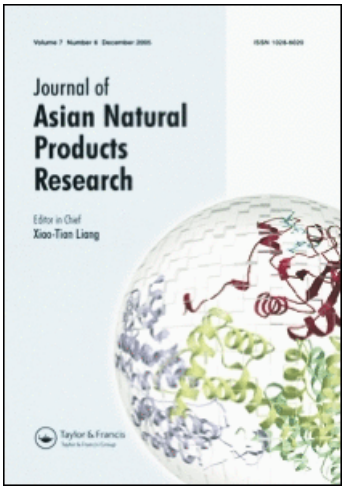

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

An investigation of the kinetic and anti-angiogenic properties of plant glycoside inhibitors of thymidine phosphorylase

S. Hussain[a]; J. Gaffney[a]; N. Ahmed[a]; M. Slevin[a]; M. Iqbal Choudhary[b]; V. U. Ahmad[b]; Z. Qasmi[b]; M. A. Abbasi[c]
[a] School of Biology, Chemistry and Health Science, Manchester Metropolitan University, Manchester, UK [b] International Center for Chemical and Biological Sciences, University of Karachi, H.E.J. Research Institute of Chemistry, Karachi, Pakistan [c] Department of Chemistry, Government College University, Center for Plant Product Research, Lahore, Pakistan

To cite this Article Hussain, S. , Gaffney, J. , Ahmed, N. , Slevin, M. , Iqbal Choudhary, M. , Ahmad, V. U. , Qasmi, Z. and Abbasi, M. A.(2009) 'An investigation of the kinetic and anti-angiogenic properties of plant glycoside inhibitors of thymidine phosphorylase', Journal of Asian Natural Products Research, 11: 2, 159 — 167
To link to this Article: DOI: 10.1080/10286020802618860
URL: http://dx.doi.org/10.1080/10286020802618860

Journal of Asian Natural Products Research
Vol. 11, No. 2, February 2009, 159–167

Taylor & Francis
Taylor & Francis Group

An investigation of the kinetic and anti-angiogenic properties of plant glycoside inhibitors of thymidine phosphorylase

S. Hussain[a], J. Gaffney[a]*, N. Ahmed[a], M. Slevin[a], M. Iqbal Choudhary[b], V.U. Ahmad[b], Z. Qasmi[b] and M.A. Abbasi[c]

[a]*School of Biology, Chemistry and Health Science, Manchester Metropolitan University, Manchester, UK;* [b]*International Center for Chemical and Biological Sciences, University of Karachi, H.E.J. Research Institute of Chemistry, Karachi, Pakistan;* [c]*Department of Chemistry, Government College University, Center for Plant Product Research, Lahore, Pakistan*

(*Received 9 July 2008; final version received 24 October 2008*)

We investigated the potential of symplocomoside (**1**) and symponoside (**2**), glycosides isolated from the bark of *Symplocos racemosa* to inhibit thymidine phosphorylase (TP) activity and associated angiogenesis. Compound **1** was a reversible, noncompetitive inhibitor of deoxythymidine binding to TP ($IC_{50} = 65.45 \pm 5.08\,\mu M$; $K_i = 62.83 \pm 2.10\,\mu M$) and **2** was a reversible, uncompetitive inhibitor ($IC_{50} = 94.17 \pm 4.05\,\mu M$; $K_i = 101.95 \pm 1.65\,\mu M$). Molecular modeling analysis indicated that both compounds bound at the active site of the enzyme but not solely to amino acid residues involved in catalysis. Both compounds were active in *in vitro* angiogenic assays inhibiting endothelial cell migration and invasion in Matrigel, but did not inhibit growth factor-induced proliferation and were not cytotoxic. Compound **1** may have potential as an anti-angiogenic and anti-tumor agent.

Keywords: thymidine phosphorylase; salirepin glycosides; angiogenesis

1. Introduction

Angiogenesis, the growth of new blood vessels from the existing vasculature, is associated with physiological and pathological processes. Angiogenesis is particularly important in the development of solid tumors that are restricted to a size of approximately $1\,mm^3$ in the absence of a blood supply.

Thymidine phosphorylase (TP, E.C.2.4.2.4), also called platelet-derived endothelial cell growth factor, is an angiogenic factor that catalyses the phosphorolysis of deoxythymidine (dThd) to thymine and 2-deoxy-D-ribose-1-phosphate [1]. TP activity is raised in the sera and tissues of cancer patients for example in colorectal, non-small cell lung, and breast cancers [2]. Increased angiogenesis and poor prognosis are associated with increased TP protein expression in colorectal and endometrial cancers [3].

The mechanism by which TP induces angiogenesis is uncertain. It appears chemotactic for endothelial cells (ECs) *in vitro* and angiogenic *in vivo*. Inhibiting TP may be a therapeutic option in cancer treatment, since tumor cells depend heavily on a salvage pathway catalyzed by TP for their supply of nucleosides and thus for proliferation. Many 5′-substituted acyluridines have been found to inhibit pyrimidine nucleoside phosphorylases but are more active against uridine phosphorylase than TP.

We have recently reported the isolation of the salirepin glycosides compounds **1** and **2**

*Corresponding author. Email: j.gaffney@mmu.ac.uk

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020802618860
http://www.informaworld.com

(Figure 1) from the bark of *Symplocos racemosa* RoxB, which inhibit the activity of TP [4]. We hypothesized that they may have anti-angiogenic properties and have determined their activity against TP using kinetic and *in vitro* angiogenic assays.

2. Results

We investigate the activity of the glycosides against TP and TP-induced EC migration and invasion, the key events in angiogenesis.

2.1 Kinetic analysis of TP

Kinetic studies showed that **1** was a more effective inhibitor of TP than **2**. Initial analysis was performed by varying [dThd] and maintaining [P_i]. The K_i (the dissociation constant of the enzyme–inhibitor complex) and IC_{50} values (the concentration causing 50% inhibition of enzyme activity) were lower for **1** than for **2** (Table 1; Figures 2(A) and 3(A)). A further analysis was performed by varying [P_i] and maintaining [dThd] and the K_i and IC_{50} values were determined for **2** (Table 1; Figures 2(C) and 3(C)). Determination of the type of inhibition indicates the mechanism and in conjunction with molecular modeling, the site of binding of the inhibitor. Competitive inhibition involves substrate and inhibitor binding at the same site, while in noncompetitive inhibition they bind at different sites in a random, reversible manner. Compound **1** showed noncompetitive inhibition and **2** showed uncompetitive inhibition that is characterized by the binding of the substrate leading to a conformational change in the enzyme allowing subsequent binding of the inhibitor. Both compounds showed reversible inhibition (Figures 2(B) and 3(B)).

2.2 Molecular modeling

Molecular modeling was used to elucidate the kinetic studies. A comparison with the data for docked thymine [5] indicated that **1** and **2** bound to the active site with limited interactions with residues involved in catalysis. Compound **1** bound to residues adjacent

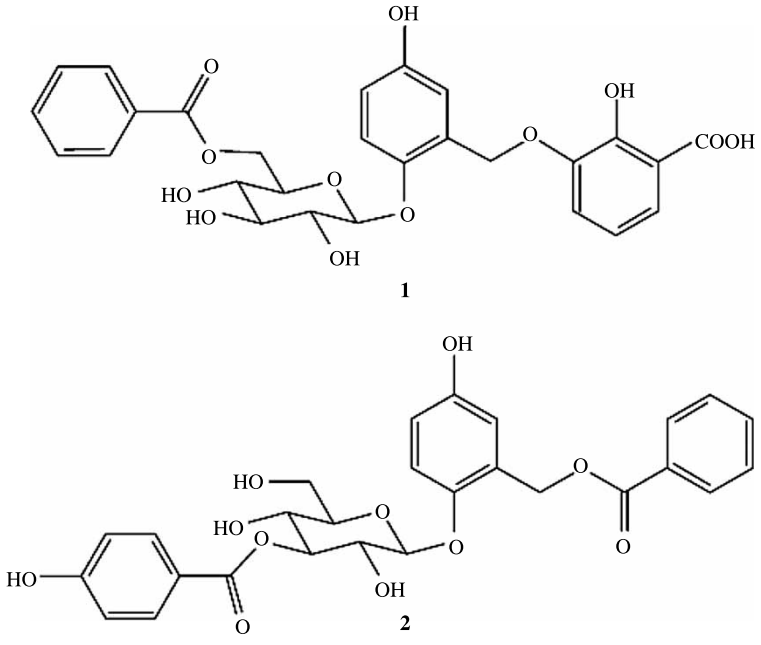

Figure 1. The structures of symplocomoside (**1**) and symponoside (**2**).

Table 1. A summary of the kinetic parameters for **1** and **2**.

Compound	Substrate	Mean ± SEM (μM) IC_{50}	Mean ± SEM (μM) K_i	Type of inhibition
1	dThd	65.45 ± 5.08	62.83 ± 2.10	NC
	P_i	94.17 ± 4.05	101.95 ± 1.65	NC
2	dThd	225.67 ± 3.79	231.73 ± 3.28	UC
	P_i	182.15 ± 2.67	165.05 ± 2.38	UC

NC, noncompetitive; UC, uncompetitive inhibition.

to the active site, His116, Gly120, Ser144, Arg146, His150, Thr151, Thr154, Gly155, and Tyr199 through hydrogen bonding and made hydrophobic contacts with residues Val208, Ile214, Leu148, Gly149, Val241, Ala240, Gly119, Asp123, Ser117, Ser126, and Gly152 (Figure 4). Compound **2** bounds among others, to residues 114–120, Lys157, Arg202, Val208, Asp209, Ile214, and Ser217 (data not shown). The method was validated by docking 7-deazaxanthine, which bound at the active site in a low-energy complex with hydrogen bonds to residues His116, Ser117, Tyr199, Ser217, and Asp233 and hydrophobic bonding to Thr118, Gly119, and Leu148.

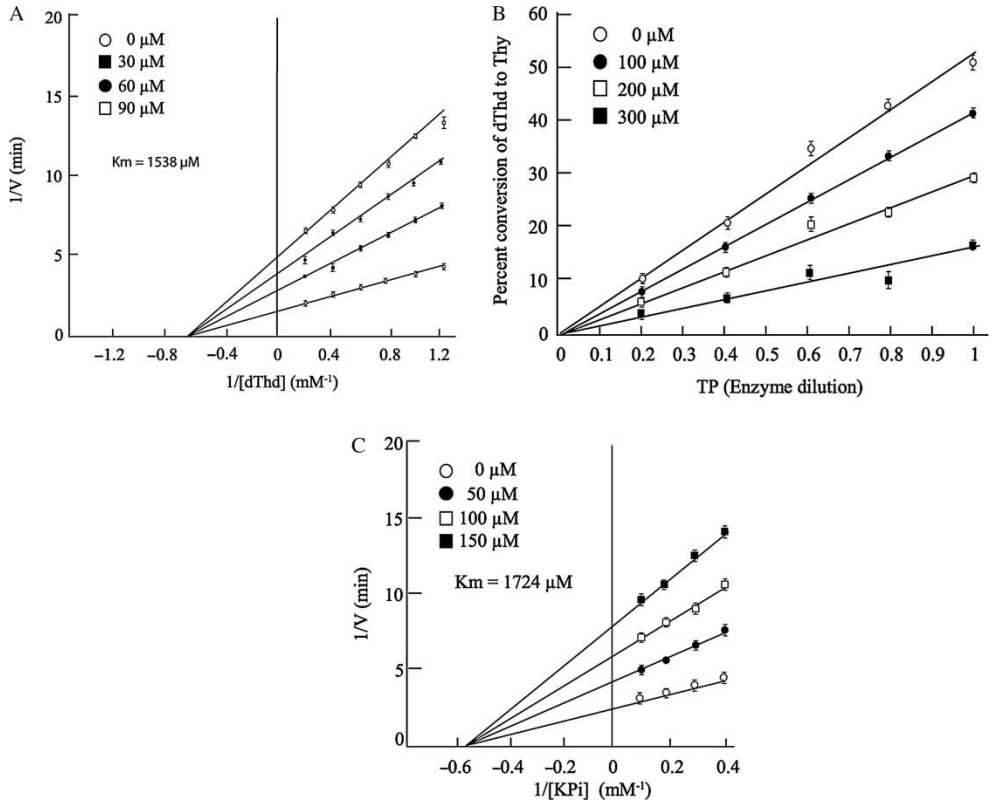

Figure 2. (A) Lineweaver–Burk plot of TP inhibition by different concentrations of **1**. (B) Reversible enzyme inhibition was determined by incubating 0.2–1 U of TP in the presence of 0–300 μM of **1**. (C) Lineweaver–Burk plot of TP inhibition by different concentrations of **1** in the presence of variable concentrations of P_i demonstrating noncompetitive enzyme inhibition.

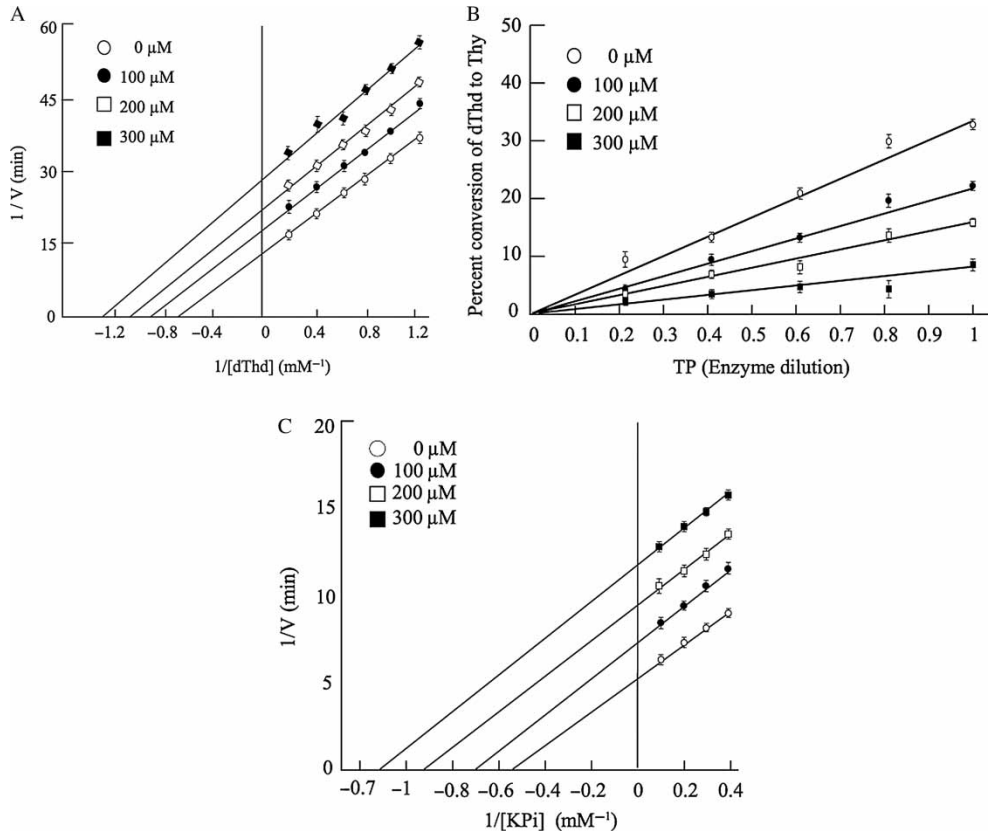

Figure 3. (A) Lineweaver–Burk plot of TP inhibition by different concentrations of **2** showing uncompetitive enzyme inhibition. (B) Reversible enzyme inhibition was determined by incubating 0.2–1 U of TP in the presence of 0–300 μM of compound **2**. (C) Lineweaver–Burk plot of TP inhibition by different concentrations of **2** in the presence of variable concentrations of P_i demonstrating uncompetitive enzyme inhibition.

The octanol–water partition coefficient showed that **1** was more hydrophobic (log $p = 3.05$) than **2** (log $p = 2.85$).

2.3 Effect of 1 and 2 on TP-induced angiogenesis

Angiogenesis was assayed by assessing EC proliferation, migration, and invasion in the presence of TP and the test compounds. Preliminary studies showed that **1** and **2** produced no significant inhibition of FGF-2- or VEGF-induced proliferation ($p > 0.05$ in all cases, results not shown). VEGF and FGF-2 are the principal *in vivo* mediators of angiogenesis and the result indicates that the test compounds did not inhibit them. However, both compounds inhibited TP-induced EC migration in a dose-dependent manner with IC_{50} values of 980 and 930 nM, respectively ($p < 0.01$; Figure 5), and invasion through a Matrigel layer ($IC_{50} = 16.6$ μM for **1**; $p < 0.01$; ANOVA, Figure 6).

Neither compounds showed significant cytotoxicity over the concentration range used (Figure 7).

3. Discussion

TP stimulates EC migration (*in vitro*) and angiogenesis (*in vivo*) [6] and overexpression accompanies tumor growth and metastasis [7]. Most clinically useful TP inhibitors are

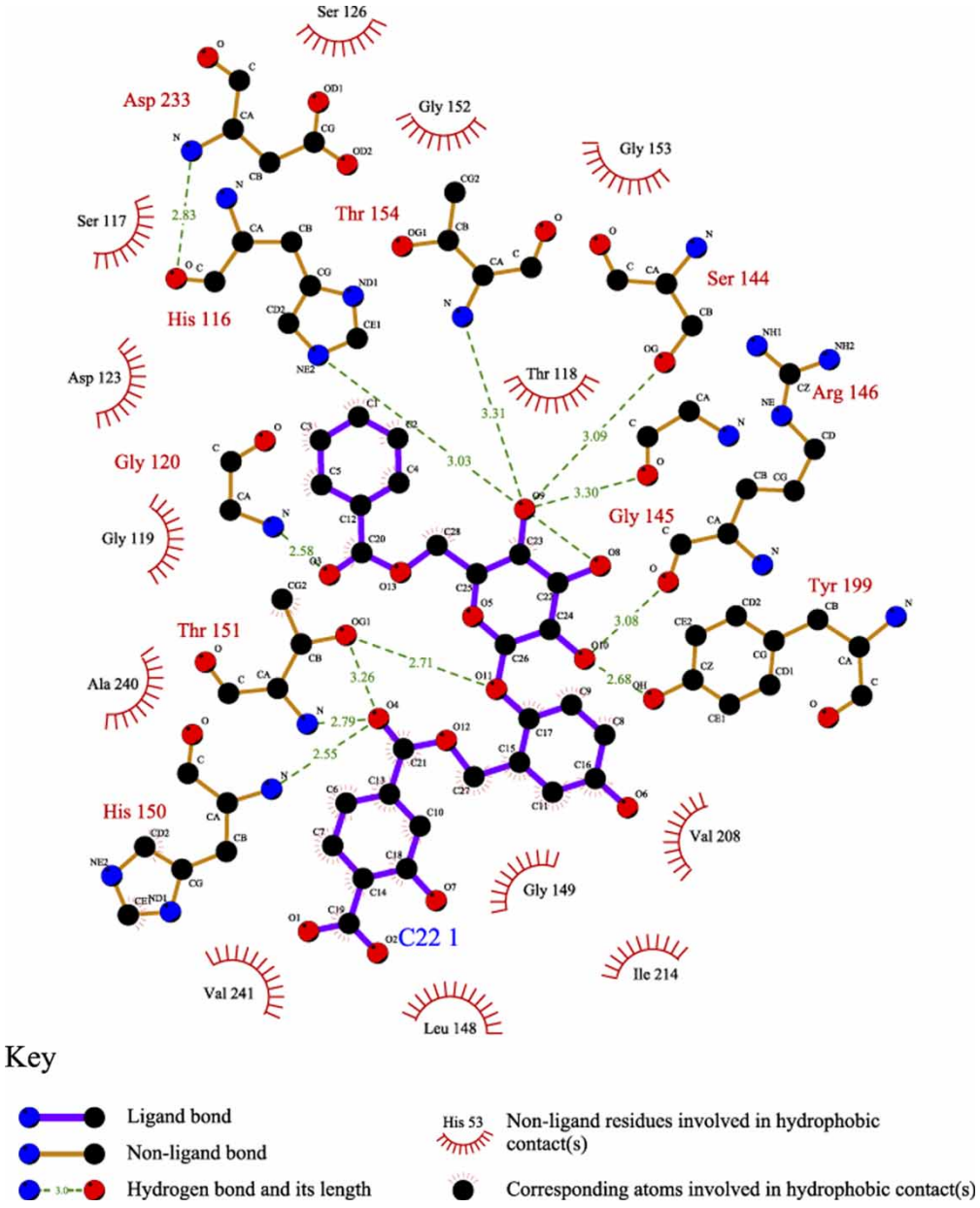

Figure 4. A ball-and-stick model of the active site of TP occupied with **1**. Hydrogen bonding and hydrophobic interactions with residues in or near the active site are shown. All distances are in angstorm.

analogs of pyrimidines, pyrimidine nucleosides, or purine bases, for example 6-(phenylalkylamino) uracil derivatives, 5-chloro-6-(aminoalkyl) uracils, 7-deazaxanthine, and 5′-*O*-trityl-inosine [8,9] with IC_{50} values of 53–110 μM and K_i values between 17.6 and 940 μM against mammalian TPs. We found that the IC_{50} values for our test compounds fell in the same range. However, the most effective inhibitor of TP is a 5-halogenated pyrimidine, TPI [10] with a K_i of 1.7×10^{-8} M.

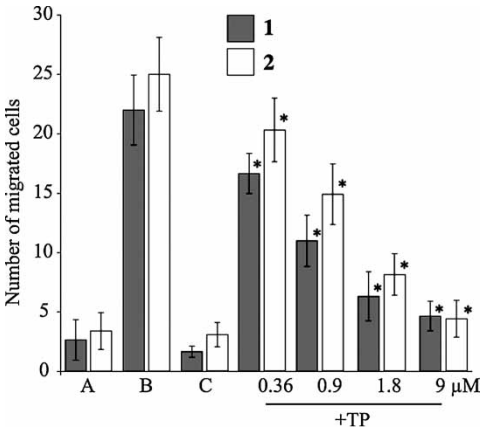

Figure 5. Effect of **1** and **2** on TP-induced human umbelical vein endothelial cell (HUVEC) migration. HUVEC were added to the upper layer of the Boyden chamber and TP and different concentrations of the test compound to the lower. The total number of migrated cells was counted. Control columns: (A) DMSO, (B) TP (100 ng/ml), and (C) compounds **1** and **2** (1.8 μM). Remaining columns show effect of test compounds on TP-induced migration. Significant differences ($p \leq 0.05$) shown by * are compared with column 2.

Molecular docking showed the test compounds interacted with some of the amino acid residues at or adjacent to the active site and acted noncompetitively or uncompetitively. The crystal structure of human TP shows that it is a dimer with an α-domain containing six α-helices, and a mixed α/β domain with a mixed β-sheet connected by three polypeptide loops [11]. The phosphate-binding site is located on the α/β and the thymidine-binding site on the α-domain [11]. Studies have suggested that binding of a phosphate residue results in the formation of a hydrogen bond between residues His116 and Gly205 causing an 8° rotation of the α/β-domain and subsequent binding of dThd allows an additional rotation to form a closed active site [11].

Our docking studies showed that the compounds bound to residues 115–120, 202, and 208, which may modulate this conformational change. The catalytic residues of TP are His116, Ser118 and 117, and Lys221 [11]. Neither compounds interacted with these residues, which is consistent with the inhibition data. However, residues Val208, Ile214, Tyr199, Arg202, and Ser217 are also located at the active site and both compounds did bind to these through hydrogen bonding and hydrophobic interactions. The reliability of our docking data was supported by the use of a known inhibitor (7-deazaxanthine) that was shown to dock to residues at or near to the active site in a low-energy conformation.

The greater effectiveness of **1** as an inhibitor of TP may relate to its increased hydrophobicity. One defect of established inhibitors of TP is the presence of ionic

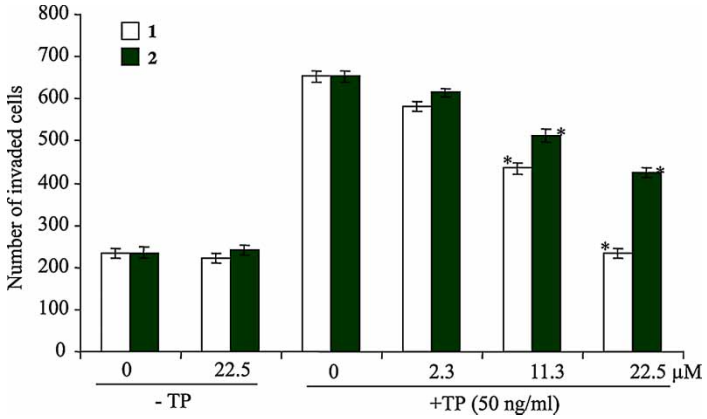

Figure 6. Effect of test compounds on HUVEC invasion in Matrigel. Compounds **1** and **2** were incubated with or without TP (control) at the concentrations shown. Values represent the mean of three experiments and values significantly different from TP alone are indicated by *.

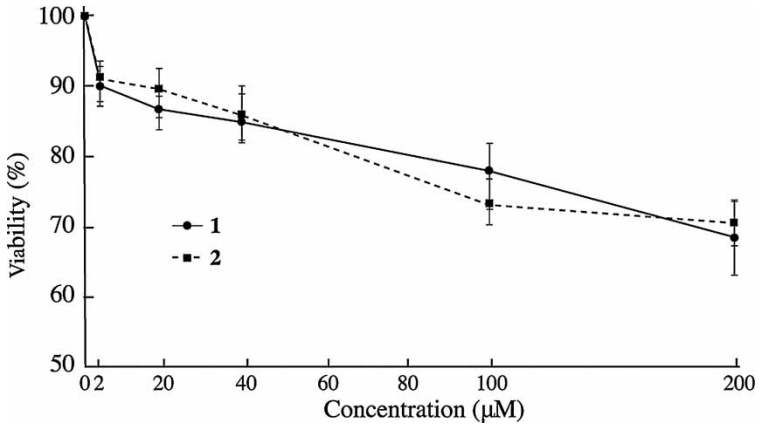

Figure 7. HUVECs (7.5×10^3 cells) were incubated with compounds **1** or **2** for 72 h and viability assessed by the MTT assay.

groups that adversely affect pharmokinetic properties, a problem not encountered by the compounds, which lack such groups.

Kinetic studies by other groups suggest that the phosphate group binds to the enzyme before dThd. Our data showed that the K_i of the inhibitors with respect to thymidine was lower than that with respect to phosphate, which may indicate that the inhibitors have a greater effect on the binding of dThd to the active site than that of P_i. This is supported by our docking studies.

We were able to show that TP was pro-angiogenic and induced HUVEC migration and invasion. Angiogenesis involves a series of coordinated events: proliferation of ECs, migration and invasion to distal sites, cell realignment and vessel formation, and production of a new basement membrane. Both compounds inhibited TP-induced EC migration and invasion in a dose-dependent manner but had no effect on FGF-2- or VEGF-induced proliferation or migration.

4. Experimental

4.1 Angiogenic inhibitors

Compounds **1** and **2** were isolated from the bark of *S. racemosa* Roxb and characterized as previously described [4].

4.2 In vitro *TP assays*

Human source TP, thymidine (dThd), and all other chemicals were purchased from Sigma (Poole, Dorset, UK). TP was assayed by the method of Krenitsky and Bushby [12]. Briefly, TP (0.05 U) and test glycosides in 5 μl of DMSO were mixed in a 96-well microplate to a final volume of 200 μl and pre-incubated for 30 min at 25°C. The reaction was initiated by the addition of dThd (20 μl; 1.5 mM in 200 mM potassium phosphate buffer, pH 7.0). The change in absorbance was monitored at 290 nm, for 10–30 min with a 96-well plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). All experiments were performed in triplicate and at least twice.

Enzyme inhibition was investigated by varying the concentration of glycosides and enzyme as described previously [13]. The IC_{50} value was determined as the mean of at least three determinations from the inhibition data using the EZ-FIT enzyme kinetic program (Perella Scientific, Inc., Amherst, MA, USA). Varying concentrations of glycosides were used in the presence of dThd (0–5.0 mM) and TP (0.002–0.01 U) in 200 μl of reaction mixture (145 μl potassium phosphate buffer, 5 μl test compound, 20 μl substrate, and 30 μl of enzyme). The concentration of inorganic phosphate

was 10 mM. The conversion of dThd to thymine was determined as described above.

The assay was also performed with inorganic phosphate (2–20 mM) in the presence of a range of concentrations of the inhibitors. The saturating concentration of dThd was fixed at 5 mM.

4.3 Cell culture

HUVECs and the appropriate medium were purchased from TCS CellWorks (Buckingham, UK) and were cultured and maintained according to the supplier's instructions at 37°C and in 2.5–15% serum and were used at passage numbers 2–10.

4.4 Angiogenesis assays

The determination of the effect of the test compounds on FGF-2- and VEGF-induced HUVEC proliferation was determined as described elsewhere [14]. Control wells were treated with 5 μl of DMSO.

The effect of **1** and **2** on HUVEC migration was measured using a Transwell chamber with 8.0 μm polycarbonate filter inserts (TCS CellWorks, Buckingham, UK) [14]. The filter was coated overnight with 0.1% gelatine; then cells (1×10^5) were placed in the upper part of the filter and test compounds and growth factors in the lower. Cells were incubated for 6 h at 37°C and the filter removed and the upper side containing nonmigrated cells wiped. Migrated cells on the lower side were fixed in 4% paraformaldehyde, stained with Giemsa and counted.

Cell invasion was assessed using a Transwell chamber with 6.5 μm polycarbonate filters coated with Matrigel (Becton-Dickinson, Oxford, UK, 30 μg/ml). Cells (1×10^5) were added to the upper chamber and test compounds with and without growth factor to the lower in serum. After 24-h incubation at 37°C, cells in the lower chamber were treated as above and counted. All experiments were performed in triplicate and at least twice.

4.5 Molecular docking studies

Molecular docking studies with inhibitors and TP were performed using the AutoDock and FlexX software programs (Scripps Research Institute, La Jolla, CA, USA) [15]. AutoDock was used to deduce the conformation and orientation of ligands in the active site of TP. The charges of ligands were calculated by using the standard RESP procedure. A radius of 6 Å was used to define active site interaction points. Computational studies were performed with the LINEX SUSE 8.2 (Kernel 2.4) operating system. To evaluate the software, a standard noncompetitive inhibitor of TP, 7-deazaxanthine was docked. The SdQSAR program (Tripos, St Louis, MO, USA) was used to determine the octanol–water partition coefficient and the relative hydrophobicity of the compounds.

4.6 Toxicity assays

The toxicity of test compounds was determined using the trypan blue and MTT assays as described elsewhere [14]. The assay was performed in triplicate and at least twice.

4.7 Statistical analysis

Statistical analysis was performed using one-way analysis of variance.

References

[1] T. Sumizawa, T. Furukawea, M. Haraguchi, A. Yoshimura, A. Takeysu, M. Ishizawa, Y. Yamada, and S.-I. Akiyama, *J. Biochem.* **114**, 9 (1993).
[2] M. Kobayashi, T. Sugimoto, A. Okabayashi, K. Okamoto, T. Namikawa, N. Tochika, T. Moriki, and K. Araki, *Med. Morphol.* **38**, 112 (2006).
[3] A. Mazurek, P. Kuc, S. Terlikowski, and T. Laudanski, *Neoplasia* **53**, 242 (2006).
[4] M. Abbasi, V. Ahmad, M. Zubair, N. Fatima, U. Farooq, S. Hussain, M. Lodhi, and M. Choudhary, *Planta Med.* **70**, 1189 (2004).
[5] V.A. McNally, K.T. Gbaj, I.J. Stratford, M. Jafar, and S. Freeman, *Bioorg. Med. Chem.* **13**, 3705 (2003).

[6] M. Haraguchi, K. Miyadera, K. Uemura, T. Sumizawa, T. Furukawa, K. Yamada, S. Akiyama, and Y. Yamada, *Nature* **368**, 198 (1994).
[7] J. Griffith and I.J. Stratford, *Br. J. Cancer* **76**, 689 (1997).
[8] F. Focher, D. Ubiali, M. Pregnolato, C. Zhi, J. Gambino, G.E. Wright, and S. Spadari, *J. Med. Chem.* **29**, 2601 (2000).
[9] J. Balzarini, A. Gamboa, R. Esnouf, S. Liekens, J. Neyts, E. De Clercq, and M.-J. Camarassa, *FEBS Lett.* **438**, 91 (1998).
[10] M. Fukushima, N. Suzuki, T. Emura, S. Yano, H. Kazuno, Y. Tada, Y. Yamada, and T. Asao, *Biochem. Pharmacol.* **59**, 1227 (2000).
[11] S. Liekens, A.-I. Hernandez, D. Ribatti, E. De Clercq, M.-J. Camarasa, M.-J. Perez-Perez, and A. Balzarini, *J. Biol. Chem.* **28**, 295 (2004).
[12] T.A. Krenitsky and S.R.M. Bushby, United States Patent 4: **178**, 212, 1–8 (Burroughs Wellcome Co., Research Triangle Park, NC, 1979).
[13] M.J. Pugmire, W.J. Cook, A. Jasanoff, M.R. Walter, and S.E. Ealick, *J. Mol. Biol.* **281**, 285 (1998).
[14] S. Hussain, M. Slevin, S. Matou, N. Ahmed, M.I. Choudhary, R. Ranjit, D. West, and J. Gaffney, *Angiogenesis* **11**, 245 (2008).
[15] C.I. Bayly, P. Cieplak, W.D. Cornell, and P.A. Kollman, *J. Phys. Chem.* **97**, 10269 (1993).