Bioorganic & Medicinal Chemistry 16 (2008) 6333–6337

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Hybrids of 1-deoxynojirimycin and aryl-1,2,3-triazoles and biological studies related to angiogenesis

Yunxue Zhao [a], Ying Zhou [b], Kathy M. O'Boyle [a], Paul V. Murphy [b,*]

[a] UCD School of Biomolecular and Biomedical Science and the UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland
[b] UCD School of Chemistry and Chemical Biology and Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

ARTICLE INFO

Article history:
Received 29 February 2008
Revised 27 April 2008
Accepted 5 May 2008
Available online 7 May 2008

Keywords:
1-Deoxynojirimycin
Aryl-triazoles
Angiogenesis
Cell cycle
Cyclin D1
Inhibitor
Cell migration
Hybrid compounds

ABSTRACT

Hybrids of 1-deoxynojirimycin (DNJ) and aryl-1,2,3-triazole have been synthesized with a view to identifying an inhibitor of both α-glucosidase and methionine aminopeptidase 2 (MetAP2). One compound was a potent inhibitor of α-glucosidase at both the enzyme and cellular level, and this agent also inhibited bovine aortic endothelial cell (BAEC) growth and tube formation. The anti-proliferative activity of this hybrid is due to its ability to induce cell-cycle arrest in the G_1phase. The novel agent caused a reduction in the expression of cyclin D1 but did not promote apoptosis or inhibit the phosphorylation of ERK1/2. These observations indicate that its mechanism of action is distinct from fumagillin and its analogues, which inhibit MetAP2. Stress-fibre assembly in BAECs was abolished by the novel agent indicating that the inhibition of BAEC tube formation observed is partially a result of a reduction in cell motility.

1. Introduction

Angiogenesis[1] provides new blood vessels to growing and developing tissue including tumours. Pathological angiogenesis occurs in tumour formation and metastasis and in a range of other 'angiogenesis-dependent diseases' that includes rheumatoid arthritis and diabetic retinopathy.[2] Accordingly, the design and synthesis of angiogenesis inhibitors is of interest and progress in this area has been recently reviewed.[3] It is emerging that angiogenesis inhibitors that target a range of angiogenic proteins and/or pathways may be less susceptible to drug resistance than those agents that target a single protein or pathway. One strategy to counteract this mode of resistance to angiogenesis inhibitors is to produce new molecules that inhibit two or more biological targets relevant to angiogenesis.

There are a number of cellular targets of interest in angiogenesis therapy, and herein we focused on two of these: methionine aminopeptidase II (MetAP2) and α-glucosidase. MetAP2 has been identified as a cellular target for angiostatic agents[4] such as fumagillin and TNP-470 as well as aryl-1,2,3-triazoles **1**.[5] Inhibitors of α-glucosidases such as *N*-methyl-1-deoxynojirimycin **2b**, castanospermine or 1-deoxymannojirimycin alter the biosynthesis of glycans on endothelial cell surfaces that are required for angiogenesis, and these glycosidase inhibitors inhibit angiogenesis.[6] With a goal to obtain novel anti-angiogenic agents that can target more than one biological process, we have designed and synthesized the hybrids **3–5** (Fig. 1) that can block both α-glucosidase and MetAP2. The design and synthesis of **3–5** and some biological properties have been outlined in a previous communication,[7] and one of these agents, **5**, has been shown to inhibit angiogenesis in vitro.[7] Cell biological studies show that **5** reduced the expression of cyclin D1, a key modulator of cell proliferation. The hybrid **5** also abolished stress-fibre assembly in BAECs that may lead to reduced cell motility.

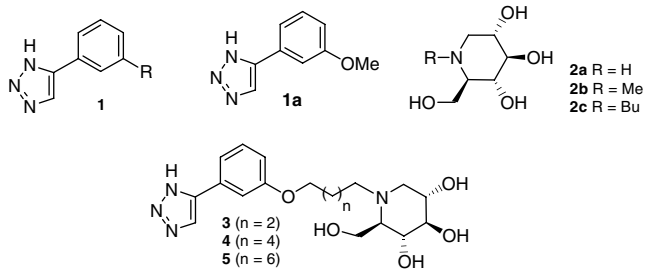

Figure 1. Structures of **1–5**.

* Corresponding author. Tel.: +353 1 7162504; fax: +353 1 7162501.
E-mail address: paul.v.murphy@ucd.ie (P.V. Murphy).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.05.012

2. Results and discussion

The design of **3**–**5** was based on combining the properties of both α-glucosidase[8] and MetAP2 in a single molecule. The ability of *N*-butyl-DNJ **2c** and other *N*-alkyl-DNJ derivatives to inhibit α-glucosidases indicated that an alkyl chain linker between the DNJ nitrogen atom and the aromatic residue would not lead to a loss of glycosidase inhibitory activity. Molecular modelling[7] indicated that the compunds could also inhibit MetAP2.

2.1. Inhibition of glycosidases

Compounds **1a**, **2a**, and **3**–**5** were evaluated for their ability to inhibit α-glucosidase from *Bacillus* stearothermophilus as described by Hakamata et al.[9] in order to test their enzyme inhibitory activity, and the results are shown in Table 1. The IC_{50} value for **2a** was 1.67 μM. The hybrid compound **5** (IC_{50} = 1.15 μM) was a more potent glycosidase inhibitor than **2a**, whereas the other compounds tested were less potent than **2a** and **5**. The modest enhancement of inhibitory properties observed for **5** indicated that the *N*-substituent may have favourable interactions with the glucosidase. The aryl-1,2,3-triazole **1a** did not inhibit the α-glucosidase, as was expected.

2.2. Inhibition of BAEC growth

The inhibition of BAEC growth by compounds was next determined using the MTT assay[10] and the results are shown in Table 1.[11] The iminosugar **2a** was inactive with no significant inhibition of proliferation being observed at concentrations up to 1 mM. However, the aromatic triazole **1a** (IC_{50} = 0.347 mM) inhibited the growth of BAECs. The inhibition of human (HUVEC) and mouse (MS-1) endothelial cell proliferation by aryl-1,2,3-triazoles is consistent[5] with an ability to inhibit MetAP2, and it can be inferred that inhibition of MetAP2 in BAECs would account for the activity observed for **1a**. It is worth noting that aryl-1,2,3-triazole derivatives had IC_{50} values of 2–30 μM as inhibitors of HUVEC and MS-1 cell growth.[5] Compounds **3**–**5** all inhibited BAEC growth with **5** being the more potent inhibitor (IC_{50} = 105 μM).

2.3. Effects on cell surface oligosaccharide structure

As hybrids **3**–**5** can inhibit α-glucosidase at the enzyme level, their effect on the bioprocessing of cell surface oligosaccharides was also investigated. Thus, BAECs were treated with compounds (50 μM) for 24 h and the binding of specific fluorescein (FITC)-labelled lectins, concanavalin A (Con A) and phytohemagglutinin-L (L-PHA), to the cells was monitored by fluorescence activated cell sorting (FACS) analysis in a flow cytometer. Con A recognizes mannose and glucose residues, whereas L-PHA recognizes complex branched chain oligosaccharides containing *N*-acetyl-lactosamine.[6a] BAECs that had been treated with **2a** for 24 h showed greater binding of Con A (Figure S1 in supporting information) than untreated control cells, indicating increased levels of glucose or mannose structures at the cell surface; this is due to inhibition by **2a** of α-glucosidases involved in glycoprotein processing in the endoplasmic reticulum (ER).[12] The treatment of BAECs with **3**–**5** led to increased binding of Con A to cell surfaces when compared to the control. As expected treatments that increased Con A binding decreased L-PHA binding; thus L-PHA binding to BAECs was reduced in cells treated with **2a** and **3**–**5**. Thus, all novel DNJ derivatives inhibited α-glucosidase at the cellular level.

Table 1
Inhibition of α-glucosidase and BAEC growth by compounds

Compound	α-Glucosidase inhibition IC_{50} (μM)	BAEC growth inhibition IC_{50} (mM)
1a	Not active	0.347
2a	1.67	Not active
3	6.07	0.797
4	2.41	0.610
5	1.15	0.105

2.4. Effects of compounds on angiogenesis in vitro

Hybrids **3**–**5** were evaluated for their ability to inhibit angiogenesis in vitro using the endothelial cell tube formation assay[13] given that they could inhibit BAEC growth and alter the biosynthesis of cell surface oligosaccharides important for angiogenesis. Endothelial cells which are induced to undergo tube formation change their architecture and form cell–cell contacts that lead to branched networks similar to capillary-like blood vessels. When BAECs were cultured on polymerized matrigel they organized into such tube-like structures (Figure S2 in supporting information). However, when BAECs were cultured on polymerized matrigel in the presence of **5** (0.2 mM, the cells failed to organize into the capillary-like structures. At these concentrations no other compounds tested inhibited tube formation and **5** was inactive at 0.1 mM.

2.5. Cell-cycle analysis

Inhibitors of MetAP2, fumagillin and TNP-470, have previously been shown to inhibit the proliferation of endothelial cells by arresting these cells at the G1 phase of the cell cycle.[14] It can be hypothesized that the aryl-1,2,3-triazole residue of **5** inhibits MetAP2 at the cellular level, which leads to the inhibition of BAEC proliferation observed for **5** (Table 1). To gain further insights into the mechanism of the anti-proliferative activity of **5** and to investigate if **5** induces G_1 arrest similar to other inhibitors of MetAP2 its effect on cell-cycle distribution was analyzed and the result of a typical experiment is shown in Figure 2. As determined by flow cytometry, exposure of BAECs to **5** (200 μM) after 24 h, resulted in an increase of the percentage of cells in the G_0/G_1 phase and a decrease in the percentage of cells in the S phase compared with control cells, consistent with an induction of G_1 arrest by **5**.

2.6. Effect of 5 on apoptosis

The inhibitor of MetAP2, TNP-470, has been reported to induce apoptosis in both tumour[15] and vascular endothelial cells.[16] The ability of **5** to promote apoptosis in BAECs was thus evaluated by treatment of cells with **5** (100 and 200 μM doses) for 24, 48, and 72 h, subsequent staining with annexin V–propidium iodide and flow cytometry analysis, in which only annexin V-stained cells were considered as apoptotic cells (data not shown). These experiments did not show any considerable promotion of apoptosis when compared to the control whereas cell growth inhibition was observed at the concentrations evaluated.

2.7. Effect of 5 on expression of cyclinD1 and the phosphorylation level of extracellular signal-regulated kinases (ERK) in BAECs

The effect of **5** on the expression of protein molecules important for cell-cycle regulation was evaluated in order to elucidate the molecular mechanisms involved in the observed cell-cycle alterations. Underlying mechanisms for other MetAP2 inhibitors have been studied previously. The inhibitor of MetAP2 and angiogenesis, TNP-470, is reported to decrease levels of cyclin D1 in tumours.[17] Increased levels of cyclin D1 are linked with the promotion of

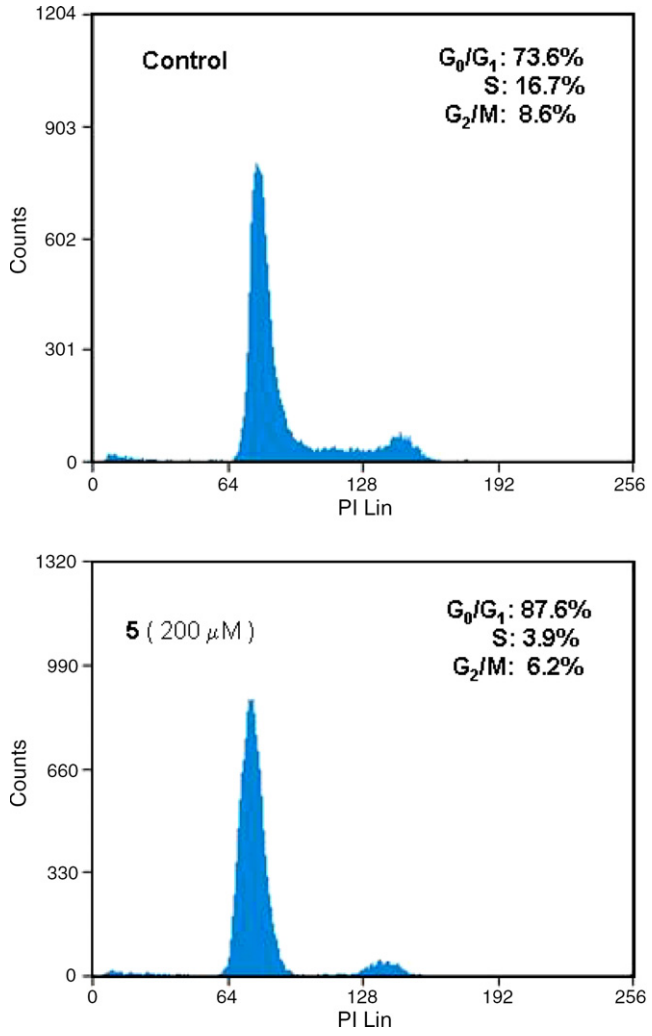

Figure 2. Effect of **5** on cell-cycle progression of BAECs. BAEC cells were treated with DMSO (control) or **5** for 24 h, respectively. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry. A total of 10,000 nuclei were analyzed from each sample, and the percentages of cells within G_0/G_1, S, and G_2/M were determined. Representative profiles are shown and the numbers given in the upper right corner of the profiles.

cell-cycle progression.[18] Compounds that reduce the expression of cyclin D1 would be expected to reduce endothelial cell growth and angiogenesis in vitro. The levels of expression of cyclin D1 in BAECs treated with **5** were established using Western blot analysis with antibodies specific for cyclin D1. The expression of cyclin D1 was found to be reduced in the presence of **5** at 100 and 200 μM doses (Fig. 3).

The phosphorylation of ERK1/2 also occurs during cell proliferation.[19] Reduction in the phosphorylation of ERK1/2 is associated with reduced proliferation, and it has been reported that fumagillin increases affinity of MetAP2 to ERK1/2 and causes the inhibition of the phosphorylation of ERK1/2.[20] There was the possibility that **5** could display similar behaviour to fumagillin. BAECs were therefore treated with **5** and the levels of both phosphorylated and non-phosphorylated ERK1/2 were established using antibodies and Western blot analysis under conditions where β-actin was used as a loading control. The hybrid compound **5** (at 100 and 200 μM doses) was found not to alter either ERK1/2 phosphorylation or the total ERK1/2 level. Thus, **5** inhibited the expression of cyclin D1 in endothelial cells but not a signalling pathway that involves ERK1/2 phosphorylation.

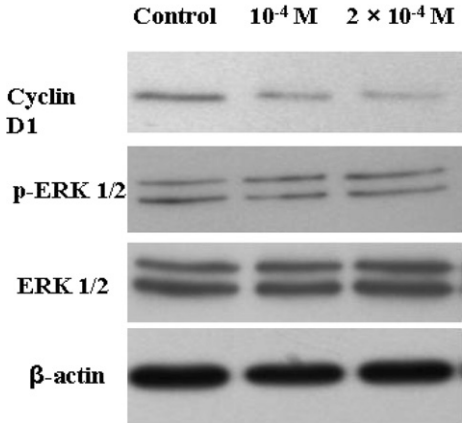

Figure 3. Western blot analyses for levels of cyclin D1 and activated Erk(phosphorylation of Erk) in BAECs. BAECs were incubated in the absence or presence of **5** at 100 and 200 μM or control for 24 h. Cell lysates were prepared from the treated cells, and Western blot analyses were performed.

2.8. Effect of 5 on stress-fibre assembly in BAECs

It is well established that actin polymerization that leads to stress-fibre assembly and depolymerization plays a crucial function in cell motility. In the presence of some angiogenesis inhibitors such as fumagillin and its analogues actin stress-fibre densities are increased, indicating that these angiogenesis inhibitors induce endothelial cells to assume an adhesive state that is not conducive to motility.[21] Other inhibitors of cell motility such as fausidil lead to a loss of stress-fibre formation.[22] It is possible that **5** also inhibited BAEC motility, given that it inhibited tube formation in vitro. The formation of the actin cytoskeleton in BAECs was assessed by staining with FITC-phalloidin, in order to specifically detect F-actin, in an attempt to identify morphological traits in response to treatment with **5**. The results (Fig. 4) show that stress-fibre assembly in BAECs was abolished by **5**, indicating that **5** inhibited BAEC tube formation, at least partially through its ability to alter the behaviour of actin.

3. Conclusions

The synthesis of novel hybrids of DNJ and aryl-1,2,3-triazoles has led **5** being identified as an inhibitor of angiogenesis in vitro. The hybrid **5** had a number of interesting biological effects. It inhibited α-glucosidase at both the enzyme and cellular level, altering the biosynthesis of oligosaccharides on endothelial cell surfaces important to angiogenesis. It inhibited the proliferation of BAECs by inducing cell-cycle arrest. The novel hybrid **5** caused a reduction in the expression of cyclin D1 but did not promote apoptosis or inhibit the phosphorylation of ERK1/2. The hybrid **5** thus displayed mechanistic differences to other classes of inhibitors of MetAP2, such as fumagillin and its analogues, which have been reported to inhibit the phosphorylation of ERK1/2 and induce apoptosis. Stress fibre assembly in BAECs was abolished by the hybrid compound indicating the inhibition of BAEC tube formation by **5** is a result of a reduction in cell motility. Hybrid **5** displays biological properties of interest in the development of multifunctional inhibitors of angiogenesis and the research provides a basis for efforts to development hybrid angiogenesis inhibitors with increased potency.

4. Experimental

4.1. Cell culture

BAE cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/mL penicil-

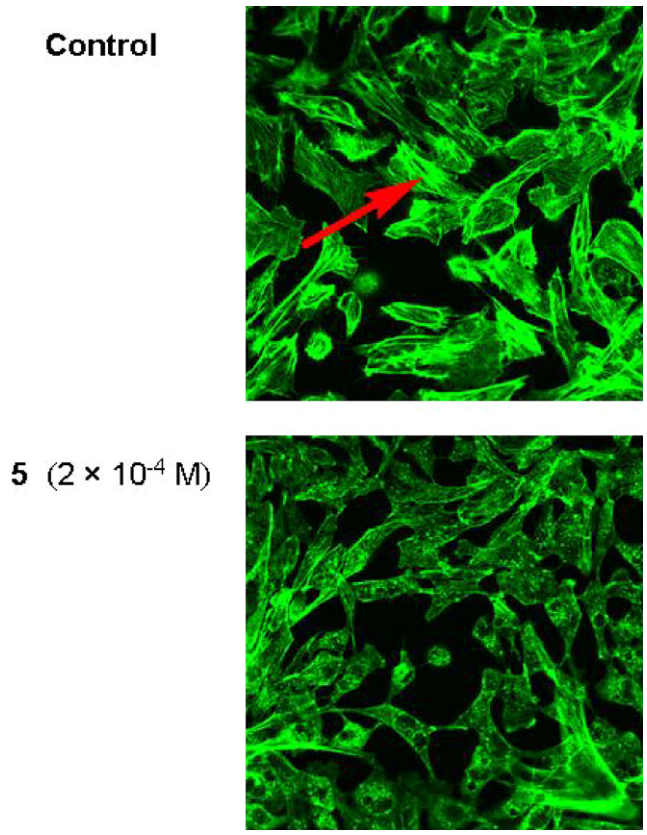

Figure 4. The effect of **5** on actin stress-fibre polymerization in BAEC was assessed by fluorescence staining with FITC phalloidin, a substance which specifically detects F-actin. In the control, stress fibres are present (red arrow). Incubation of BAECs with **5** (200 μM) leads to marked disruption of stress-fibre assembly.

lin, and 100 μg/mL streptomycin and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Subcultures were created by passaging using a trypsin/EDTA mixture in phosphate buffered saline.

4.2. Flow cytometry analysis of cell surface oligosaccharides

Cells were stained with fluorescin-labelled plant lectins and analyzed by flow cytometry. Cultured cells were treated with compounds (50 μM) for 24 h in 6-well plate. Cells were then harvested and washed with DPBS. Approximately 5×10^5 cells were resuspended in test tubes and incubated in DPBS/1% BSA with 200 μl 2 μg/mL of Con A-FITC or L-PHA-FITC for 1 h at 4 °C. Cells were washed twice with DPBS, and fluorescence histogram profiles were determined using flow cytometric FACS analysis. Each experiment was carried out at least twice with each compound.

4.3. Inhibition of proliferation of BAECs

BAECs (3×10^3 cells) were seeded on 96-well microtiter plates in 1640 medium with 10% FBS and incubated overnight. The compounds (10^{-6}–10^{-3} M) were then added to the cells and cultured for another 48 h. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] reagent was added directly to the cell supernatant so as to give a final concentration of 0.5 mg/mL of MTT. After 3 h at 37 °C the cell culture medium was removed. Formazan crystals in adherent cells were dissolved in 200 μl DMSO, and the absorbance of the formazan solution was measured at 570 nm.[10] Each compound was tested in quadruplicate and the experiments were repeated at least twice.

4.4. Tube-formation assay

Tube-structure formation on Matrigel was conducted and modified as described previously.[13] Briefly, 70 μl growth factor-reduced Matrigel was added to 96-well plates at 4 °C and then allowed to polymerize at 37 °C for 1 h. Cultured cells were treated with compounds (0.2 mM) for 24 h in 6-well plate. BAE cells were then harvested and suspended at a concentration of 3×10^4cells/0.1 ml in RPMI 1640 containing 10 ng/ml bFGF and 0.2 mM compounds. Control cells were re-suspended with 10 ng/mL bFGF alone. Cells were carefully layered on top of the polymerized gel and incubated for 8 h at 37 °C in 5% CO_2. Tube formation was observed and photographed under a microscope. At least five visual fields were counted and the average number of tubes per field was calculated using light microscope. The experiments were repeated at least twice for each compound.

4.5. Cell-cycle analysis

Cells were seeded on 6-well plates in 1640 medium with 10% FBS overnight. Then the compounds were added to the cells and cultured for another 24 h. Cells were collected with trypsin–EDTA and then washed three times with DPBS. The cells were resuspended and fixed for at least 2 h at −20 °C with 70% Ethanol. After washing twice with DPBS, cells were incubated at 25 °C with 200 μg/ml RNase A for 30 min. The resulting cells were incubated with 50 μg/ml propidium iodide for 30 min at 4 °C. The treated cells were subjected to flow cytometry and the percentage of cells at each phase of the cell cycle was analyzed. Each experiment was repeated at least twice.

4.6. Quantitative apoptosis assay

The ability of **5** to induce apoptotic death of BAEC was quantified by annexin V and PI staining and flow cytometry, as described recently.[23] Briefly, after treatment with **5** (0, 100, and 200 μM for 24, 48, and 72 h), cells were collected and washed with PBS twice, and subjected to annexin V and propinium iodide staining using annexin V FITC apoptosis kit following the step-by-step protocol provided by the manufacturer. After staining, flow cytometry was performed for the quantification of apoptotic cells. Each group was tested in triplicate and the experiments were repeated at least twice in each case.

4.7. Immunoblot analysis

BAEC cells were treated with **5** (0, 100, and 200 μM) for 24 h in 6-well plate for the western blot studies. Cells were washed twice with ice-cold PBS and lysed with RIPA buffer with protease inhibitors. After centrifuged at 13,000 rpm for 15 min, protein concentrations of the lysates were determined by micro-BCA protein assay kit. The total cellular protein extracts were boiled with 2× Laemmli sample buffer and separated by SDS–PAGE and transferred to PVDF membrane. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature and incubated with antibodies against cyclin D1, Erk, phosphor-Erk, and β-actin overnight at 4 °C. Blots were washed three times in TBS-T buffer, followed by incubation with the appropriate HRP-linked secondary antibodies for 1 h at room temperature. The specific proteins in the blots were visualized using the enhanced chemiluminescence reagent. The experiments were repeated at least twice in each case.

4.8. Immunofluorescence

Stress fibre of BAEC cells were detected using fluorescent phalloidin and analyzed by confocal microscopy. Cells were seeded to

the glass coverslips in cell culture dish plate (3.5 cm) overnight. Then compound **5** ws added and cultured for 24 h. After fixing with 4% paraformaldehyde, cells were treated with 0.1% Triton X-100 and blocked with 1% BSA. Then, cells were incubated with FITC-conjugated phalloidin (2 μg/ml) for 30 min and examined under Zeiss confocal laser scanning microscope (40×, oil). The experiments were repeated at least twice in each case.

4.9. Statistical analysis

The statistical analysis was done using Student's t test. $P < 0.05$ was accepted as significant. The number of replicates and statistical significance are indicated in methods and in the figure legends.

Acknowledgment

Funding was provided by Science Foundation Ireland (03/IBN/B352).

Supplementary data

Synthesis of **3**–**5** and Figures S1 and S2. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.012.

References and notes

1. (a) Liekens, S.; de Clercq, E.; Neyts, J. *Biochem. Pharm.* **2001**, *61*, 253–270; (b) Carmeliet, P.; Jain, R. K. *Nature* **2000**, *407*, 249–257.
2. Hanahan, D. *Nat. Med.* **1998**, *4*, 13–14.
3. Folkman, J. *Nat. Rev. Drug Disc.* **2007**, *6*, 273–286.
4. Griffith, E. C.; Su, Z.; Niwayama, S.; Ramsay, C. A.; Chang, Y.-H.; Liu, J. O. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15183–15188.
5. Kallander, L. S.; Lu, Q.; Chen, W.; Tomaszek, T.; Yang, G.; Tew, D.; Meek, T. D.; Hofmann, G. A.; Schulz-Pritchard, C. K.; Smith, W. W.; Janson, C. A.; Ryan, M. D.; Zhang, G.-F.; Johanson, K. O.; Kirkpatrick, R. B.; Ho, T. F.; Fisher, P. W.; Mattern, M. R.; Johnson, R. K.; Hansbury, M. J.; Winkler, J. D.; Ward, K. W.; Veber, D. F.; Thompson, S. K. *J. Med. Chem.* **2005**, *48*, 5644–5647.
6. (a) Pili, R.; Chang, J.; Partis, R. A.; Mueller, R. A.; Chrest, F. J.; Passaniti, A. *Cancer Res.* **1995**, *55*, 2920–2926; (b) Nguyen, M.; Folkman, J.; Bischoff, J. *J. Biol. Chem.* **1992**, *267*, 26157–26165.
7. Zhou, Y.; Zhao, Y.; O'Boyle, K. M.; Murphy, P. V. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 954–958.
8. For reviews on glycosidase inhibition see: (a) Winchester, B.; Fleet, G. W. J. *Glycobiology* **1992**, *2*, 199–210; (b) Ganem, B. *Acc. Chem. Res.* **1996**, *29*, 340–347; (c) Heightman, T. D.; Vasella, A. T. *Angew. Chem. Int. Ed.* **1999**, *38*, 750–770; (d) Lillelund, V. H.; Jensen, H. H.; Liang, X.; Bols, M. *Chem. Rev.* **2002**, *102*, 515–553; (e) Greimel, P.; Spreitz, J.; Stutz, A. E.; Wrodnigg, T. M. *Curr. Top. Med. Chem.* **2003**, *3*, 513–523; (f) Asano, N.; Nash, R. J.; Molyneaux, R. J.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2000**, *11*, 1645–1680.
9. Hakamata, W.; Nakanishi, I.; Masuda, Y.; Shimizu, T.; Higuchi, H.; Nakamura, Y.; Saito, S.; Urano, S.; Oku, T.; Ozawa, T.; Ikota, N.; Miyata, N.; Okuda, H.; Fukuhara, K. *J. Am. Chem. Soc.* **2006**, *128*, 6524–6525.
10. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
11. For other selected carbohydrate derived compounds that inhibit BAEC growth see (a) Rawe, S. L.; Zaric, V.; O'Boyle, K. M.; Murphy, P. V. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1316–1319; (b) Murphy, P. V.; Pitt, N.; O'Brien, A.; Enright, P. M.; Dunne, A.; Wilson, S. J.; Duane, R. M.; O'Boyle, K. M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3287–3290.
12. Hughes, A. B.; Rudge, A. J. *Nat. Prod. Rep.* **1994**, *11*, 135–162.
13. Akalu, A.; Roth, J. M.; Caunt, M.; Policarpio, D.; Liebes, L.; Brooks, P. C. *Cancer Res.* **2007**, *67*, 4353–4363.
14. Ingber, D.; Fujita, T.; Kishimoto, S.; Sudo, K.; Kanamaru, T.; Brem, H.; Folkman, J. *Nature* **1990**, *348*, 555–557.
15. Parangi, S.; O'Reilly, M.; Christofori, G.; Holmgren, L.; Grosfeld, J.; Folkman, J.; Hanahan, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2002–2007.
16. Yoshida, T.; Kaneko, Y.; Tsukamoto, A.; Han, K.; Ichinose, M.; Kimura, S. *Cancer Res.* **1998**, *58*, 3751–3756.
17. Kalebic, T.; Tsokos, M.; Helman, L. J. *Int. J. Cancer* **1996**, *68*, 596–599.
18. For a recent review on the functions and regulation of cyclin D1 see: Tashiro, E.; Tsuchiya, A.; Imoto, M. *Cancer Sci.* **2007**, *98*, 629–635.
19. Morrison, D. K.; Davis, R. J. *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 91–118.
20. Datta, B.; Majumdar, A.; Datta, R.; Balusu, R. *Biochem.* **2004**, *43*, 14821–14831.
21. Keezer, S. M.; Ivie, S. E.; Krutzsch, H. C.; Tandle, A.; Libutti, S. K.; Roberts, D. D. *Cancer Res.* **2003**, *63*, 6405–6412.
22. Yin, L.; Morishige, K.-I.; Takahashi, T.; Hashimoto, K.; Ogata, S.; Tsutsumi, S.; Takata, K.; Ohta, T.; Kawagoe, J.; Takahashi, K.; Kurachi, H. *Mol. Cancer Ther.* **2007**, *6*, 1517–1525.
23. Singh, R. P.; Dhanalakshmi, S.; Agarwal, C.; Agarwal, R. *Oncogene* **2005**, *24*, 1188–1202.