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A Furan-Based Lewis-Y-(CD174)-Saccharide Mimetic Inhibits Endothelial Functions and *In Vitro* Angiogenesis

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Angiogenesis is a fundamental process underlying cancer progression and autoimmune disease. Lewis Y is known as a regulated glycan-structure supporting human endothelial function and angiogenesis.
Objectives. We hypothesize that Lewis Y based analogues interfere with Lewis Y mediated endothelial functions and angiogenesis. We therefore evaluated the ability of 3, 4-bis [(b-D-galactopyranosyl)osy]-methyl-furan (BGF) a furan-based Lewis-Y saccharide mimetic to inhibit human endothelial adhesion, migration and *in vitro* angiogenesis.
Material and Methods. The ability of BGF and additional furan-based saccharide-mimetics was investigated to inhibit adhesion and migration of human bone marrow endothelial cells (HBMEC). Influence of BGF was tested on a multicelluar *in vitro* – angiogenesis assay in the presence of VEGF.
Results. BGF significantly inhibited HBMEC adhesion and migration stimulated by TNF-alpha by up to 70%. The anti-adhesive effect of BGF was particularly evident when HBMEC adhesion and migration was tested on collagen as extracellular matrix with weaker effect when laminin and fibronectin were used as an extracellular matrix. BGF was ineffective when HBMEC were stimulated with VEGF. The inhibition of endothelial function translated into a significant inhibitory effect of BGF in the multicellular *in vitro* angiogenesis-assay. BGF reduced the angiogenesis index compared to the positive controls by 32%.
Conclusions. We identified the ability of the furan-based Lewis Y saccharide mimetic BGF as a specific modulator of TNF-alpha activated endothelial function and *in vitro* angiogenesis. BGF and other related glycan analogues should further be explored for their ability to down modulate endothelial activation in TNF-alpha driven pathophysiologic conditions in autoimmune disease and cancer indications (**Adv Clin Exp Med 2015, 24, 5, 759–768**).

Key words: cancer, angiogenesis, Lewis Y mimetic, endothelial.

Angiogenesis is a central mechanism underlying malignant and auto-immune disease [1]. A key event in the vascularization process is the activation of resting endothelial cells by pro-angiogenic molecules resulting in a sequence of molecular and cellular events that lead to a neo-vascular structure that supports tumor cell metastasis and proliferation [2, 3]. There is a general recognition of glycosylation for cell proliferation, cell adhesion and migration but the role of glycosylation and glycan molecules specifically in endothelial biology and tumor angiogenesis has been investigated only by a limited number of publications. Surface expression of glycans and their role in endothelial cell adhesion and angiogenesis is of particular relevance in this context.

It was described that the tetra-sacharide structure Lewis Y or CD174 is expressed after TNF-alpha induced activation of endothelial cells [4, 5]. Upregulation of Lewis Y on capillaries was observed in the context of tumor angiogenesis indicating that Lewis Y is also highly upregulated *in vivo* in pathophysiologic conditions as tumor angiogenesis [4]. These and functional data

suggest a functional role of Lewis Y in endothelial adhesion and *in vitro* angiogenesis.

Lewis Y or CD174 was initially recognized as blood group antigen [6]. CD174 tetrasacharide structure is composed of 2 alpha-L-fucose molecules, alpha-D-galactose and N-acetyl-glucosamine (Fig. 1). Carrier molecules of Lewis Y can be glycoproteins and glycolipids. Surface expression of Lewis Y and the very similar blood group H Typ 2 (CD173) antigen are both regulated by fucosyltransferase FUT1 and FUT2. FUT1/FUT2 transcription and as a consequence Lewis Y expression is a tightly regulated process [6, 7].

Outside of the hematopoietic system, Lewis Y can be expressed by a variety of tissues as epithelial cells [8]. Endothelial expression and upregulation of fucosylated glycans was initial discovered by Kannagi et al. and Garcia-Vallejo et al. [5, 7, 9]. Lewis Y is upregulated on endothelial cells *in vitro* by TNF-alpha but not by VEGF or bFGF which indicated a TNF-alpha-specific signaling pathway that leads to the upregulation of Lewis Y. On endothelial cells several cell surface receptor as ICAM-1 and αvβ3 integrin which are involved in adhesion events were discovered as carrier for Lewis Y [10, 11].

The discovery of the exact function of Lewis Y in the various tissues but in particular for the angiogenesis process is ongoing. Lewis Y is not only upregulated under tumor and inflammatory conditions but also in the embryonic development as another circumstantial evidence that Lewis Y has a role in multicellular processes of development and proliferation [11]. The use of glycan analogues is an important approach to interfere with glycan related adhesion mechanisms to study the physiological role on cell surface expressed glycolipids or glycoproteins. Moreover, this concept has potential relevance for the development of glycan analogues as therapeutic agents.

The potential of the synthetic Lewis Y analogue 3, 4-bis [(b-D-galactopyranosyl)osy]-methyl-furan (BGF) to inhibit tumor adhesion as well as migration was initially demonstrated by Kim et al. using the B16F10 melanoma cells [12].

The ability to inhibit tumor as well as endothelial cell adhesion and migration was recently demonstrated for another newly synthesized glycan analogue based on a bis-hydroxymethylate cyclohexan core which was found to bind to integrin αvβ3 [13].

We explored if the Lewis Y analogue BGF might be able to influence endothelial functions as endothelial adhesion, migration and *in vitro* angiogenesis.

Material and Methods

The retrovirally immortalized human bone marrow-derived endothelial cells (HBMEC-60) were kindly provided by Dr. E. van der Schoot (Sanguin, Amsterdam, the Netherlands) and originally described by Rood et al. In our study, we used the cloned HBMEC-60 cell line. These cells are characterized by expression of von-Willebrand factor (vWF) and VE-cadherin (CD144). E-selectin (CD62e) and VCAM-1 (CD106) are not expressed on unstimulated HBMEC-60, stimulation with IL-1 beta upregulates CD54 and CD106 [14].

HBMEC-60 were grown under standard cell culture condition at 36°C and humidified atmosphere with 5% CO2 in endothelial cell growth medium (Promocell, Heidelberg, Germany) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Biochrom KG, Berlin, Germany), 1 μg/mL hydrocortisone, 0.1 ng/mL human epidermal growth factor (EGF) (Promocell, Heidelberg, Germany) and 1 ng/mL human basal fibroblast growth factor (Biozol, Germany), amphotericin B 50 ng/mL and gentamycin 50 mg/mL. Cells for these assays were mycoplasm-free as verified by DAPI-staining of DNA and a PCR-based mycoplasm test (Venor GeM-OneStep, Minerva Biolabs, Berlin, Germany). Cells were stimulated with recombinant TNF-a (Promocell) at a concentration of 40 ng/mL or VEGF at 20 ng/mL (Promocell).

3,4-bis([(β-D-galactopyrano-syl)oxy]methyl) furan (BGF) as well as 3,4-Bis-Xyl-Furan (GM133) and 3,4-Bis-Glu-Furan (GM139) were synthesized as described previously [14]. Chemical structures of the analogues investigated in our assays are displayed in Fig. 1.

Adhesion Assay

HBMEC-60 cells were stimulated for 48 h with 40 ng/mL TNF before the beginning of the adhesion assay. Cells were washed and were pre-incubated with respective glycan analogues in phosphate-buffered saline solution (PBS, Promocell, Heidelberg, Germany) in the indicated concentration on ice for 15 min. Cells were washed subsequently and re-suspended in ECG medium without supplements. As a next step cells were seeded in 96-well plates pre-coated with extracellular matrix proteins as indicated (fibronectin, laminin, collagen) in a cell density of 2.5×10^4 cells in 150 μL per well in the presence or absence of analogues as indicated. Adhesion assay was performed at a cell culture condition at 37°C for 30 min. Experiments were performed with 3–6 replicates. The supernatant with non-adherent cells was finally

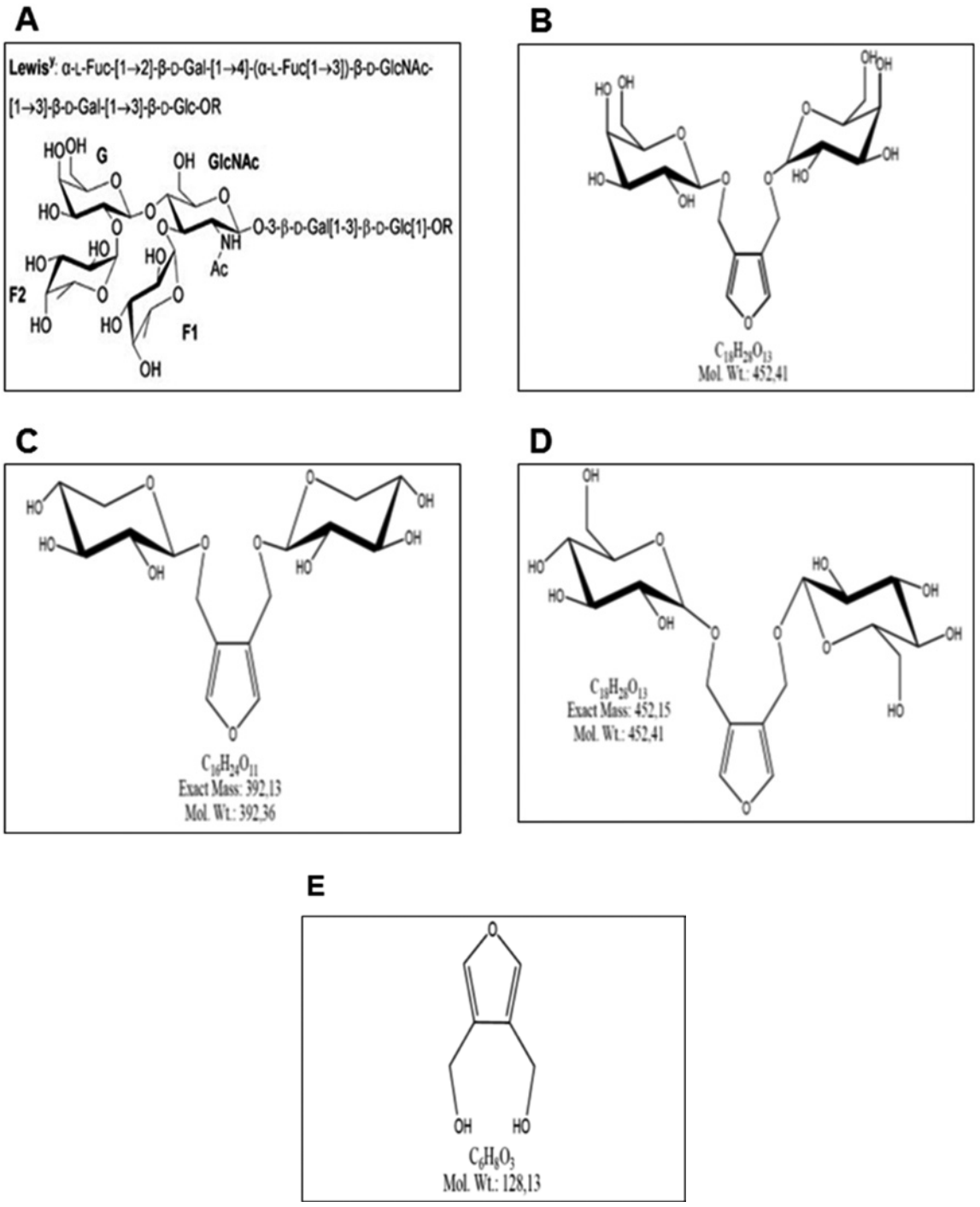

Fig. 1 A–E. Structure of Lewis Y and furan-based Lewis Y saccharide mimetics

Tetrasacharide structures possibly relevant for adhesion and binding to ECM (Gal-Fuc1-Fuc2- und GlcNAc-) are highlighted (G, F1, F2, GlcNAc). These tetrasacharide structures were added to a furan backbone to generate various furan-based Lewis Y saccharide mimetics (Kim et al 2005). A – Lewis Y structure, B – BGF; 3,4-Bis-Gal-Furan; C – GM133; 3,4-Bis-Xyl-Furan; D – GM139; 3,4-Bis-Glu-Furan; E – 3,4-Bishydroxymethyl-Furan (control).

removed and exchanged with fresh supplement--free ECG medium. Images of adherent cells were captured using an AxioCam MRm Camera System (Zeiss, Jena, Germany) and number of adherent cells were counted using a Cell Counter Program of Image J.

Migration Assay

HBMEC-60 cells were stimulated for 48 h with 40 ng/mL TNF before the beginning of the migration assay. Cells were washed and incubated in EC-GS-medium with only 5% FBS for 12 h before the start of the experiments. Polycarbonate transwells

with 8 μm pore size (Corning Costar) were coated on the lower side with laminin, fibronectin or collagen type I (10 μg/mL in PBS each for 1 h at 37°C) and were inserted into 12-well plates. Endothelial cells were plated at a density of 4×10^4 cells/mL into the upper compartment of the transwells in endothelial cell medium with 40 ng/mL TNF-alpha or 20 ng/mL VEGF. Then BGF or 3,4-bis(hydroxymethyl)furan (control) or medium only were added. After incubation for 60 min at 37°C non-adherent cells were removed, and cells ticking on the transwells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at rt, washed again and stained in the dark with Hoechst 33342 DNA dye (Invitrogen), washed twice with PBS at rt and then stored at 4°C until photographical documentation of the microscopy image and further counting of adherent stained cells. The experiments were performed in triplicates and in three independent experiments for each extracellular matrix protein.

In Vitro-Angiogenesis Assay/Tubing Assay

Essentially the *in vitro* angiogenesis assay TCM provided by Cellworks (Buckingham, UK) was used and performed as per the manufacturer recommendations. This test system was developed based on experimental data that the resulting tubule formation resembles *in vivo* angiogenesis and, therefore, is termed *in vitro* angiogenesis [14]. Using this test system, cocultures of human fibroblasts and human umbilical cord vein endothelial cells (HUVEC) were incubated in 24-well plates in endothelial growth medium including growth factors and test substances. Medium and test substances were changed on day 4 and 7 of cell culture. Where indicated VEGF was added at a concentration of 20 ng/mL on day 1, 4, 7 to support *in vitro* angiogenesis. On day 9 the medium was removed and the cells were washed and fixed in 70% (v/v) EtOH for 30 min at room temperature. Subsequently, cells were incubated with methyl alcohol and 30% H_2O_2 to block non-specific binding of visualization reagents. Cells were then washed with PBS. This was followed by a 30 min incubation step using an anti-CD31 antibody (DAKO, Hamburg, Germany) which specifically binds to endothelial cells in this experimental setting. After washing a secondary goat anti-mouse IgG antibody coupled to streptavidine-horse-radish-peroxidase (HRP) was incubated for 20 min and subsequently with AEC + substrate for 14 min (DAKO, Hamburg, Germany). After additional washing steps, a counterstaining with hematoxylin was performed. Cell cultures and tube formation was investigated with an Olympus IX70 microscope with integrated digital camera (Olympus, Germany). Digital images were further analysed using Angiosys 1.0 software to evaluate tubule length, tubule network formation as junctions between tubes, number of tubules (Cellworks, Buckingham, UK). For a quantitative comparison of cultures an angiogenesis index was calculated that captured and integrated all relevant aspects of *in vitro* angiogenesis (tubule number, tubule length, network formation/junctions). To integrate all of these parameters in the index the results of the individual measurements were related to the mean value of the positive control for each parameter by setting the mean value of positive control for each parameter (junctions/length/ /number of tubules) as 100%.

Statistical Analysis

Kruskal-Wallis and Wilcoxon rank sum tests were used to assess significance in comparing inhibitors to control mimetic. Inhibition of BGF compared to positive controls was assessed using a *t*-test with pairwise comparison. A Welch two sample *t*-test was utilized to analyze the difference between BGF and control. All statistical tests were used with the number of cells actually counted or obtained by quantifying the results of the test systems. Data was then presented either as mean of absolute numbers or as percentages (see figures and figure legends). All values are shown as mean of at least three triplicates. If applicable results were corrected for multiple testing using the Benjamini-Hochberg method. In all statistical tests, an effect was considered statistically significant if the p value of its corresponding statistical test was not greater than 5%. All statistical computations were performed using R v. 3.1.1 (A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014).

Results

The Glycan Analogue BGF Inhibits Endothelial Adhesion

To investigate if the Lewis Y saccharide-mimetic BGF could inhibit the adhesion of human endothelial cells, BGF was investigated in increasing concentrations in an adhesion assay using HBMEC-60 stimulated with TNF-alpha as described in material and methods. Stimulation with TNF was used as TNF-alpha is known to activate the Lewis Y expression [4, 8]. Adhesion of HBMEC-60 was tested in the context of various ECM proteins (fibronectin,

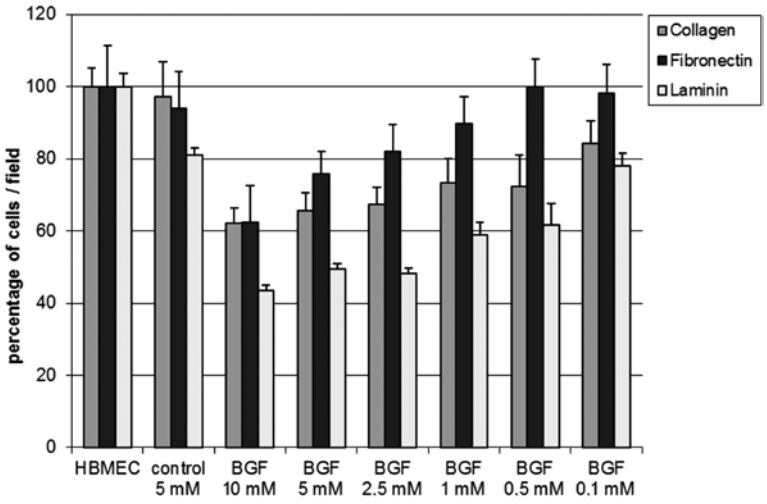

Fig. 2. BGF induced inhibition of adhesion of HBMEC-60 to extracellular matrix proteins

HBMEC-60 cells were stimulated with TNF incubated for 30 min with BGF (3,4-Bis-Gal-Furan) or 3,4-bis(hydroxymethyl)furan (control) or medium only (HBMEC) at the concentrations indicated. The cells were then seeded onto plates coated with fibronectin, collagen or laminin. After 30 min nonadherent cells were removed, and adherent cells were fixed, washed and stained with Hoechst dye; the number of adherent and spread cells was then counted using an Olympus IX70 microscope. At least three areas for each well were counted for each experiment. The values shown are the mean of three replicates. Statistical significance of inhibitors compared to control mimetic was analyzed using Kruskal-Wallis chi-square test and p-values were adjusted for multiple testing using the Benjamini-Hochberg method. BGF/collagen: $p < 0.01$ for all test conditions. BGF/fibronectin: $p = 0.03$ (2.5 mM), $p < 0.01$ (5 and 10 mM). BGF/laminin: $p = 0.016$ (1mM), $p = 0.011$ (2.5 mM to 10 mM).

collagen, laminin) and with increasing concentrations of BGF starting with 0.1 mM. We observed a dose dependent inhibition of the HBMEC-60 adhesion (Fig. 2). The adhesion inhibition reached statistical significance in the context of all ECM molecules with 5 mM or higher compared to the control substance (Fig. 1E). The maximum inhibition was achieved with 10 mM of BGF. The maximum inhibition of adhesion was achieved with 10 mM BGF was in the range between 40% and 66% and was depended on the experimental condition and utilized ECM molecules (Fig. 2 and 3A). The ability of BGF to block adhesion was dependent on the ECM molecules used in the experimental condition. Whereas BGF could inhibit collagen mediated adhesion significantly even at the lowest tested concentration of 0.1 mM ($p < 0.01$), BGF concentrations of 1 mM or 0.5 mM were required for fibronectin oder laminin-mediated adhesion.

Next, we investigated the anti-adhesive effect of furan-based Lewis Y analogues that had a modified structure compared to BGF (Fig 1). For this comparison we used the concentration of 5 mM and 10 mM that led to best inhibition in the concentration-dependent adhesion assays investigating BGF (Fig 3A, 3B). In the assay shown in Fig. 3 using the 10 mM BGF concentration 69% (collagen), 68% (fibronectin) and 42% (laminin) inhibition compared to the control analogue was achieved. GM133 (10 mM) reached 47% for fibronectin and collagen dependent adhesion and 43% laminin-dependent adhesion. For GM139 (10 mM) we obtained 53% (collagen). 54% (fibronectin), 40% (laminin). At the next lower concentration of 5 mM the modified Lewis Y saccharide--mimetics GM133 and GM139 lost their ability to significantly inhibit adhesion whereas BGF confirmed the inhibitory potential (Fig. 3B). Taken together the adhesion data comparing BGF with GM133 and GM139 did not indicate that the modification increased but rather decreased the anti-adhesive properties.

The Glycan Analogue BGF Inhibits Endothelial Migration

After confirming the ability of BGF to significantly inhibit adhesion we investigated BGF in migration assays. Similar to the adhesion assay the migration assays were conducted in the presence of 3 different ECM components (fibronectin, collagen, laminin) and with TNF-alpha as the stimulating cytokine.

Overall, we observed a dose dependent inhibition of the HBMEC-60 migration by BGF (Fig. 4). Collagen-mediated migration was particularly sensitive to inhibition by BGF as BGF in the lowest concentration of 0.1 mM ($p < 0.02$) resulted already in a significant inhibition of migration. In

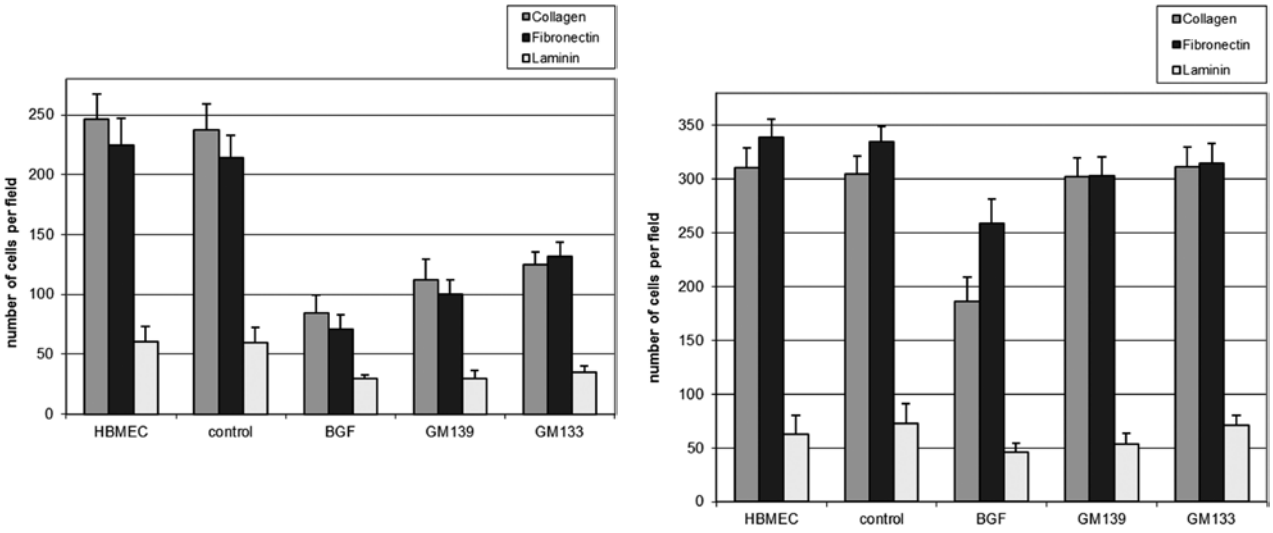

Fig. 3A + 3B. Comparison of BGF with 2 modified furan-based saccharide mimetics to inhibit adhesion of HBMEC-60 to extracellular matrix proteins

HBMEC-60 cells were stimulated with TNF-α then incubated with 3,4-Bis-Gal-Furan (BGF), 3,4-Bis-Xyl-Furan (GM133), 3,4-Bis-Glu-Furan (GM139) or 3,4-bis(hydroxymethyl)furan (control) or medium only (HBMEC) at a concentration of 10 mM (A) or 5 mM (B). The cells were then seeded onto plates coated with fibronectin, collagen or laminin. After 30 min nonadherent cells were removed, and adherent cells were fixed, washed and stained with Hoechst dye; the number of adherent and spread cells was then counted using an Olympus IX70 microscope. At least three areas for each well were counted for each experiment. The values shown are the mean of three replicates. Statistical significance of inhibitors compared to control mimetics was analyzed using Wilcoxon rank sum test and p-values were adjusted for multiple testing using the Benjamini-Hochberg method. BGF – $p < 0.001$ (for 5 and 10 mM) for all ECM test conditions. GM139 (10 mM) and GM133 (10 mM): $p < 0.05$ for all ECM test conditions; GM139 (5 mM)/laminin: $p = 0.01$; GM133 and GM139: all other experimental conditions p-value not significant.

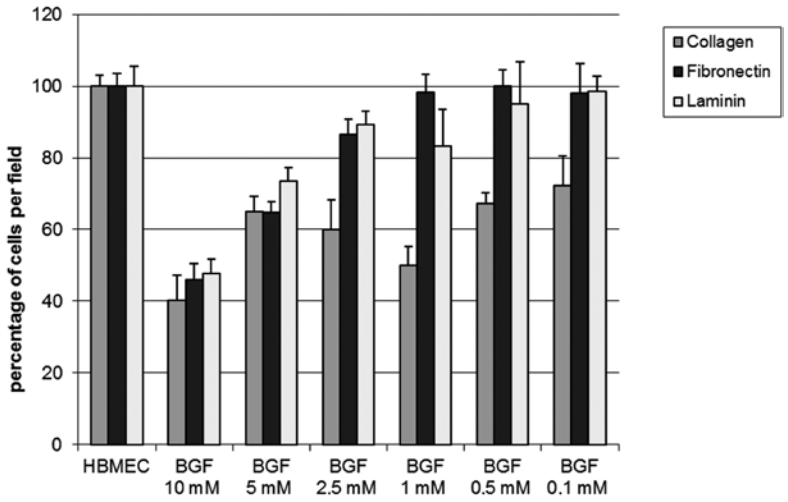

Fig. 4. Effect of BGF on transmigration of HBMEC-60 activated by TNF-α

HBMEC-60 cells were stimulated with TNF-α for 30 min. Cells were then seeded into transwell inserts coated with extracellular matrix proteins in the presence of BGF, 3,4-bis(hydroxymethyl)furan (control), or medium only (HBMEC), as indicated. After incubation for 30 min non-adherent cells were washed away, and adherent cells were fixed, stained and quantified. The experiments were performed in triplicates and in three independent experiments for each extracellular matrix protein. Statistical significance of inhibition of BGF compared to positive control was assessed using a t-test with pairwise comparison. BGF for all tested concentrations on collagen: $p \leq 0.02$, for 5 and 10 mM on fibronectin: $p < 0.02$, for 10 mM on laminin: $p = 0.028$.

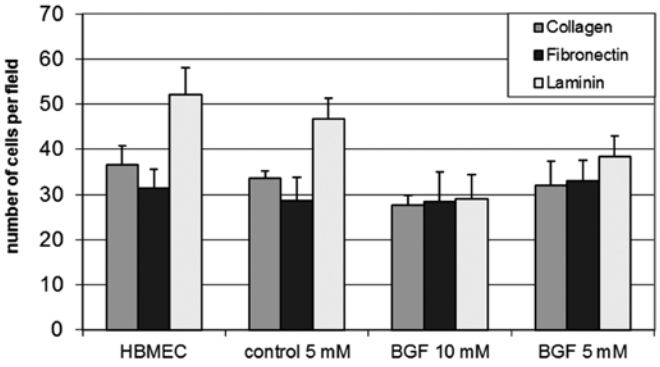

Fig. 5. Effect of BGF on transmigration of HBMEC-60 cells activated by VEGF

HBMEC-60 cells were stimulated with VEGF for 30 min. Cells were then seeded into transwell inserts coated with extracellular matrix proteins in the presence of BGF, 3,4-bis(hydroxymethyl)furan (control), or medium only (HBMEC), as indicated. After incubation for 30 min non-adherent cells were washed away, and adherent cells were fixed, stained and quantified. The experiments were performed in triplicates and in three independent experiments for each extracellular matrix protein. Statistical significance of inhibition of BGF compared to positive control was assessed using a *t*-test with pairwise comparison. BGF 10 mM/collagen: $p = 0.048$, BGF 5 mM/collagen p = non significant (ns.); BGF 10 mM and 5 mM)/fibronectin: NS; BGF 10 mM/laminin: $p < 0.05$, 5 mM: ns.

contrast, for the inhibition of fibronectin and laminin-mediated migration approx. 10-25-fold higher concentrations of BGF were required. To inhibit fibronectin and collagen mediated adhesion concentrations of 2.5 mM were required. Across all ECM glycoproteins used the highest inhibition of migration was achieved with 10 mM, which decrease the migration to approx. 60% of the level observed with control analogue.

We next investigated if the effect of BGF inhibition was maintained if other cytokines were used for stimulating migration. We, therefore, investigated HBMEC-60 migration after stimulation with VEGF at a concentration of 10 ng/mL knowing that VEGF is not effective in stimulating Lewis Y expression [4]. As demonstrated in Fig. 5 we found that BGF was not able to suppress HBMEC-60 migration in the context of fibronectin and collagen after stimulation with VEGF. Only in the context of laminin BGF was able to exert a low level of inhibition of migration of approx. 40% at the highest concentration.

Inhibition of *In Vitro* Angiogenesis by BGF

As a further step to investigate the ability of BGF to influence and inhibit endothelial function we explored the effect of BGF in an *in vitro* angiogenesis assay. In this test system fibroblasts and umbilical vein cord endothelial cells are cultured on a mix of extracellular matrix proteins including laminin in the presence of angiogenic cytokines including VEGF. After 9 days in culture formation of capillary-like structures and capillary networks are observed and can be quantified. BGF was used in these experiments at a concentration of 5 mM which was found to be inhibitory in the adhesion and migration assays.

Examples of the capillary network formation in the presence of medium only (no stimulation of angiogenesis), VEGF 20 ng/mL in the presence of control analogue or BGF are demonstrated in Fig. 6. Comparison between medium control (Fig. 6A) and VEGF with or without control substance (Fig. 6B, 6C) reveals the strong induction of angiogenesis in this model by VEGF. Addition of BGF diminished all aspects of tubule formation (Fig. 6D) but could not reduce the angiogenesis-response completely.

We further quantitatively evaluated the effect of BGF in the angiogenesis assay. For this purpose Angiosys 1.0 software captured the following key parameters of the *in vitro* angiogenesis: tubules, total tubule length, network formation/total number of tubule junctions. As described in material and methods the angiogenesis index was used to reflect on the overall ability of BGF to reduce *in vitro* angiogenesis. For this purpose data on tubule length, number of tubules and junctions was normalized to the mean of positive control and pooled for each experimental condition.

As displayed in Fig. 7, the presence of VEGF increased angiogenesis 4-5-fold. Similar results were obtained in the absence of the control substance as expected. Compared to the control substance incubation in the presence of BGF significantly ($p = 0.012$) reduced *in vitro* angiogenesis by 32%. Angiogenesis index was still substantially higher compared to the negative control as an indicator that BGF could not completely suppress angiogenesis.

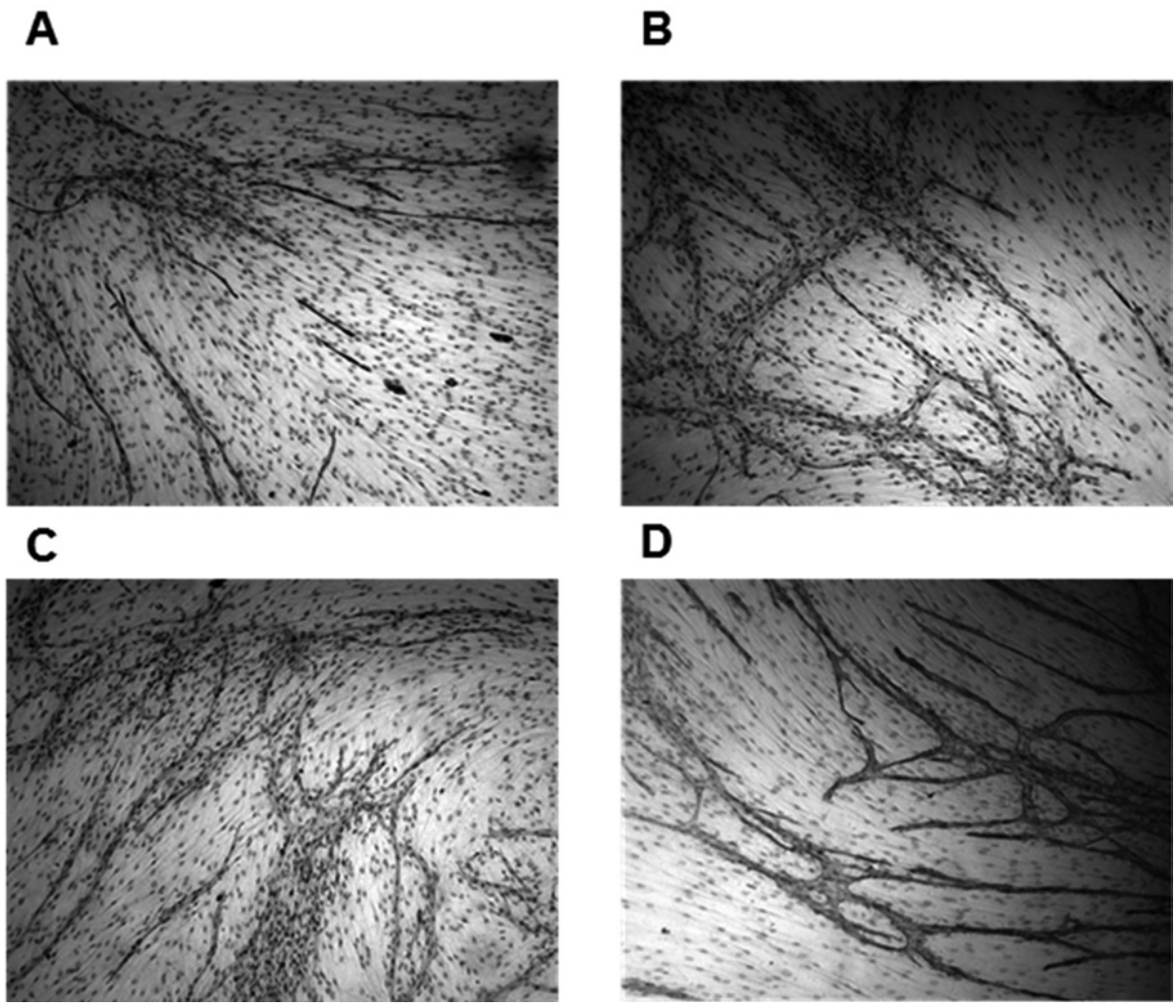

Fig. 6. Tubing Assay: inhibition of *in vitro* angiogenesis by BGF

Saccharide mimetics or control substances were added on day 0, 4, 7 to the *in vitro* angiogenesis assay. On day 9 the medium was removed and the endothelial cells were stained with a monoclonal antibody against the cell surface antigen CD31 to allow evaluation of tubule formation. The following conditions were investigated and representative examples were digitally captured using an Olympus IX70 microscope equipped with a digital camera: medium only (A), VEGF-activated angiogenesis + control substance (B), VEGF alone (C), VEGF + BGF (5 mM) (D). The assay was performed in three independent experiments with the same results in terms of the BGF effect.

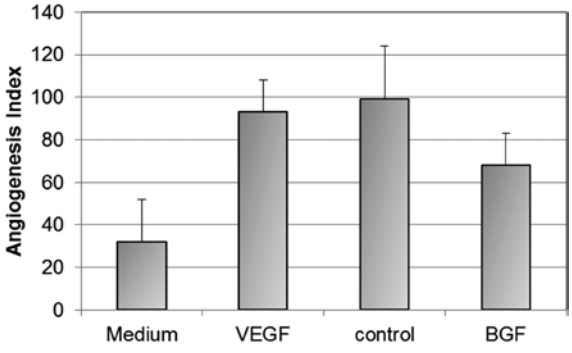

Fig. 7. Quantitative evaluation of the inhibition of *in vitro* angiogenesis by BGF

Tubing Assays were performed as indicated in material and method and legend of Fig. 6. On day 9 – as the end of the assay period – digital images of the tubule formation was obtained and quantitatively analyzed using Angiosys 1.0 software capturing the following parameters: total tubule length (in nm), number of tubules and junctions between tubules. Data on tubule length, number of tubules and junctions were normalized to the mean of positive control and pooled for each experimental condition: medium, VEGF, VEGF + control mimetic, VEGF + BGF (5 mM). Standard deviations are displayed. A Welch two sample *t*-test was utilized to analyze the difference between BGF and control which indicated a p value of 0.012.

Discussion

There is increasing evidence that the glycan structure Lewis Y has a role in endothelial functions. We demonstrate and comprehensively analyze the ability of a Lewis Y furan-based saccachride mimetic (BGF) to inhibit endothelial adhesion and *in vitro* angiogenesis.

Endothelial cells use a variety of ECM-receptors during the complex angiogenesis process [15]. A critical step for angiogenesis is the adhesion and migration of endothelial cells to ECM in particular laminin, fibronectin and collagen [17]. Our data demonstrates the ability of BGF to inhibit endothelial adhesion to all of these ECM molecules. We, therefore, conclude that BGF interferes with the adhesive properties of various adhesion receptors known to support the binding of endothelial cells to extracellular matrix. Taking adhesion and migration assays together, we have identified the highest efficiency of BGF to inhibit binding and migration in the context of collagen. BGF was less efficient to inhibit endothelial cell adhesion and migration to laminin and fibronectin. This conclusion is based on our finding that approx. 10–25-fold higher concentrations of BGF were required for inhibition of fibronectin and laminin-mediated adhesion and migration compared to the collagen-mediated adhesion and migration.

Our findings are in line with the concept that inhibition of BGF is tightly coupled to the relevance of Lewis Y for endothelial migration and adhesion.

Lewis Y expression has so far been recognized on a small set of cell surface receptors involved in adhesion to ECM or intercellular adhesion. In particular, regulated Lewis Y expression was so far identified for integrins alphavbeta3 and alpha5beta1 and the intercellular adhesion molecule-1 (ICAM-1) [10, 11, 16]. Zhu et al. have demonstrated that in contrast to ICAM-1, VCAM-1, P- and E-selectins do not express Lewis Y. This demonstrates that Lewis-Y dependent support of adhesion is restricted to a specific set of receptors and not a ubiquitous adhesion concept. Importantly, cytokine-regulated Lewis Y expression on integrins αvβ3 and α5β1 increased binding to ECM [10, 16].

In line with this data our working hypothesis is that Lewis Y is upregulated on endothelial cells specifically by TNF-alpha on a specific set of integrin receptors. The upregulation of Lewis Y results in an improved binding to ECM molecules and migration of endothelial cells. As a consequence of increased adhesion and migration Lewis Y supports angiogenesis. By mimicking Lewis Y, BGF interferes with the Lewis Y mediated improved ECM binding of ECM receptors, which in turn leads to the observed BGF induced decrease in adhesion, migration and angiogenesis.

Our finding that BGF is not able to completely block the adhesion of HBMEC-60 supports the concept that Lewis Y expression is an enhancer of the adhesive function of ECM receptors. For this reason mice deficient in FUT1 and FUT2 expression do not show phenotypic abnormalities in regards to embryonic and vascular development [17].

In contrast to TNF-alpha endothelial adhesion and migration stimulated by VEGF could not be inhibited by BGF, which is in line with previous data that VEGF did not induce Lewis Y expression on endothelial cells [4]. Despite the substantially lower activity of BGF to inhibit VEGF-induced adhesion, the inhibition of angiogenesis in the 9 day angiogenesis assay triggered by the addition of VEGF was significant. We conclude that due to the long culture duration in the angiogenesis-assay VEGF-induced endothelial and fibroblast activation led to the secondary development of cytokines as TNF-alpha and others that were able to upregulate Lewis-Y.

Further modifications of the furan-based structure of BGF (Fig. 1) by removing an OH-Group on the galactosyl-group (GM compounds, Fig. 3A, 3B) were not able to generate superior anti-adhesive or anti-migratory results in our test systems. Additional chemical modifications could be explored to further improve the activity of BGF. Developing saccharide mimetics for *in vivo* applications is possible and might be explored in the future as well for the Lewis Y analogue BGF or similar compounds [18]. A particularly interesting aspect is that Lewis Y inhibitors will not serve as universal angiogenesis inhibitors but rather show selectivity for pathophysiological situations with TNF-alpha over expression which is known e.g. for autoimmune disease and certain cancer types. Therefore, future research will concentrate on the question if the inhibition of Lewis Y will be of special advantage to suppress angiogenesis and endothelial activation in TNF-alpha dependent disease as rheumatoid arthritis or specific cancer types.

Taken together, our data supports the role for Lewis Y in angiogenesis. Furthermore, we can demonstrate that a furan-based Lewis Y analogue is able to block endothelial adhesion, migration and *in vitro* angiogenesis. Our data provides a rationale for developing Lewis Y specific glycan analogues based on a furan backbone as selective modulators of TNF-alpha-driven angiogenesis for pathophysiological conditions as cancer or autoimmune disease.

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