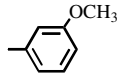
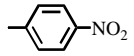
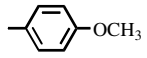
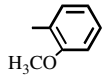
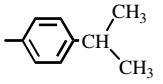
Scheme 1. Chemical structures of biscoumarins **1**–**12**.

68.05 ± 1.2 μM), and **5** (IC_{50} = 73.1 ± 0.5 μM) have pyridinyl, pyrol, and 3,4-dimethyl phenyl at position 11 of biscoumarin moiety, respectively. Compounds **6**–**24** were also found to have a varying degree of activity.

These results clearly indicated that for the snake venom NPP1 enzyme inhibition, the biscoumarins unsubstituted at C-11 are more suitable as substitution at C-11 decreases the inhibitory potential of compounds.

2.2. Structure–activity relationship studies for human recombinant NPP inhibition

Compounds **1**–**27** were also screened against the human recombinant NPP1. Compounds **3**, **4**, **6**–**16**, **18**–**24,** and **26** demonstrated a varying degree of inhibitory potential against this enzyme. The most active compound of the series was 4-hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(2-chlorophenyl) methyl]-2*H*-chromen-2-one (**14**) (IC_{50} = 164 ± 0.40 μM) having a 2-chlorophenyl substituent at C-11 of coumarin moiety, whereas closely related compound **11** having a 3-chlorophenyl substituent at the same position showed a drastic decline in the activity, indicating that the chloro substitution on phenyl ring plays an adverse role in activity. The compound **18** having a 2-bromophenyl at C-11 showed less activity than its chloro analog which further prove that a chlorine at C-2′ of C-11 substituent is important for activity. Compounds **3** (IC_{50} = 261 ± 1.50 μM), **4** (IC_{50} = 250 ± 2.50 μM), and **7** (IC_{50} = 322 ± 1.2 μM) were also significantly active against the human NPP1. Compounds **3** and **7**, two closely related compounds, possess marked difference in activity apparently due to attachment of the pyridine substituent. The activity of compound **4** may be due to a pyrole ring, attached to C- 11.

Compounds	R
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-ethoxyphenyl) methyl]- 2*H*-chromen-2-one (**13**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (2-chlorophenyl) methyl]-2*H*-chromen-2-one (**14**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-aminophenyl) methyl]-2*H*-chromen-2-one (**15**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (1*H*-indol-2-yl) methyl]-2*H*-chromen-2-one (**16**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3,4,5-trimethoxy phenyl) methyl]-2*H*-chromen-2-one (**17**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (2-bromophenyl) methyl]-2*H*-chromen-2-one (**18**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-methoxyphenyl) methyl]-2*H*-chromen-2-one (**19**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-nitrophenyl) methyl]-2*H*-chromen-2-one (**20**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-methoxyphenyl) methyl]-2*H*-chromen-2-one (**21**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (2-thienyl) methyl]-2*H*-chromen-2-one (**22**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-pyridinyl) methyl]-2*H*-chromen-2-one (**23**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (3-thienyl) methyl]-2*H*-chromen-2-one (**24**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (2-methoxyphenyl) methyl]-2*H*-chromen-2-one (**25**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (4-isopropylphenyl) methyl]-2*H*-chromen-2-one (**26**)	
4-Hydroxy-3-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl) (2-pyridinyl) methyl]-2*H*-chromen-2-one (**27**)	

Scheme 2. Chemical structures of biscoumarins **13**–**27**.

3. Conclusion

In conclusion, a large number of biscoumarin derivatives, presented in Tables 1 and 2, were found to be potential inhibitors of snake venom and human NPP1 enzymes. Kinetic analysis of these compounds showed that the binding of inhibitor with the enzyme had no effect on the binding of substrate. Non-competitive inhibition exhibited by these compounds was confirmed by the Lineweaver–Burk plots and their secondary replots as the V_{max}, observed in the presence of these compounds, was less than that observed in the absence of these compounds, while K_m values remain the same.

The cytotoxicity of the most active compounds on human neutrophils was also evaluated by measuring the reduction of WST-1 salt. WST-1 Tetrazolium salt readily reduced to Formazan dye in the presence of cellular enzymes of metabolically active neutrophils. Five concentrations within the ranges 12.5–200 μg/mL of compounds **1**, **3**, **4**, **6**, **7**, **16**, **24**, and **27** were used to determine the viability of cells. Compounds **3**, **4**, **6**, **7**, **16**, and **24**, which showed good to moderate inhibitory effects against both the enzymes, were found to be non-toxic, as evident by >90% viability of cells in the presence of these compounds up to 200 μg/mL. Compound **27** which showed a weak inhibitory activity against the enzymes of both the origins showed ED_{50} value of 85.55 μg/mL, whereas compound **1**, which showed highest activity against the snake venom enzyme but weak activity against the NPP1 of human origin, has an ED_{50} value of 64.3 μg/mL. Graphical presentations are given in Figure 5.

4. Experimental

All reagents were purchased from Aldrich and Sigma. The water used was redistilled and deionized.

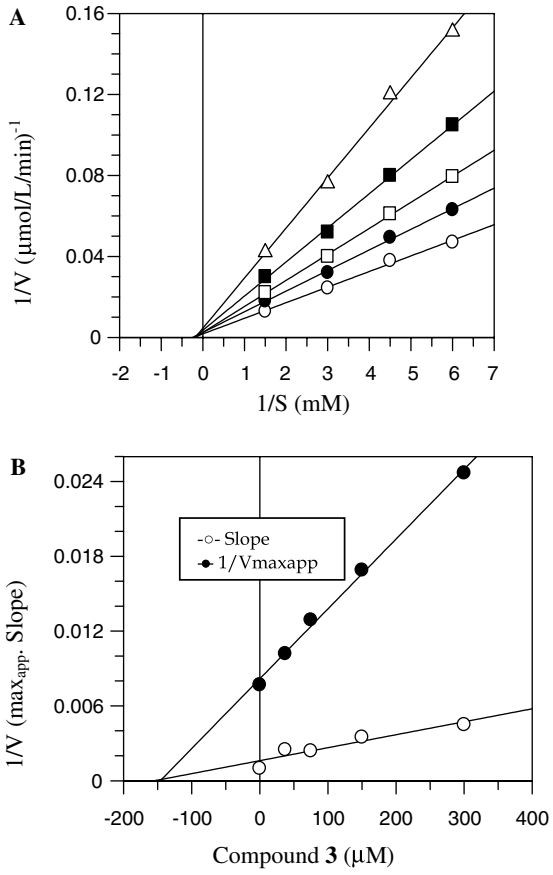


Figure 1. Steady State Inhibition of Snake Venom Phosphodiesterase by Compound **3**. (A) Lineweaver–Burk plot in absence (○) and presence of 37.5 μM; (●), 75 μM (□) and 150 μM (■) and 300 μM (△) of compound **3**, (B) respective secondary replots of the Lineweaver–Burk plot, that is, $1/V_{maxapp}$ and slope versus various concentrations of compound **3**. Each point in the graph represents the mean of three experiments.

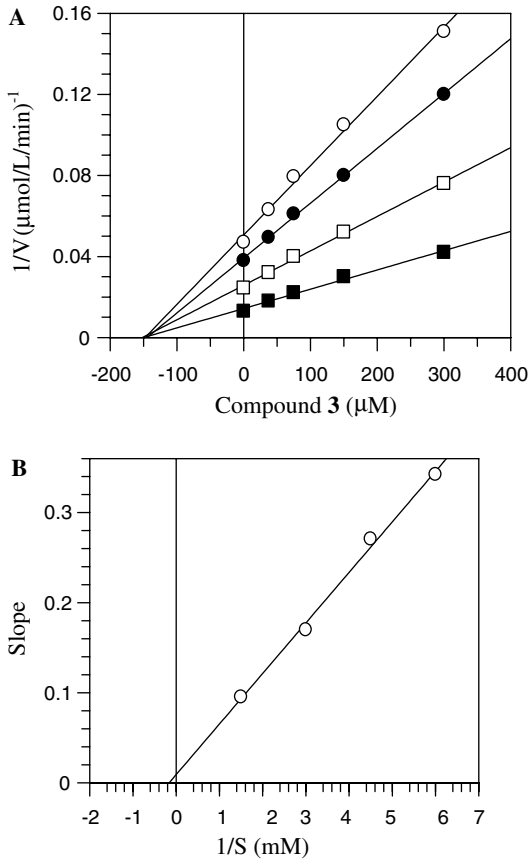


Figure 2. Steady State Inhibition of Snake Venom Phosphodiesterase by Compound **3**. (A) Dixon plot at four fixed *bis*-(*p*-nitrophenyl) phosphate concentrations: (○) 1.5 mM; (●) 3.0 mM; (□) 4.5 mM and (■) 6.0 mM, (B) Secondary replots of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

4.1. Enzyme inhibitory assays

4.1.1. Snake venom. Activity against the snake venom phosphodiesterase I (Sigma P 4631) (EC 3.1.4.1) was assayed by using the reported method[11] with the following modifications. 33 mM Tris–HCl buffer, pH 8.8, 30 mM Mg-acetate with 0.000742 U/well final concentrations using microtiter plate assay and 0.33 mM *bis*-(*p*-nitro phenyl) phosphate (Sigma N-3002) as a substrate. Cysteine and EDTA[12–14] (E. Merck) were used as positive controls (IC_{50} = 748 μM ± 0.15, 274 μM ± 0.07, respectively). After 30 min of incubation, the enzyme activity was monitored spectrophotometrically at 37 °C on a microtiter plate spectrophotometer (Spectra Max, Molecular Devices) by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 410 nm. All the reactions were performed in triplicate and the initial rates were measured as the rate of change in OD/min (optical density/min) and used in subsequent calculations.

4.1.2. Human recombinant NPP1 (PC-1). Recombinant water-soluble human NPP1 (catalytic extracellular domain) was received from Professor J. W. Goding, Monash University, Melbourne, Australia, as a gift. NPP1 was assayed by hydrolysis of *p*-nitrophenyl phenyl phosphonate (Sigma N-2881), as described by Kelly et al.[15] The assay was performed in a buffer consisting of 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, and 1 mM $CaCl_2$. Enzyme activity was monitored spectrophotometrically at 37 °C on a microtiter plate spectrophotometer (Spectra Max, Molecular Devices) at 405 nm by following the release of *p*-nitrophenol. All assays were conducted in triplicate.

4.1.3. Enzyme kinetic studies. Kinetic parameters were calculated using EZ-Fit Enzyme Kinetics Program (Perrella Scientific Inc., Amherst, USA). The assay conditions for measurement of the residual activities of all inhibitors were identical, except that fixed concentrations of inhibiting compounds were used in the assay medium. Dissociation constants (K_i values) were determined by the interpretation of Dixon plots, Lineweaver–Burk plots and their secondary plots using initial velocities measured over substrate concentrations between 0.166 and 0.666 mM for snake venom, and 1.0 and 2.5 mM for human recombinant NPP1.

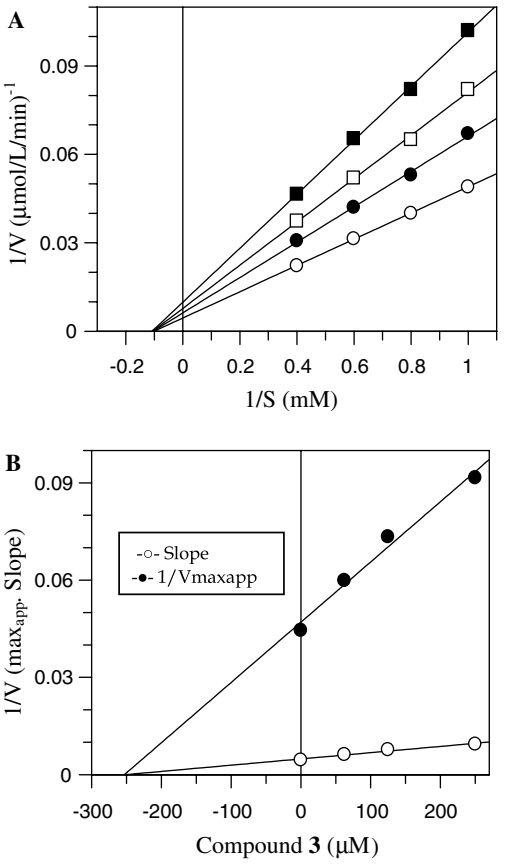


Figure 3. Steady State Inhibition of Human PC-1 by Compound **3**. (A) Lineweaver–Burk plot in absence (○) and presence of 62.5 µM (●), 125 µM (□) and 250 µM; (■) of compound **3**, (B) respective secondary replots of the line weaver Burk plot, that is, $1/V_{maxapp}$ and slope versus various concentrations of compound **3**. Each point in the graph represents the mean of three experiments.

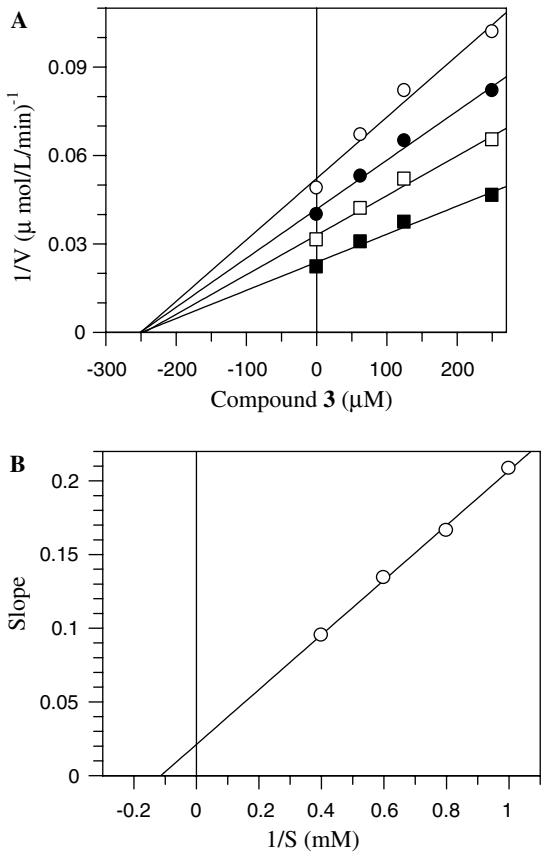


Figure 4. Steady State Inhibition of Human PC-1 by Compound **3**. (A) Dixon plot at four fixed *p*-nitrophenyl phenyl phosphonate concentrations: (○) 0.4 mM; (●) 0.6 mM; (□) 0.8 mM and (■) 1.0 mM, (B) Secondary replots of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

4.1.4. Determination of type of inhibition. Two different methods were applied to monitor the effect of the inhibitor on both K_m and V_{max} values. This was done first by plotting the reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as Lineweaver–Burk plot,[16] and second by the Dixon plot in which the reciprocal of the rate of the reactions was plotted against the inhibitor concentrations.[17] The secondary re-plot of the Lineweaver–Burk plot was also constructed as $1/V_{max}$ or slope against different concentrations of the respective inhibitor. The secondary re-plot of Dixon plot was constructed as the slope of each line of substrate concentration in original Dixon plot against the reciprocals of the substrate concentrations.

The types of inhibition were determined by the graphical views of Dixon plots, Lineweaver–Burk plots and their secondary plots. K_i Values (the constant of dissociation of the enzyme-inhibitor complex into free enzyme and inhibitor) were determined by the interpretation of Dixon plot, Lineweaver–Burk plot and its secondary replots by using initial velocities.

4.1.5. Statistical analysis. Assays were conducted in triplicate at each concentration of the potential inhibitors. Graphs were plotted using GraFit program (Leather barrow, R. J. GraFit; 4.09 ed; Erithacus Software Ltd.; Tains, UK). Values of the correlation coefficient, slope, intercept, and their standard errors were obtained by the linear regression analysis using the same software. The correlation coefficient for all the lines of all graphs was found to be >0.99. Each point in the constructed graphs represents the mean of three experiments.

4.2. Cell viability assay

4.2.1. Isolation of human neutrophils. Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated by the method of Siddiqui et al.[18] Briefly, whole blood was mixed with Ficoll paque with the ratio of 1:3 and left for the sedimentation of unwanted red blood cells. After 30 min, buffy coat was formed and RBCs were sedimented in the bottom.This buffy coat was layered on the Ficoll (3 mL) in clean glass centrifuge tubes and centrifuged for 30 min at 1500 rpm. After discarding the supernatant, unwanted RBCs were further lysed by mixing with hypotonic ammonium chloride solution (0.83%) and kept for 10 min then centrifuged and washed

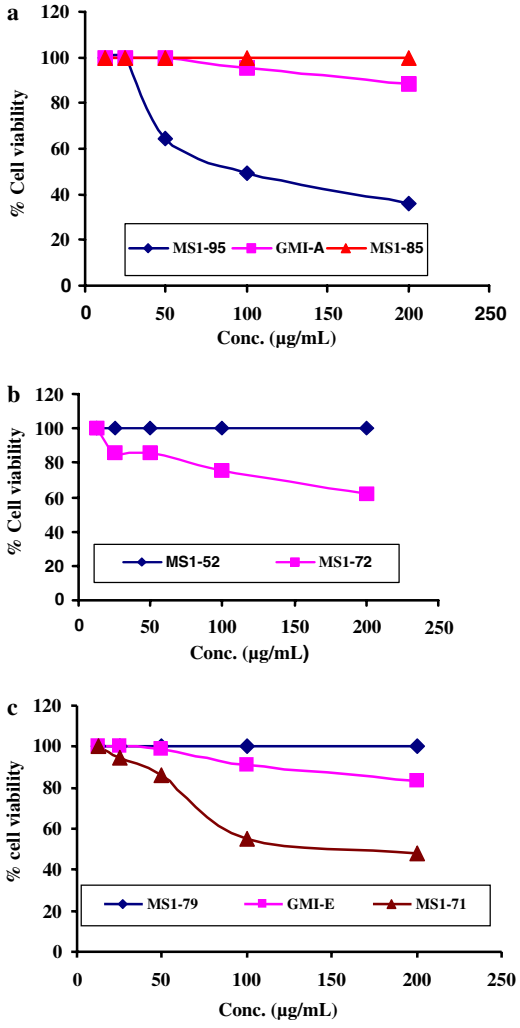


Figure 5. % Cell viability of freshly isolated human neutrophils (1×10^7 cells/mL) in the presence of various concentrations (200–12.5 µg/mL) of compound (a) **1**, **3**, **4**, (b) **6**, **7** (c) **17**, **26**, and **30**.

the pallet with modified hank, solution (MHS) and resuspended the cell at 1×10^7 cells/mL concentration.

4.2.2. Assay procedure. The modified method of Berridge et al.[9] was used. Briefly, the human isolated neutrophils (1×10^7 cells/mL) were incubated with the test compounds for 30 min, WST-1 (0.25 mM) was added, and the plate was incubated in shaking water bath at 37 °C. After 3 h incubation, absorbance at 450 nm was measured with microplate reader (SpectraMAX 340, MolecularDevices). The OD is the mean of five experimental replicates. Percentage Cell Viability was calculated by using the following formula:

$$\% \text{ Viability of cells} = \{(\text{OD test compound} \times 100/\text{OD control}) - 100\}$$

Acknowledgments

We are extremely grateful to Professor J. W. Goding, Monash University, Melbourne, Australia, for a generous gift of recombinant water-soluble human NPP1 (catalytic extra cellular domain) and also for reviewing this manuscript. We also acknowledge Higher Education Comission (HEC), Pakistan, for the financial assistance under 'National Research Program for Universities'. We gratefully acknowledge the help of the Fatmid Foundation for providing human blood.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.07.037.

References and notes

1. Campbell, H. A.; Link, K. P. *J. Biol. Chem.* **1941**, *138*, 21.
2. Khan, K. M.; Iqbal, S.; Lodhi, M. A.; Maharvi, G. M.; Ullah, Z.; Choudhary, M. I.; Atta-ur-Rahman; Perveen, S. *Bioorg. Med. Chem.* **2004**, *12*, 1963.
3. Li, S. S.; Gao, Z.; Feng, X.; Hecht, S. M. *J. Nat. Prod.* **2004**, *67*, 1608.
4. Razzel, W. E. Phosphodiesterases. In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1963; Vol. 6, pp 236–258.
5. Goding, J. W.; Terkeltaub, R.; Maurice, M.; Deterre, P.; Sali, A.; Belli, S. I. *Immunol. Rev.* **1998**, *161*, 11.
6. Johnson, K.; Hashimoto, S.; Lotz, M.; Pritzker, K.; Goding, J.; Terkeltaub, R. *Arthritis Rheum.* **2001**, *44*, 1071.
7. Okawa, A.; Nakamura, I.; Goto, S.; Moriya, H.; Nakamura, Y.; Ikegawa, S. *Nat. Genet.* **1998**, *19*, 271.
8. Rutsch, F.; Vaingankar, S.; Johnson, K.; Goldfine, I.; Maddux, B.; Schauerte, P.; Kalhoff, H.; Sano, K.; Boisvert, W. A.; Superti-Furga, A.; Terkeltaub, R. *Am. J. Pathol.* **2001**, *158*, 543.
9. Berridge, M. V.; Tan, A. S.; McCoy, K. D.; Wang, R. *Biochemica* **1996**, *4*, 15.
10. Francoeur, A. M.; Assalian, A. *Biochemica* **1996**, *3*, 19.
11. Mamillapalli, R.; Haimovitz, R.; Ohad, M.; Shinitzky, M. *FEBS Lett.* **1998**, *436*, 256.
12. Razzell, W. E.; Khorana, H. G. *J. Biol. Chem.* **1959**, *234*, 2105.
13. Nakabayashi, T.; Matsuoka, Y.; Ikezawa, H.; Kimura, Y. *Int. J. Biochem.* **1994**, *26*, 171.
14. Lopez-Gomez, J.; Costas, M. J.; Ribeiro, J. M.; Fernandez, A.; Romero, A.; Avalos, M.; Cameselle, J. C. *FEBS Lett.* **1998**, *421*, 77.
15. Kelly, S. J.; Dardinger, D. E.; Butler, L. G. *Biochemistry* **1975**, *14*, 4983.
16. Dixon, M. *Biochem. J* **1953**, *55*, 170.
17. Segal, I. In *Enzyme Kinetics*, 2nd ed.; John Wiley and Sons: New York, USA, 1993; pp 125–143.
18. Siddiqui, R. A.; English, D.; Cui, Y.; Martin, M. I.; Wentlands, J.; Akard, L. *J. Leukocytes Biol.* **1995**, *58*, 189.