Inhibition of Endothelial Cell Proliferation by per-*O*-acetylated Mannose Conjugates

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Abstract. *Background: The inhibition of angiogenesis, defined as the process of new blood vessel formation, represents a promising strategy for treating cancer. Materials and Methods: The inhibitory properties of two N-(per-O-acetylated-β-D- mannopyranosyl)-thiophene-2-carboxamides derivatives (AMTCs,* ***[1],[2]****), N-(2,3,4,6-tetra-O-ethoxycarbonyl-β-D-mannopyranosyl)- thiophene-2-carboxamide* ***[3]*** *and of 2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl-acetamide* ***[4]*** *on the growth of bovine aortic endothelial cells (BAECs) induced by basic fibroblast growth factor (bFGF) were assessed using a [^{3}H]thymidine incorporation assay. The cellular uptake of AMTCs and the non-acetylated homologue (MTC) into BAEC were compared using mass spectrometry analysis of cell lysates. Results: AMTCs* ***[1],[2]****), at 80 μM, reversed the increase of [^{3}H]thymidine incorporation induced by bFGF, suggesting that these compounds inhibited bFGF-induced proliferative response in BAECs. The acetamide* ***[4]*** *was inactive showing the importance of the thiophene carboxamide for biological activity. The results of a study of AMTC uptake into BAEC suggest that AMTC is rapidly converted to its non-acetylated counterpart. Conclusion: The promising results obtained with AMTCs as inhibitors of BAEC growth could lead to the development of novel angiogenesis inhibitors.*

Cancer is the second leading cause of death in the Western world. Despite advances in diagnosis and treatment, the overall survival of patients still remains poor. At present, in many cancer types, animal studies and clinical studies have established that the progression and the growth of tumors is associated with aberrant angiogenesis (the formation of new, abnormal blood vessels) (1, 2). A growing number of angiogenic factors have been shown to regulate tumor angiogenesis. Among them, vascular endothelial growth factor (VEGF) and acidic or basic fibroblast growth factor (aFGF and bFGF) are considered the main effectors, stimulating proliferation of the endothelial cells (ECs) that comprise the lining of the blood vessels to form a new blood supply (3, 4).

Although many anticancer agents, targeting tumor cells, have been shown to improve patient survival, their dose-limiting toxicity and the emergence of drug resistant clones remain obstacles to their use. Current research is investigating novel therapeutic strategies aimed at inhibiting the proliferation of stimulated ECs, which are genetically stable, a readily accessible target, proliferate much more rapidly during angiogenesis than in normal tissues and their unique phenotype makes them susceptible to a wide range of drugs (5). Our recent studies have focused on the synthesis and design of novel N-glycosyl-thiophene-2-carboxamide derivatives as modulators of EC growth (6, 7). We previously reported that acetylated sugar improved the potential of the compounds to inhibit thymidine incorporation during DNA synthesis in bovine aortic ECs (BAEC) compared to their non-acetylated homologues (8). The aim of this study was to investigate the capacity of a range of per-*O*-acetylated-*N*-D-mannopyranosyl-thiophene-2-carboxamide (AMTC) derivatives (Figure 1) to inhibit bFGF-stimulated DNA synthesis in BAEC and to analyse their cellular uptake in comparison to their non-acetylated homologue.

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Materials and Methods

Synthesis of D-mannopyranosylamide derivatives. N-(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-thiophene-2-carboxamide **[1]** and *N*-(β-D-mannopyranosyl)-thiophene-2-carboxamide **[1a]** were prepared as described elsewhere (8, 9). *N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-5-ethyl-thiophene-2-carboxamide **[2]** was prepared by the reaction of *N*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl-amine (10) with 5-ethyl-thiophene-2-carbonyl chloride in presence of aqueous Na_2CO_3 in dichloromethane as

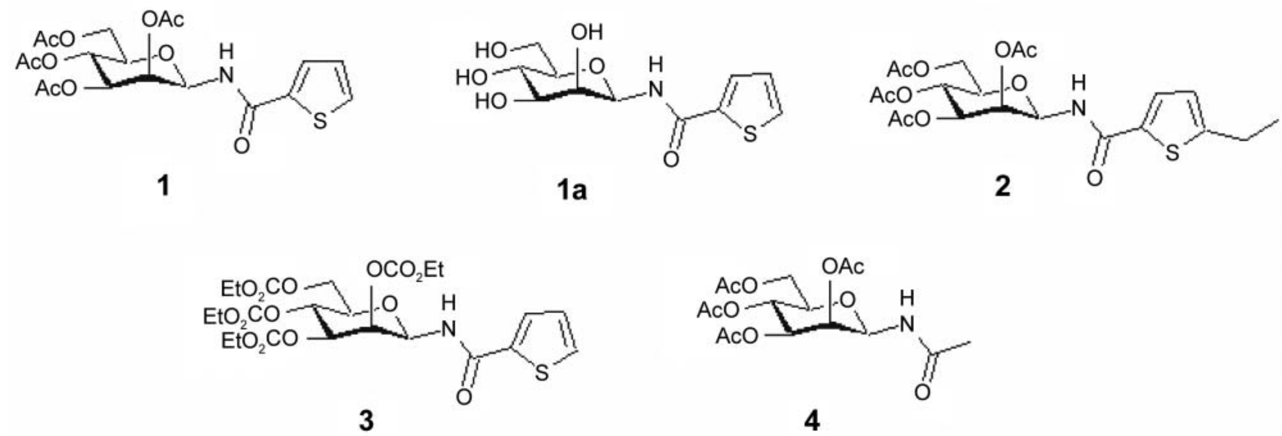

Figure 1. *Structures of compounds used in this study. 1: N-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-thiophene-2-carboxamide; 1a: N-(β-D-mannopyranosyl)-thiophene-2-carboxamide; 2: N-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-5-ethyl-thiophene-2-carboxamide; 3: N-(2,3,4,6-tetra-O-ethoxycarbonyl-β-D-mannopyranosyl)-thiophene-2-carboxamide; 4: N-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-acetamide.*

Table I. *ESI-MS data from the analysis of culture medium and cell lysate from BAECs treated with* **[1]** *or* **[1a]** *after different incubation times.*

Mass spectrometry analysis				
Time after incubation (h)	Peaks corresponding to mannose conjugates detected in culture medium (*m/z*)		Peaks corresponding to mannose conjugates detected in cell lysate (*m/z*)	
	Cells treated with **[1]**	Cells treated with **[1a]**	Cells treated with **[1]**	Cells treated with **[1a]**
2	480 [M**1**+Na$^+$]; 438 [M**1**-OAc+Na$^+$]	312 [M**1a**+Na$^+$]	not detected	not detected
4-6	480; 438; 396 [M**1**-2OAc+Na$^+$]	312	312	not detected
24	480; 438; 396; 354 [M**1**-3OAc+Na$^+$]	312	312	not detected
48	480; 438; 396; 312	312	312	not detected

described elsewhere (9) for the structurally related glucose analogue. *N*-(2,3,4,6-Tetra-*O*-ethoxycarbonyl-β-D-mannopyranosyl)-thiophene-2-carboxamide **[3]** was prepared by reaction of **[1a]** with excess ethyl chloroformate in dichloromethane in the presence of pyridine and *N,N*-dimethyl-4-aminopyridine (catalyst). *N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-acetamide **[4]** was prepared by reaction of N-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl-amine with acetic anhydride in pyridine. ^{1}H nuclear magnetic resonance (NMR), IR and mass spectrometry analysis confirmed the structure of the final products and their high purity. The compounds were dissolved in dimethyl sulfoxide and added to the culture medium so that the final concentration of the vehicle was 0.02%.

Cell culture. BAECs were purchased from the Coriell Institute for Medical Research (USA) and were cultured as described elsewhere (9). Cells used for the experiments were between passages 5 and 9.

[^{3}H]-Thymidine incorporation assay. Thymidine incorporation into DNA was measured in order to determine the effect of the compounds on DNA synthesis. Confluent BAECs were trypsinised, suspended in RPMI (Sigma-Aldrich, UK) with 5% foetal bovine serum (FBS) and seeded at 4.0x10^4 cells per well into 24-well plates. After a 24-h adhesion period, the cells were starved with 0.5% FBS-RPMI medium for 24 h. BAECs were treated with human recombinant bFGF (Sigma-Aldrich, UK) with or without increasing concentrations of test compounds in RPMI medium supplemented with 1% FBS for 48 h at 37˚C. During the final 4 h, the culture was pulsed with 2 μCi/mL [^{3}H]thymidine (Methyl-^{3}H, 6.7 Ci/mmole, PerkinElmer, UK) and incorporated radioactivity was determined as described elsewhere (8, 9).

Analysis of the compounds in the culture medium and in the cell extract by mass spectrometry. In order to study the cellular uptake of the mannose conjugates, cells were seeded (4.0x10^4 per well) one day before the addition of the compounds. BAECs were incubated in RPMI with 10% FBS containing 80 μM of **[1]** or the non-acetylated analogue **[1a]** and the culture medium was collected at five different time points. Subsequently, BAECs were washed twice with PBS, trypsinised and lysed with 1 mL distilled water per sample for at least 30 min followed by 2 min sonication. The samples were then stored at –20˚C before being subjected to electrospray ionisation-mass spectrometry (ESI-MS) analysis. The culture medium and the total cellular extract were analysed for the detection of the sugar analogues using the mass spectrometer (Quattro micro™, Waters MS Technologies, Manchester, UK) with an electrospray source.

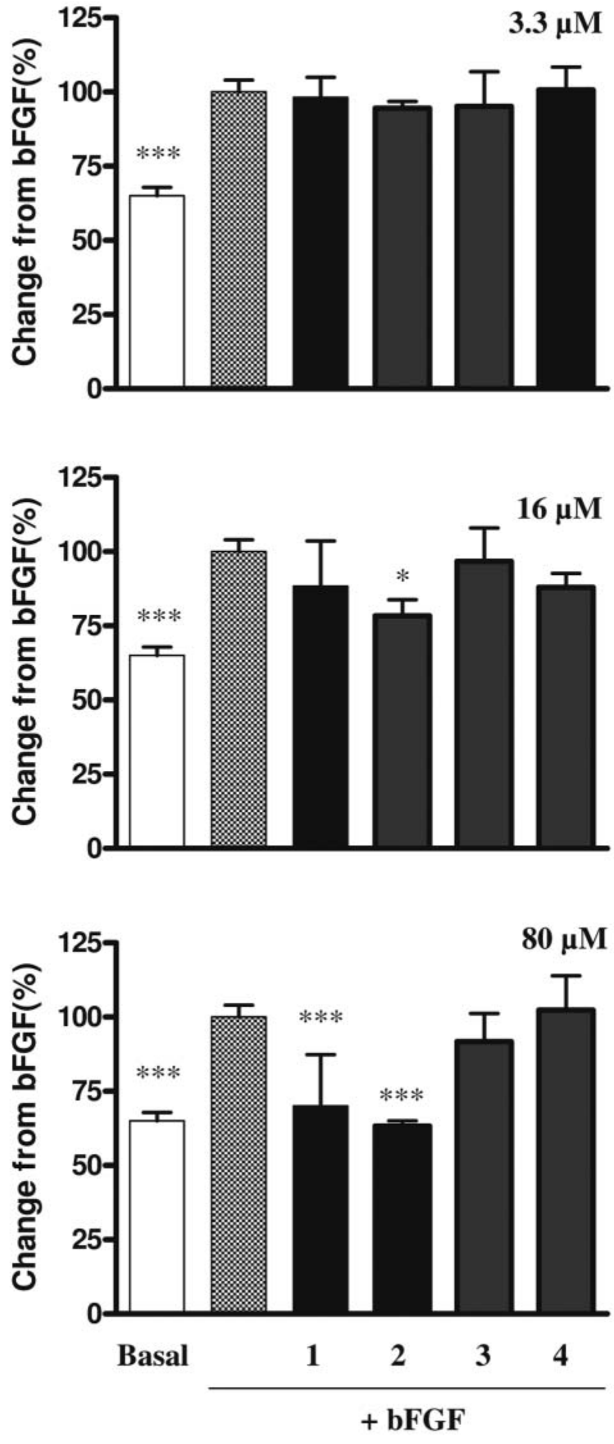

Figure 2. *Effect of mannose derivatives on bFGF-induced DNA synthesis in BAECs, applied at (A) 3.3 µM, (B) 16 µM and (C) 80 µM. bFGF (10 ng/mL)-stimulated BAECs display significantly increased [^{3}H]thymidine incorporation compared to untreated (basal) cells over 48 h. Each result is the mean and SEM of triplicate values from three separate experiments. For each condition, data are shown normalized to cells incubated with bFGF with no drug. *p<0.05, ***p<0.001 versus bFGF. Comparisons between treatments were assessed using one way Anova followed by Bonferroni's test.*

Results

Effect of the mannose derivatives on DNA synthesis induced by bFGF. The ability of four different sugar derivatives (Figure 1) to affect DNA synthesis activity induced by bFGF was assessed using the [^{3}H]thymidine incorporation assay. In the presence of FGF (10 ng/mL), the [^{3}H]thymidine incorporation significantly increased by 45±3% ($p<0.001$, ANOVA) compared to the basal (untreated cells) (Figure 2). The addition of AMTCs **[1]** and **[2]** reduced the [^{3}H]thymidine incorporation induced by bFGF in a concentration-dependent manner. No inhibitory activity was observed at the lowest concentration of 3.3 µM tested for compounds **[1]** and **[2]**. The addition of compound **[2]** at a concentration of 16 µM significantly inhibited bFGF-induced DNA synthesis by 86.6±5%, whereas compound [1] induced a weaker inhibitory effect (76.6%±15%). At a concentration of 80 µM, both **[1]** and **[2]** significantly reversed bFGF-induced DNA biosynthesis to basal levels after 48 h treatment (95±17% and 98.23±2% inhibition of [^{3}H]thymidine, respectively). By contrast, the per-*O*-ethoxycarbonyl derivative **[3]** and the acetamide **[4]** showed no inhibitory activity on bFGF-induced DNA synthesis at any concentration tested (Figure 2).

Acetylated versus non-acetylated ManTC cellular uptake analysed by mass spectrometry. We have previously observed that the acetylation of the sugar group improved the inhibitory activity of sugar thiophene carboxylamide on DNA synthesis induced by bFGF (8). To investigate the difference in the potency of AMTCs (*e.g.* **[1]**) *versus* MTCs (*e.g.* **[1a]**) their cellular uptake were analysed. Mass spectrometry analysis was conducted in both culture media and cell extracts after different time of exposure to either **[1]** or **[1a]** (each at 80 µM) and the results are summarised in Table I. Only the non-acetylated form of compound **[1a]** [m/z=312 (M+Na$^+$)] could be detected in the cell lysates (Table I and Figure 3) after 4-48 h exposure with the tetra-*O*-acetate **[1]**. In the culture medium, after 2 h exposure to **[1]**, two compounds were detected, namely **[1]** [m/z=480 (M+Na$^+$)] and an analogue resulting from the removal of one acetyl group [m/z=438]. After 24 h **[1]** was still present in the culture medium and successive degradation products resulting from the loss of more than one acetyl group were increasingly detected in the culture medium (Table I and Figure 3). After 48 h exposure to **[1]**, the non-acetylated parent compound **[1a]** (**[1]**-4OAc) was also detected in addition to the intermediate degradation products (1-OAc, 1-2OAc, 1-3OAc). By contrast, **[1a]** could not be detected in the cell lysate (up to 48 h, Table I) after exposure of cells to **[1a]**; it was detected in the culture medium at all times.

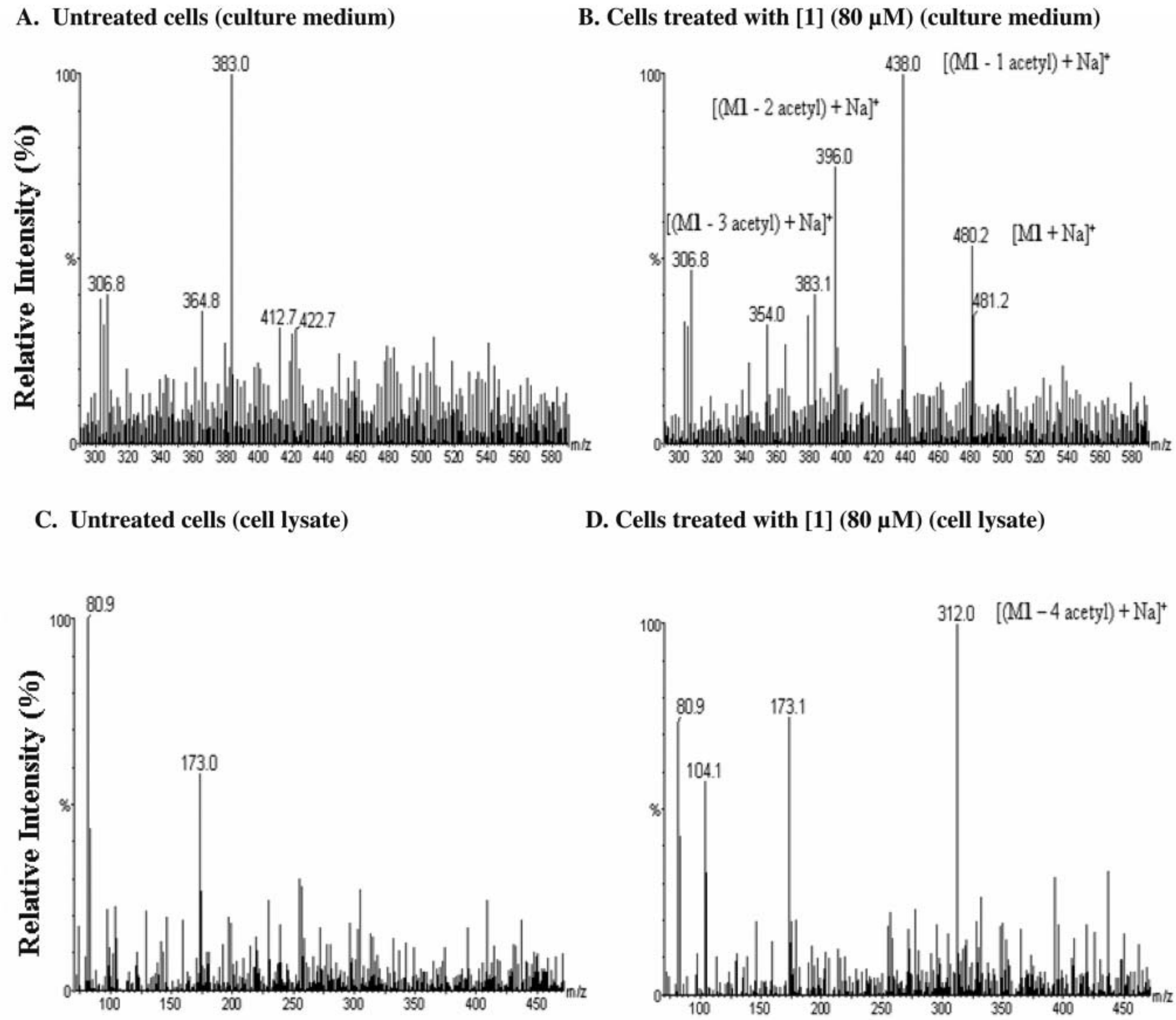

Figure 3. *Mass spectra of culture medium of (A) untreated cells and (B) cells treated with **[1]** after 24 h, and of cell lysate of (C) untreated cells and (D) cells treated with **[1]** after 24 h.*

Discussion

To assess the activity of a range of compounds on BAEC growth induced by bFGF, we used the [^{3}H]thymidine incorporation assay, which measures the DNA synthesis activity of the cells. We have previously shown that acetylated glycosyl-thiophene carboxamide derivatives were able to inhibit [^{3}H]thymidine incorporation induced by bFGF over a period of 48 h (8). We described herein that the addition of either AMTC **[1]** or its analogue **[2]** inhibited, at similar levels, bFGF-induced proliferative responses of BAEC over 48 h. Thus, the variation in the sugar moiety may not affect the inhibitory activity of the compound. However, the replacement of the thiophene carboxamide group with acetamide (as presented in **[4]**) abolished the inhibition of [^{3}H]thymidine incorporation, suggesting that the thiophene carboxamide is important for inhibiting DNA synthesis, driven by bFGF in BAECs. In addition, the replacement of acetates with ethoxycarbonyl groups also led to inactive compounds, suggesting that only acetylation of the sugar may be specifically important for the inhibitory activity of the compounds.

It was shown that per-*O*-acetylation of monosaccharides improves their membrane permeability (11-13). Prior to the present study, the cellular uptake of *N*-(2,3,4,6,-tetra-*O*-acetylated-β-D-glycosyl)-thiophene-2-carboxamides had not yet been investigated. We thus analysed the cellular uptake of **[1]** and compared it to its non-acetylated analogue **[1a]**. Using mass spectrometry analysis, we demonstrated, that the non acetylated counterpart of **[1]** rapidly appeared in the cell lysate (Table I and Figure 3D). Therefore, it is believed that **[1]** may act like a pro-drug to release the active, deacetylated form inside the cells, which then inhibits DNA synthesis. This interpretation is supported by the fact that cell lysate from cells treated with the free sugar analogue **[1a]**) did not accumulate at detectable levels inside cells. This result supported other research work which demonstrated that the biological activity of acetylated sugar derivatives is enhanced by the presence of increased amounts of the compound released in the cell (14). On the other hand, **[1]** was progressively deacetylated over 48 h in the culture medium in the presence of cells, suggesting a half-life under these conditions of at least 12 h. Previously, Eklund *et al.* (15), however, reported that the shorter half-life of acetylated mannose analogues compared to their ethoxycarbonyl derivatives was correlated with poor biological activity of acetylated sugars. This differs from our results which indicate that **[1]** is active and **[3]** non-active in inhibiting DNA synthesis. Our data suggest that BAECs may be less permeable to **[3]** than **[1]** or, alternatively, **[3]** is not capable of releasing the active form of the compound. These initial experiments provide evidence that acetylated sugar thiophene carboxamide derivative acting as pro-drugs to release the active form in cells are inhibitors of DNA synthesis in BAECs. Work investigating the possible mechanism of action of AMTCs is in progress with a view to finding new synthetic compounds with improved inhibitory activity against activated ECs.

Acknowledgements

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