Biochemical and Biophysical Research Communications 404 (2011) 86–89

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Novel glycosaminoglycan biosynthetic inhibitors affect tumor-associated angiogenesis

Karthik Raman [a], Masayuki Ninomiya [b], Thao Kim Nu Nguyen [a], Yasuhiro Tsuzuki [c], Mamoru Koketsu [b], Balagurunathan Kuberan [a,d,e,*]

[a] Department of Bioengineering, University of Utah, Salt Lake City, UT 84112, USA
[b] Department of Materials Science and Technology, Faculty of Engineering, Gifu University, Gifu 501-1193, Japan
[c] Department of Chemistry, Faculty of Engineering, Gifu University, Gifu 501-1193, Japan
[d] Interdepartmental Program in Neuroscience, University of Utah, Salt Lake City, UT 84112, USA
[e] Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Article history:
Received 29 October 2010
Available online 19 November 2010

Keywords:
Heparan sulfate
Angiogenesis
Xyloside
Proteoglycan
Inhibitor
Matrigel

ABSTRACT

Heparan sulfate proteoglycans (HSPGs) are essential players in several steps of tumor-associated angiogenesis. As co-receptors for several pro-angiogenic factors such as VEGF and FGF, HSPGs regulate receptor–ligand interactions and play a vital role in signal transduction. Previously, we have employed an enzymatic strategy to show the importance of cell surface HSPGs in endothelial tube formation *in vitro*. We have recently found several fluoro-xylosides that can selectively inhibit proteoglycan synthesis in endothelial cells. The current study demonstrates that these fluoro-xylosides are effective inhibitors of endothelial tube formation *in vitro* using a matrigel based assay to simulate tumor-associated angiogenesis. These first generation scaffolds offer a promising stepping-stone to the discovery of more potent fluoro-xylosides that can effectively neutralize tumor growth.

1. Introduction

Inhibiting tumor angiogenesis is a powerful approach to mitigate cancer growth [1]. Heparan sulfate proteoglycans (HSPGs), cell-surface and ECM proteins containing highly sulfated glycosaminoglycan (GAG) chains, play vital roles throughout the various stages of angiogenesis and tumor growth [2–4]. They act as co-receptors for a variety of pro-angiogenic factors including VEGF and FGF [5–7]. As co-receptors, HSPGs facilitate receptor–ligand interactions and signal transduction. HS chains require certain sulfation patterns in order to bind to growth factors [8]. In particular, the binding of HS and FGF2 requires *N*-sulfated glucosamine units and 2-*O* sulfated iduronic acid units [9]. Furthermore, to bind to FGF receptor, HS chains require 6-*O* sulfated glucosamine residues and 2-*O* sulfated iduronic acid along with *N*-sulfated glucosamine [10,11]. Thus, only HS chains containing such a sulfation pattern can potentiate FGF/FGFR mediated signaling.

Xylosides containing certain hydrophobic aglycone groups can act as acceptors for GAG biosynthesis in the Golgi [12–14]. The primed GAGs are then secreted outside the cell and can have a variety of biological consequences by competing with endogenous proteoglycan chains [15]. Previously, it was found that β-D-xylopyranoside virtually eliminated the invasion of wound microvascular endothelial cells into fibrin gels [16]. Xylosides have also shown efficacy in preventing tumor progression [17–19]. It is also possible to inhibit proteoglycan synthesis by utilizing fluorine-containing xylosides [20].

Previously, we have shown that cell surface HS is essential for tube formation *in vitro* using heparitinase I and III [21]. Recently, we found that several novel fluoro-xylosides selectively inhibited GAG synthesis *in vitro* in endothelial cells (Table 1) [22]. Based on these results, we hypothesized that these fluoro-xylosides would be effective inhibitors of endothelial tube formation as well. In this article, we utilize the matrigel tube formation assay to show the anti-angiogenic efficacy of these novel fluoro-xylosides.

2. Methods

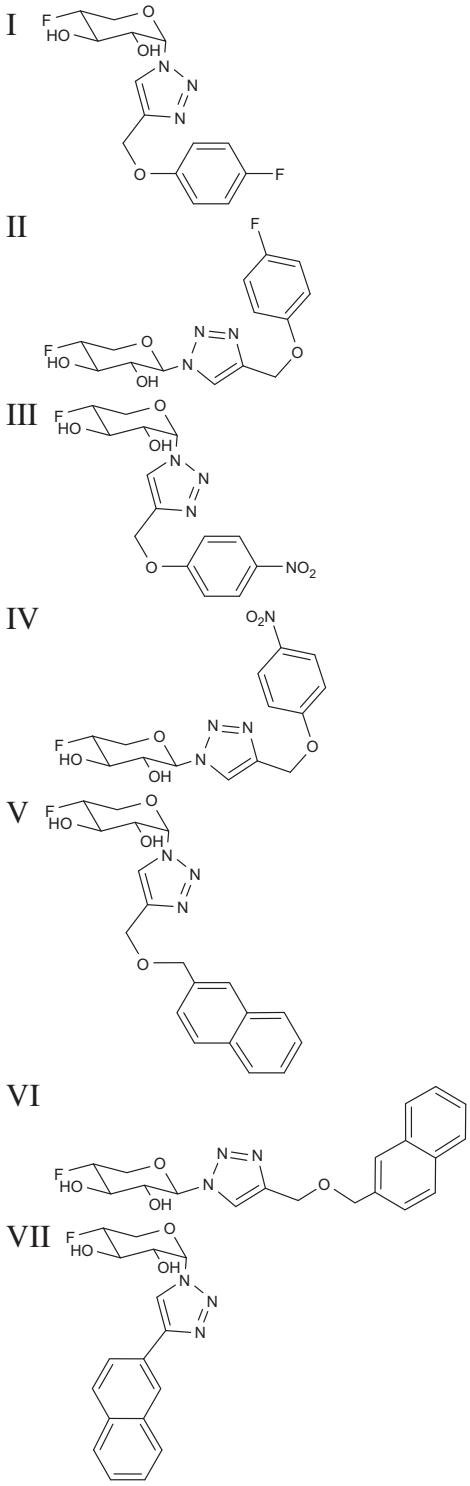
2.1. Cell culture

Bovine lung microvascular endothelial cells of passage 4–8 (a generous gift from Dr. Randall Dull) were cultured in MCDB-131 Complete media (Vec Technologies) in a humidified 37 °C incubator. Cells were split 24 h prior to conducting tube formation assays in order to keep them in the log phase of growth.

* Corresponding author. Address: University of Utah, 30 S 2000 E, Skaggs Hall Room 307, Salt Lake City, UT 84112, USA. Fax: +1 801 585 9119.
E-mail address: kuby@pharm.utah.edu (B. Kuberan).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.069

Table 1
Fluoro-xylosides tested for their ability to inhibit tube formation of BLMVEC *in vitro*.



2.2. Tube formation assay

Reduced growth factor basement membrane matrix (RGF-BME, Trevigen) was thawed overnight at 4 °C in a frost free refrigerator. Fifty microliters of RGF-BME were then added to wells of a chilled 96 well plate using chilled pipette tips. The 96 well plates were then incubated in a humidified incubator for 1 h. Concurrently, BLMVEC were suspended by incubation with Tryp LE Express (Invitrogen). 1×10^5 cells were then added to each well along with MCDB-131 complete media and various fluoro-xylosides. The plates were then incubated at 37 °C for 16 h prior to Calcein staining and imaging.

2.3. Calcein staining

Media was removed from each well containing cells by gentle dabbing with a paper towel. The wells were then washed twice with PBS and then 100 μl of 2 μM Calcein AM was added to each well. Cells were then stored for 30 min in the incubator. After incubation in the calcein AM working solution, the cells were washed once again with PBS and imaged with an Olympus IX81 microscope attached to a color CCD Filter and a GFP emission filter using 485 nm excitation/520 nm emission.

3. Results and discussion

Tube formation experiments were performed on reduced growth factor basement membrane extract (matrigel) which simulates angiogenesis near the tumor microenvironment (Fig. 1). Since BLMVEC spontaneously form tubes on RGF-BME, wells without any compounds were used as positive controls. Sulforaphane (provided by the manufacturer) was used at 20 μM as a negative control.

Initially tube formation experiments were performed at a 300 μM concentration of each fluoro-xyloside as this concentration has previously been shown to inhibit GAG biosynthesis [22]. As shown in Fig. 1, only xylosides III and IV were able to inhibit tube formation at 300 μM concentration. No other fluoro-xylosides tested had any effect on tube formation at this concentration.

Based on these initial results, two other concentrations of xylosides III and IV were tested for their ability to inhibit tube formation in order to understand the dose-dependent nature of these small molecule drug candidates (Fig. 2). Xylosides III and IV did not inhibit tube formation at 150 μM concentration whereas they strongly inhibited tube formation at 600 μM concentration. At this concentration, the extent of inhibition of tube formation is comparable to the Sulforaphane negative control.

Angiogenesis is a complex multistep process whereby blood vessels sprout from existing vessels. It requires a multitude of molecular players including integrins, ECM components, proteases, and growth factors.

Several potent anti-cancer agents such as Bevacizumab (Avastin) have utilized this knowledge to attack tumors in the past [23]. However, drugs such as Avastin, which act only on singular molecular targets, may not be as efficacious as drugs that can affect multiple targets. The fluoro-xylosides presented in this paper represent a novel and powerful tool to inhibit angiogenesis because of their ability to target GAG biosynthesis and hence affect the multitude of interactions that are affiliated with cell-surface GAGs and proteoglycans.

In this paper, we have shown two fluoro-xylosides (III and IV) that are potent inhibitors of endothelial tube formation *in vitro*. There is a direct correlation between the most potent inhibitors of tube formation and the most potent inhibitors of GAG synthesis [22]. Since we have previously shown that cell surface heparan sulfates are essential players in the process of tube formation, it is likely that these fluoro-xylosides prevent tube formation by inhibiting GAG production [21]. Not only are these fluoro-xylosides ideal drug candidates due to their small size and their ability to penetrate cells, they are also excellent chemical biology tools to probe proteoglycan biology.

It can be argued that these first generation fluoro-xylosides are ineffective because of their high dosage requirements (300 μM). However, there are several methods of improving their potency.

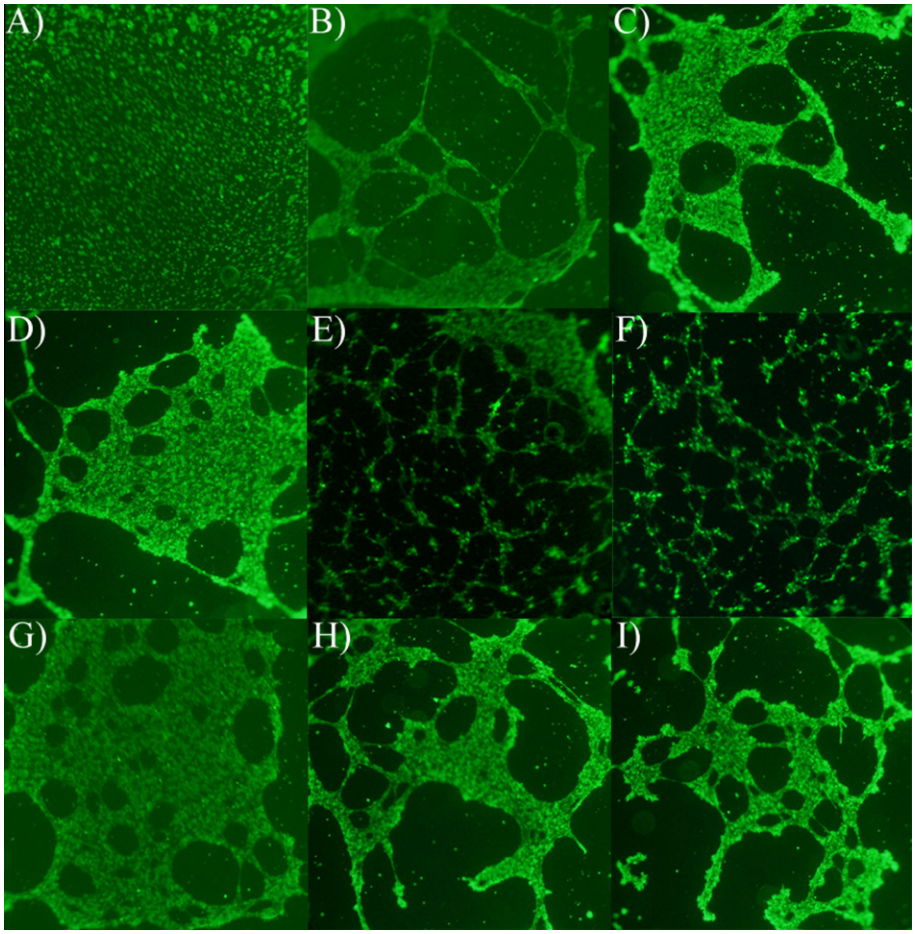


Fig. 1. Several fluoro-xylosides were added to BLMVEC on RGF matrigel at 300 μM concentrations. Representative images are: (A) 20 μM sulforaphane control. (B) Positive control. (C) Xyloside I. (D) Xyloside II. (E) Xyloside III. (F) Xyloside IV. (G) Xyloside V. (H) Xyloside VI. (I) Xyloside VII. These experiments were performed three times in duplicate wells.

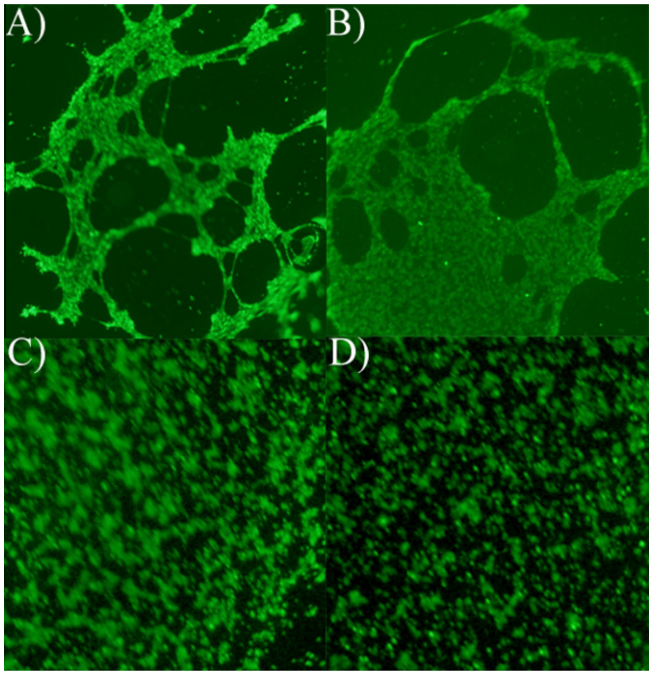


Fig. 2. Dose-dependent inhibition of tube formation by xylosides III and IV. Representative images are: (A) Xyloside III 150 μM. (B) Xyloside IV 150 μM. (C) Xyloside III 600 μM. (D) Xyloside IV 600 μM. These experiments were performed three times in duplicate wells.

Our lab has previously shown that varying the aglycone moiety attached to the xyloside can greatly affect its ability to prime distinct GAGs [12]. Additionally, several methods exist for targeting activated endothelial cells in the tumor microenvironment [24,25]. Future studies will utilize this information to design more potent fluoro-xylosides and test them *in vivo*. In conclusion, we have found novel fluoro-xylosides that inhibit GAG production in endothelial cells and also inhibit tumor-associated angiogenesis.

Acknowledgments

This work was supported by the National Institutes of Health Grant (GM075168), Human Frontier Science Program grant and American Heart Association National Scientist development Award to B.K. T.N. acknowledges a graduate fellowship support from Vietnam Education Foundation.

References

[1] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.
[2] K. Raman, B. Kuberan, Chemical tumor biology of heparan sulfate proteoglycans, Curr. Chem. Biol. 4 (2010) 20–31.
[3] R.D. Sanderson, Y. Yang, T. Kelly, V. MacLeod, Y. Dai, A. Theus, Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: growth regulation and the prospect of new cancer therapies, J. Cell. Biochem. 96 (2005) 897–905.
[4] R. Sasisekharan, S. Ernst, G. Venkataraman, On the regulation of fibroblast growth factor activity by heparin-like glycosaminoglycans, Angiogenesis 1 (1997) 45–54.

[5] J. Folkman, M. Klagsbrun, J. Sasse, M. Wadzinski, D. Ingber, I. Vlodavsky, A heparin-binding angiogenic protein–basic fibroblast growth factor–is stored within basement membrane, Am. J. Pathol. 130 (1988) 393–400.
[6] M. Klagsbrun, Mediators of angiogenesis: the biological significance of basic fibroblast growth factor (bFGF)-heparin and heparan sulfate interactions, Semin. Cancer Biol. 3 (1992) 81–87.
[7] H. Nakato, K. Kimata, Heparan sulfate fine structure and specificity of proteoglycan functions, Biochim. Biophys. Acta 1573 (2002) 312–318.
[8] C.I. Gama, L.C. Hsieh-Wilson, Chemical approaches to deciphering the glycosaminoglycan code, Curr. Opin. Chem. Biol. 9 (2005) 609–619.
[9] S. Guimond, M. Maccarana, B.B. Olwin, U. Lindahl, A.C. Rapraeger, Activating and inhibitory heparin sequences for FGF-2 (basic FGF). Distinct requirements for FGF-1, FGF-2, and FGF-4, J. Biol. Chem. 268 (1993) 23906–23914.
[10] N. Sugaya, H. Habuchi, N. Nagai, S. Ashikari-Hada, K. Kimata, 6-*O*-sulfation of heparan sulfate differentially regulates various fibroblast growth factor-dependent signallings in culture, J. Biol. Chem. 283 (2008) 10366–10376.
[11] L. Lundin, H. Larsson, J. Kreuger, S. Kanda, U. Lindahl, M. Salmivirta, L. Claesson-Welsh, Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis, J. Biol. Chem. 275 (2000) 24653–24660.
[12] X.V. Victor, T.K. Nguyen, M. Ethirajan, V.M. Tran, K.V. Nguyen, B. Kuberan, Investigating the elusive mechanism of glycosaminoglycan biosynthesis, J. Biol. Chem. 284 (2009) 25842–25853.
[13] F.N. Lugemwa, J.D. Esko, Estradiol beta-D-xyloside, an efficient primer for heparan sulfate biosynthesis, J. Biol. Chem. 266 (1991) 6674–6677.
[14] M.A. Bourdon, T. Krusius, S. Campbell, N.B. Schwartz, E. Ruoslahti, Identification and synthesis of a recognition signal for the attachment of glycosaminoglycans to proteins, Proc. Natl. Acad. Sci. USA 84 (1987) 3194–3198.
[15] N.B. Schwartz, L. Galligani, P.L. Ho, A. Dorfman, Stimulation of synthesis of free chondroitin sulfate chains by beta-D-xylosides in cultured cells, Proc. Natl. Acad. Sci. USA 71 (1974) 4047–4051.
[16] C.A. Henke, U. Roongta, D.J. Mickelson, J.R. Knutson, J.B. McCarthy, CD44-related chondroitin sulfate proteoglycan, a cell surface receptor implicated with tumor cell invasion, mediates endothelial cell migration on fibrinogen and invasion into a fibrin matrix, J. Clin. Invest. 97 (1996) 2541–2552.
[17] S. Brule, V. Friand, A. Sutton, F. Baleux, L. Gattegno, N. Charnaux, Glycosaminoglycans and syndecan-4 are involved in SDF-1/CXCL12-mediated invasion of human epithelioid carcinoma HeLa cells, Biochim. Biophys. Acta 1790 (2009) 1643–1650.
[18] K. Raman, B. Kuberan, Click-xylosides mitigate glioma cell invasion in vitro, Mol. BioSyst. 6 (2010) 1800–1802.
[19] K. Mani, M. Belting, U. Ellervik, N. Falk, G. Svensson, S. Sandgren, F. Cheng, L.A. Fransson, Tumor attenuation by 2(6-hydroxynaphthyl)-beta-D-xylopyranoside requires priming of heparan sulfate and nuclear targeting of the products, Glycobiology 14 (2004) 387–397.
[20] D.R. Garud, V.M. Tran, X.V. Victor, M. Koketsu, B. Kuberan, Inhibition of heparan sulfate and chondroitin sulfate proteoglycan biosynthesis, J. Biol. Chem. 283 (2008) 28881–28887.
[21] K. Raman, B. Kuberan, Differential effects of Heparitinase I and Heparitinase III on endothelial tube formation in vitro, Biochem. Biophys. Res. Commun. 398 (2010) 191–193.
[22] Y. Tsuzuki, T.K.N. Nguyen, D.R. Garud, B. Kuberan, M. Koketsu, 4-Deoxy-4-fluoro-xyloside derivatives as inhibitors of glycosaminoglycan biosynthesis, Bioorg. Med. Chem. Lett. 20 (2010) 7269–7273.
[23] L.S. Rosen, Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers, Cancer Control 9 (2002) 36–44.
[24] V.M. Tran, X.V. Victor, J.W. Yockman, B. Kuberan, RGD-xyloside conjugates prime glycosaminoglycans, Glycoconj. J. 27 (2010) 625–633.
[25] S.D. Rosen, Ligands for L-selectin: homing, inflammation, and beyond, Annu. Rev. Immunol. 22 (2004) 129–156.