Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 5793–5798
Bioorganic & Medicinal Chemistry

Phenolic glycosides, a new class of human recombinant nucleotide pyrophosphatase phosphodiesterase-1 inhibitors

Muhammad Iqbal Choudhary,* Naheed Fatima, Muhammad Athar Abbasi, Saima Jalil, Viqar Uddin Ahmad and Atta-ur-Rahman

Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical Sciences, University of Karachi, Karachi 75270, Pakistan

Received 22 July 2004; revised 26 August 2004; accepted 26 August 2004
Available online 17 September 2004

Abstract—Cytotoxicity and kinetic studies of phenolic glycosides, benzoyl salireposide (**1**) and salireposide (**2**), isolated from *Symplocos racemosa*, were performed against phosphodiesterase I enzyme from snake venom and human nucleotide pyrophosphatase phosphodiesterase-1. Lineweaver–Burk and Dixon plots and their secondary replots showed that these compounds are pure non-competitive inhibitors of both enzymes. K_i Values of compounds **1** and **2** were found to be 360 and 1000 μM, respectively, against human nucleotide pyrophosphatase phosphodiesterase, and 525 and 1100 μM, respectively, against snake venom phosphodiesterase. IC_{50} values of compounds **1** and **2** are 90 μM ± 0.04 and 383 μM ± 0.03, respectively, against human nucleotide pyrophosphatase phosphodiesterase and 171 μM ± 0.02 and 544 μM ± 0.021, respectively, against snake venom phosphodiesterase. Both compounds were found to be nontoxic up to concentration of 500 μM/mL as >90% cells were viable after 3 h of incubation. These compounds are potential candidates for the therapy of arthritis.

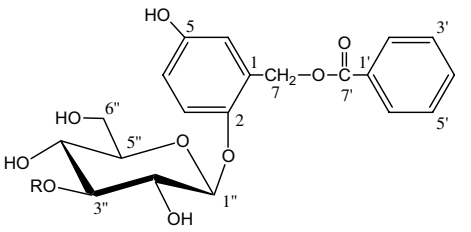
1. Introduction

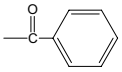
Nucleotide pyrophosphatases/phosphodiesterases (EC 3.1.4.1 NPP1) catalyze the release of nucleoside-5′-monophosphates from a variety of nucleotides or nucleotide derivatives.[1] They exist both as membrane proteins with an extracellular active site or as soluble proteins in body fluids. They are widely distributed in mammalian intestinal mucosa, mammalian liver cells, serum, snake venom, and various plants.[1–3] NPPs release nucleoside 5′-monophosphates from various pyrophosphate bonds (e.g., nucleoside diphosphates and triphosphates, NAD, FAD, UDP-glucose) and phosphodiester bonds (oligonucleotides and some artificial substrates like di-*p*-nitrophenyl phosphate, *p*-nitrophenyl ester of TMP).[2,3] NPP1 or PC-1 (plasma cell membrane glycoprotein) is a key regulator of calcification of bone and other tissues. Over-expression of NPP1 has been associated with chondrocalcirosis,[4] while under-expression causes severe periarticular calcification in mice[5,6] and the syndrome of idiopathic infantile arterial calcification in humans.[7] Recently benzoyl salireposide (**1**) and salireposide (**2**) were isolated from *Symplocos racemosa* and identified as new snake venom phosphodiesterase (SVPDE1) inhibitors by us.[8] The plant *S. racemosa* (Symplocaceae) was collected from Abbot Abad, Pakistan, and identified by Dr. Manzoor Ahmed, Taxonomist at the Department of Botany, Post-Graduate College, Abbot Abad, Pakistan. A voucher specimen (No.6453) has been deposited at the herbarium of the Department of Botany, Post Graduate College, Abbot Abad, Pakistan.

In continuation of our ongoing work on the phosphodiesterase I inhibition by natural products, we now describe the detail SVPDE and human NPP1 (PC-1) inhibitory activities, inhibition kinetics, and cytotoxicity of two benzoyl glycosides **1** and **2**. In the present study, measurement of cell viability was carried out by reduction of WST-1 salt to yellow colored Formazan dye in presence of viable human neutrophils. Neutrophils are defensive cells against bacterial and viral infections or any type of external factors like proteins, allergens, etc. They provide a good model for cytotoxicity assessment.[9,10]

Keywords: PC-1; Inhibition; Kinetics; Phenolic glycosides; Cytotoxicity.
* Corresponding author. Tel.: +92 21 9243211/9243224; fax: +92 21 9243190/91; e-mail: hej@cyber.net.pk

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.08.035



1 R= 

2 R= H

2. Results and discussion

Recently phenolic glycosides benzoyl salireposide (**1**) and salireposide (**2**) were isolated from the plant *S. racemosa* Roxb. and identified as the natural inhibitors of PDE-1 from snake venom.[8] We now report the inhibitory activity of compounds **1** and **2** against nucleotide pyrophosphatase phosphodiesterase along with kinetic studies against both enzymes.

The K_i values (the dissociation constant of the enzyme–inhibitor complex into free enzyme and inhibitor) of compounds **1** and **2** are 360 μM ± 0.2 and 1000 μM ± 0.14, respectively, against human PC-1, and 525 μM ± 0.06 and 1100 μM ± 0.2 were against snake venom phosphodiesterase **I**. The IC_{50} values (the concentrations of test compounds that inhibits the enzyme activity by 50%) of compounds **1** and **2** are 90 μM ± 0.04 and 383 μM ± 0.03, respectively, against human PC-1. Compounds **1** and **2** were found to be noncompetitive inhibitors of both human PC-1 and snake venom phosphodiesterase I enzyme.

Both compounds exhibited a pure noncompetitive type inhibition as they decrease the V_{max} values without affecting the affinity of the enzyme towards the substrate (K_m values). Secondary replots of Lineweaver–Burk plots show linear lines rather than hyperbolic lines as expected in the case of partial noncompetitive inhibition. The graphic analysis of steady-state inhibition data of compounds **1** and **2** for noncompetitive inhibition against human PC-1 and snake venom phosphodiesterase are shown in Figures 1–4.

Structures of both compounds are different only by an additional benzoyl group at C-3″ of the glucose, Which apparently plays an important role in the inhibitory potential of these compounds against NPP1, with enhanced inhibition.

We have also evaluated the cytotoxicity of these compounds on human neutrophils by measuring 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

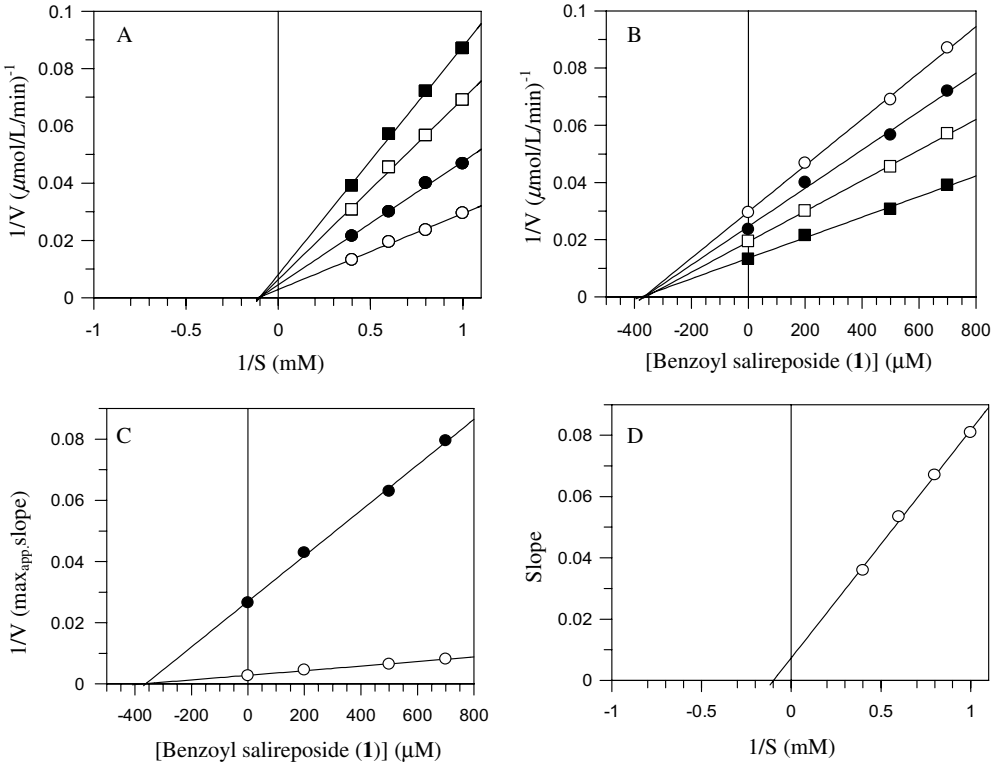


Figure 1. Steady state inhibition of human PC-1 by benzoyl salireposide (**1**). (A) Lineweaver–Burk plot in absence (○) and presence of 200 μM (●), 500 μM (□), and 700 μM (■) of compound **1**. (B) Dixon plot at four fixed *p*-nitrophenyl phenylphosphonate concentrations: (○) 0.4 mM; (●) 0.6 mM; (□) 0.8 mM and (■) 1.0 mM. (C) Respective secondary replots of the Lineweaver–Burk plot, that is $1/V_{max_{app}}$ and slope versus various concentrations of compound **1**. (D) Secondary replot of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

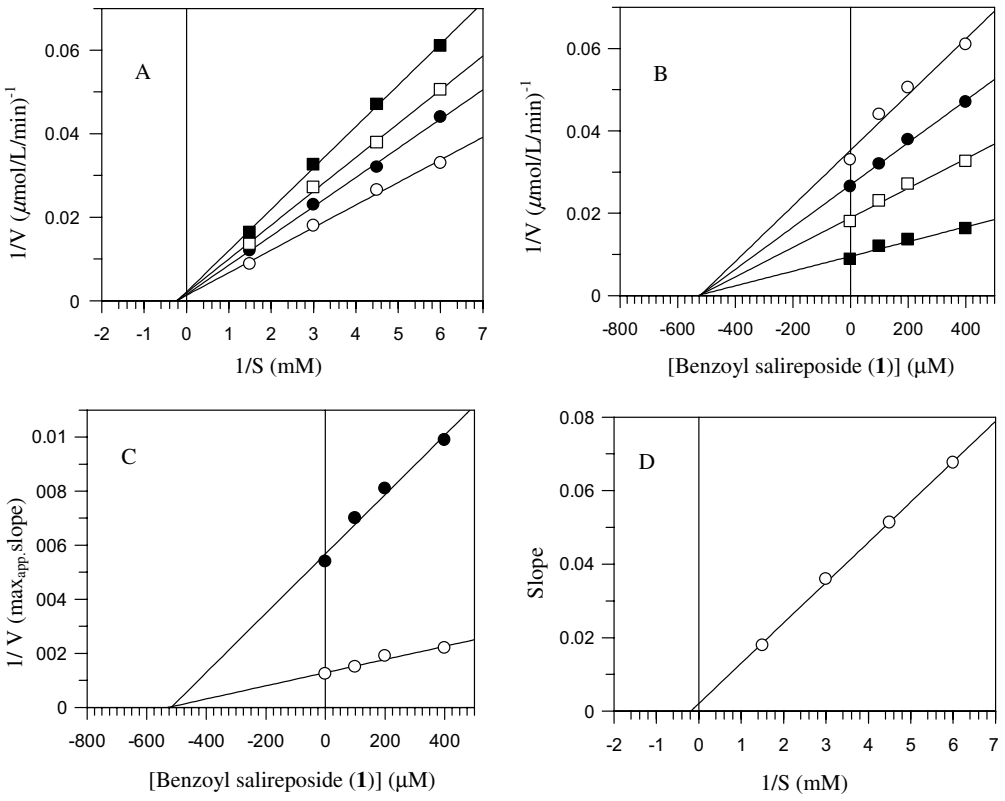


Figure 2. Steady state inhibition of snake venom phosphodiesterase by benzoyl salireposide (**1**). (A) Lineweaver–Burk plot in absence (○) and presence of 100 μM (●), 200 μM (□), and 400 μM (■) of compound **1**. (B) Dixon plot at four fixed bis-(*p*-nitrophenyl) phosphate concentrations: (○) 1.5 mM; (●) 3.0 mM; (□) 4.5 mM and (■) 6.0 mM. (C) Respective secondary replots of the Lineweaver–Burk plot, that is $1/V_{max_{app}}$ and slope versus various compound concentrations. (D) Secondary replots of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

ranges 500–25 μM/mL of compounds **1** and **2** were used to determine the viability of cells (Fig. 5). Both compounds were found to be nontoxic as neutrophils showed >90% viability up to 500 μM/mL.

3. Experimental

All reagent grade chemicals were obtained from Aldrich and Sigma Chemical used without further purification. The used water was redistilled and ion free.

3.1. Enzyme inhibitory assays

3.1.1. Snake venom phosphodiesterase. Activity against snake venom phosphodiesterase I (Sigma P 4631) (EC 3.1.4.1) was assayed by using the reported method[11] with the following modifications. Tris–HCl buffer (33 mM) pH 8.8, 30 mM Mg-acetate with 0.000742 U/well final concentrations using microtitre plate assay and 0.33 mM bis-(*p*-nitro phenyl) phosphate (Sigma N-3002) as a substrate. Cysteine and EDTA[1,12–14] (E. Merck) were used as positive controls (IC_{50} = 748 μM ± 0.15, 274 μM ± 0.07, respectively). After 30 min incubation, the enzyme activity was monitored spectrophotometrically at 37 °C on a microtitre plate spectrophotometer (Molecular Devices) by following the release of *p*-nitro phenol from *p*-nitro phenyl phosphate at 410 nm. All the reactions were performed in triplicate and the initial rates was measured as the rate of change in OD/min (optical density/min) and used in subsequent calculations.

3.1.2. Human recombinant NPP1 (PC-1). Recombinant water soluble human NPP1 (catalytic extracellular domain) was the gift of Professor J. W. Goding, Monash University, Melbourne, Australia. 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 microtitre plate spectrophotometer (Molecular Devices) at 405 nm by following the release of *p*-nitrophenol. Assays were conducted in triplicate.

3.1.3. 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 were drawn over substrate concentrations between 0.166 and 0.666 mM for snake venom and 1.0 and 2.5 mM for human recommended PC-1.

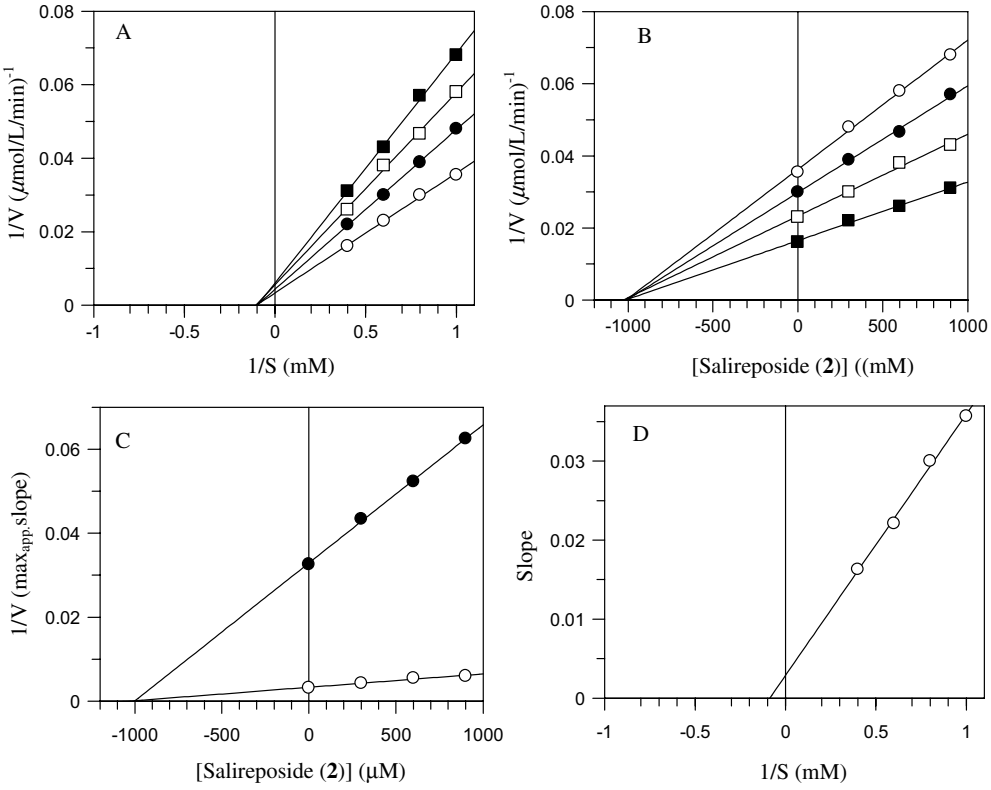


Figure 3. Steady state inhibition of human PC-1 by salireposide (**2**). (A) Lineweaver–Burk plot in absence (○) and presence of 300 μM (●), 600 μM (□), and 900 μM (■) of compound **2**. (B) Dixon plot at four fixed *p*-nitrophenyl phenyl phosphonate concentrations: (○) 0.4 mM; (●) 0.6 mM; (□) 0.8 mM and (■) 1.0 mM. (C) Respective secondary replots of the Lineweaver–Burk plot, that is $1/V_{max_{app}}$ and slope versus various concentrations of compound **2**. (D) Secondary replots of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

3.1.4. 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 firstly by plotting the reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as Lineweaver–Burk plot[16] and secondly by the Dixon plot in which the reciprocal of the rate of the reactions was plotted against the inhibitor concentrations.[17] The secondary replot of the Lineweaver–Burk was also constructed as $1/V_{max}$ or slope against different concentrations of the respective inhibitor. The secondary replot 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.

3.1.5. Statistical analysis. Assays were conducted in triplicate at each concentration of the 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 >0.99, at each point in the constructed graphs represents the means of three experiments.

3.2. Cell viability assay

3.2.1. 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 and left for the sedimentation of unwanted red blood cells. After 30 min, buffy coat was layered on the Ficoll (3 mL) in the centrifuge tube and centrifuged for 30 min at 1500 rpm. After discarding the supernatant, unwanted RBCs was further lysed by mixing it with hypotonic ammonium chloride solution (0.83%), centrifuged, and wash the neutrophils with MHS (Modified Hank's solution) and resuspended at 1×10^7 cells/mL.

3.2.2. Assay procedure. We used the modified method of Berridge et al.[19] 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 incubated in shaking water bath at 37 °C. After 3 h incubation, absorbance at 450 nm was measured with spectraMAX 340 (Molecular Devices) micro plate reader.

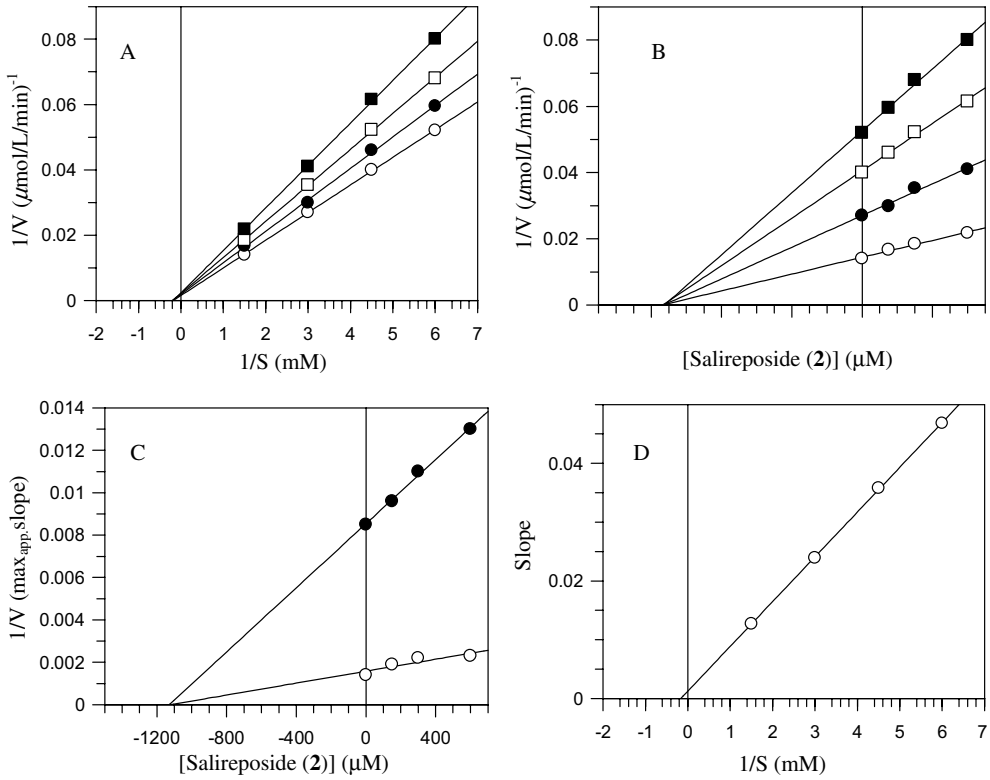


Figure 4. Steady state inhibition of snake venom phosphodiesterase I by salireposide (**2**). (A) Lineweaver–Burk plot in absence (○) and presence of 150 μM (●), 300 μM (□), and 600 μM (■) of compound **2**. (B) Dixon plot at four fixed bis-(*p*-nitrophenyl) phosphate concentrations: (○) 1.5 mM; (●) 3.0 mM; (□) 4.5 mM and (■) 6.0 mM. (C) Respective secondary replots of the Lineweaver–Burk plot, that is $1/V_{max_{app}}$ and slope versus various compound concentrations. (D) Secondary replots of the Dixon plot: slope versus reciprocal of substrate concentrations. Each point in the graph represents the mean of three experiments.

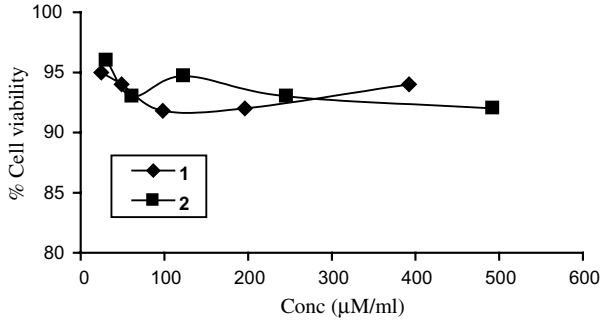


Figure 5. Graph shows % cell viability of freshly isolated human neutrophils (1×10^7 cells/mL) in the presence of various concentrations (500–25 μM/mL) of compounds **1** and **2**.

The OD is the mean of the five experimental replicates. Percentage cell viability was calculated by using the following formula:

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

Acknowledgements

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 the Third World Academy of Sciences for the financial assistant. We gratefully acknowledge the help of the Fatmid Foundation for the blood samples and Ms. Talat Makhmoor for the establishment of cytotoxic assay.

References and notes

1. Razzell, W. E. *Meth. Enzymol.* **1963**, *6*, 236.
2. Bollen, M.; Gijsbers, R.; Ceulemans, H.; Stalmans, W.; Stefan, C. *Crit. Rev. Biochem. Mol. Biol.* **2000**, *35*, 393.
3. Goding, J. W.; Grobben, B.; Slegers, H. *Biochim. Biophys. Acta* **2003**, *1638*, 1.
4. Johnoson, K.; Hashimoto, S.; Lotz, M.; Pritzker, K.; Goding, J.; Terkeeltaub, R. *Arthritis Rheum.* **2001**, *44*, 1071.
5. Okawa, A.; Nakamura, I.; Goto, S.; Moriya, H.; Nakamura, Y.; Ikegawa, S. *Nat. Genet.* **1998**, *19*, 271.
6. Sali, A.; Favaloro, J. M.; Terkeltaub, R.; Goding, J. W. Paper presented at: Proceedings of the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases, Diepenbeek, Belgium,1999; Shaker Publishing BV: Maastricht, The Netherlands.
7. 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.

8. Ahmad, V. U.; Abbasi, M. A.; Hussain, H.; Akhtar, M. N.; Farooq, O.; Fatima, N.; Chaudhary, M. I. *Phytochemistry* **2003**, *63*, 217.
9. Berridge, M. V.; Tan, A. S.; McCOY, K. D.; Wang, R. *Biochemica* **1996**, *4*, 14.
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. Segal, I. *Enzyme Kinetics*, 2nd ed.; John Wiley and Sons: New York, USA, 1993; pp 125–143.
17. Dixon, M. *Biochem. J.* **1953**, *55*, 170.
18. Siddiqui, R. A.; English, D.; Cui, Y.; Martin, M. I.; Wentlands, J.; Akard, L. *J. Leukocytes Biol.* **1995**, *58*, 189.
19. Berridge, M. V.; Tan, A. S.; Mccoy, K. D.; Wang, R. *Biochemica* **1996**, *4*, 14.