Synthesis and cellular uptake of carbamoylated mannose derivatives

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ABSTRACT

A series of 3-carbamoyl- and 2,3-dicarbamoyl-mannose derivatives were synthesized, conjugated to a fluorescent dye (Cy5GE, AF 647 or NBD) and their cellular uptake in A549 and THP-1 cell lines was studied by FACS. In contrast to earlier studies on carbamoyl mannosides, the observed uptake was not related to carbamoyl group on the mannose residue but rather to the cyanine dye attached, a trend previously observed for Cy5-fructose conjugates. The NBD-conjugates however, showed a temperature and concentration dependent uptake in case of mannose conjugates. These results suggest a profound impact of the dye which should be taken into consideration when studying the uptake of small molecules by dye conjugation.

1. Introduction

The selective uptake of diagnostics and cytostatics in cancerous tissue is one of the main challenges in cancer diagnosis and therapy, respectively. A number of cancers can be targeted by utilizing their aberrant carbohydrate uptake and metabolism e.g. overexpressing of glucose transporter GLUT-1 [1] and the enzymes involved in the anaerobe glycolysis related to the Warburg effect [2–4]. For example, positron emission tomography (PET) imaging using 2-deoxy-2,18-fluoro-D-glucose (FDG) [5] is frequently used to diagnose cancers that display a heightened glucose metabolism. In addition, it was discovered that the carbohydrate portion of bleomycin, a natural product used to treat various cancers, enables its selective uptake in cancerous cells [6]. The carbohydrate portion of bleomycin is an unusual disaccharide consisting of 3-carbamoyl-mannose (1→3)-α-linked to l-gulose (Fig. 1).

Hecht and co-workers showed that microbubbles coated with bleomycin where adherent to MCF-7 human breast carcinoma cells whilst microbubbles coated with bleomycin lacking the disaccharide were not [7]. Critically, non-cancerous MCF-10A breast cells were not affected. Subsequent studies showed that the disaccharide portion itself was sufficient to enable selective uptake using disaccharides conjugated to fluorescent dyes to assay cellular uptake [8]. Cellular uptake of the disaccharide conjugates was temperature dependent and much higher at 37 °C compared to 4 °C indicating ATP-dependent uptake. Furthermore, the induction of increased glycolysis flux in healthy cells using mitochondrial complex 1 inhibitor rotenone [9] led to enhanced uptake. Conversely, inhibition of glucose transporter GLUT1 with cytochalasin B and phloretin led to a decrease in uptake hinting at the involvement of GLUT transporters. A later study with six different...
cancerous cell lines showed that the minimal epitope for the targeting and uptake in cancer cells is the 3-carbamoyl mannose residue [10]. When the 3-carbamoyl group was removed, the cellular uptake was significantly lower indicating that the carbamoyl group is crucial for the recognition and internalization [10]. Finally the position and substitution (Fig. 1, highlighted in blue) of the carbamoyl functionality in the bleomycin disaccharides was explored [11]. From these studies it became clear that N-methyl carbamoyl group at the 2- or 3-position of mannose showed even greater uptake than the parent 3-carbamoyl mannose.

Intrigued by these results we set out to investigate the uptake of a 2,3-dicarbamoyl mannose reasoning that there may be a synergistic effect when installing two carbamoyl groups at once. In addition, as determination of the uptake by confocal microscopy on fixed cells is laborious and does not allow for high throughput screening, we opted for a more convenient method on living cells. Herein we report the synthesis of 3-carbamoyl- and 2,3-dicarbamoyl-mannose derivatives and their conjugation to three different fluorescent dyes. Cellular uptake was assayed by fluorescence activated cell sorting (FACS).

2. Results and discussion

2.1. Chemical synthesis

To prepare carbamoylated mannose derivatives 9–10 in a divergent manner from known thioglycoside 1 we developed the synthetic route shown in Scheme 1. Deacetylation of 1 followed by installation of a 4,6-benzylidene afforded 3 in 48% yield after crystallization. Acetylation of the 2,3-diol was carried out to ensure α-selectivity in the ensuing glycosylation. Glycosylation of 2-(2-azidoethoxy)-ethanol using 4 [12] and the NIS/TfOH promoter system afforded mannoside 5 in 64% [13]. Subsequent deacetylation afforded 2,3-diol 6 which was used to prepare carbamoylated derivatives 7–8. An unmodified mannose derivative 10 [14] used for control experiments was prepared first by global deprotection of 6 using Pd/C and H2. 3-carbamoyl-mannose derivative 7 was prepared by a regioselective reaction with benzylisocyanate. Removal of the protecting groups was achieved using hydrogenolysis (Pd/C, H2) to afford 3-carbamoyl-mannose derivative 9 [15]. 2,3-dicarbamoylation using benzylisocyanate proved more difficult to achieve. Fortunately, using trichloroacetyl (TCA) isocyanate dicarbamoylation was possible and after removal of the TCA-groups using K2CO3 and MeOH 8 was obtained. Hydrogenolysis (Pd/C, H2) of 8 finally afforded deprotected compound 11.

With compounds 9–11 in hand we prepared conjugates with a fluorescent label to assay their cellular uptake (see Scheme 2). To this end, we selected three fluorescent dyes to investigate the influence of the presence of the carbamoyl groups. The synthesis of the carbohydrate conjugates 9–12 was accomplished as follows: (i) Cy5-OSu, 0.2 M aq. phosphate buffer; (13, 79%; 14, 62%; 15, 32%; 16, 26%; (ii) AF 647-OSu, 0.2 M aq. phosphate buffer; 17, 55%; 18, 69%; 19, 48%; 20, 51%; (iii) NBD-Cl, 0.3 M aq. NaHCO3; 21, 15%; 22, 25%; 23, 7%; 24, 10%.

Scheme 1. Synthesis of mannoside conjugates 9–11: (i) MeOH, K2CO3; 2, 99%; 6, quant.; (ii) PhCH(O)Me2, HBF4· Et2O, DMF; 3, 48%; (iii) pyridine, Ac2O; 4 [12], 92%; (iv) HOCl2H2OCl2H2N2, NIS, TIOH, DCM; 5, 64%; (v) DMF, benzyl isocyanate; 7, 23% (vi) N,N-diisopropylethylamine, trichloroacetyl isocyanate, DCM vii) MeOH, K2CO3; 8, 32% over two steps; viii) H2O, tert-butanol, EtOAc, Pd/C, H2; 9, 35%; 10, 39%; 11, 42%;

Scheme 2. Dye conjugations to carbohydrate conjugates 9–12: (i) Cy5<sup>560</sup>-OSu, 0.2 M aq. phosphate buffer; 13, 79%; 14, 62%; 15, 32%; 16, 26%; (ii) AF 647-OSu, 0.2 M aq. phosphate buffer; 17, 55%; 18, 69%; 19, 48%; 20, 51%; (iii) NBD-Cl, 0.3 M aq. NaHCO3; 21, 15%; 22, 25%; 23, 7%; 24, 10%.
the dye structure on the uptake. In addition to mannose derivative 10 we also prepared glucose derivative 12 [16] (see supporting information, S8–S9) as a control for the cell experiments. Although most uptake studies involving 3-carbamoyl mannosides published used the Cy5** dye (structure presented supporting information, S10) [17], we switched to more available dyes since Cy5** has a restricted availability. Fluorescent dyes Cy5GE and Alexa Fluor 647 (AF 647), also used in related experiments by Bhattacharya et al. [15], were conjugated to amines 9–12 using their corresponding N-hydroxysuccinimide esters. HPLC purification afforded pure conjugates used for cellular uptake studies. The nitrobenzoxadiazole (NDB) [18] dye is also frequently used to track the cellular uptake [19,20] and hence we prepared conjugates of this dye as well by reacting 4-chloro-7-nitrobenzofurazan with amines 9–12 to afford conjugates 20–24.

2.2. Biological assays

Next, we investigated the cellular uptake of 13–24 in two cancer cell lines (Fig. 1). We selected two cancer cell-lines (A549 and THP-1), derived from lung cancer and acute monocytic leukemia patients, respectively (see supporting information for specific culture conditions). The concentration of conjugates 13–24 was set at 2μM after FACS signal optimization. Cells were incubated for 1 h at 37°C before being rinsed with PBS twice and released from the surface by trypsin containing buffer (A549) or mechanical stress (THP-1). Phosphate buffered albumin (PBA, 1% BSA, 0.02% NaN₃ in PBS) was added followed by propidium iodide (PI) staining (0.1% PI in PBA) to assess cell viability. Cellular uptake was assayed using FACS based on at least 5000 live cells per measurement. To ensure cell viability during the FACS experiments, cells were stained with trypan blue after 2 h incubation in buffer. No significant cell death was observed. In addition, the cells were isolated, resolved in medium and showed adherence and growth at normal speed.

The uptake of the conjugates showed a striking difference depending on the fluorescent group (Fig. 2, A–E). Conjugates containing Cy5GE (Fig. 2, A–C) and AF 647 (figure, D–E) showed a high uptake regardless of the sugar attached. Internalization of the cyanine-dyes was confirmed by confocal microscopy studies on conjugate 12–16 (see supporting information), which showed a non-even distribution throughout the cells, suggesting lysosomal uptake. To assess whether the internalization is largely mediated by an active process or passive diffusion, the uptake was studied at low temperature. The uptake of all conjugates was lowered by incubation at 4°C, most pronounced in the AF 647 conjugates (17–20), hinting at a specific form of uptake. In contrast, previous studies with the monosaccharide 3-carbamoyl mannose-Cy5** conjugate showed temperature independent uptake [15], suggesting a different mechanism of internalization. Critically, the expected difference in uptake between 3-carbamoyl mannose vs mannose itself was not observed and both showed comparable uptake. Additionally, the glucose control as well as the 2,3-dicarbamoyl mannose-Cy5** conjugate showed temperature independent uptake [15], suggesting a different mechanism of internalization. In general, the NDB dye (Fig. 2, C) is less bright than dyes Cy5GE and

![Cellular uptake of fluorescent glycosyl conjugates 14–24 in A549 (A–C) and THP-1 (D–E) cancer cell lines. Cells were incubated at warm (37°C) or cold (4°C) temperature (final concentration in all temperature experiments was set at 2μM). Fluorescence was normalized according to the blank (H₂O).](image-url)
AF 647 and hence provided only a weak signal at 2μM concentration. We therefore performed experiments at increasing concentrations of the NDB conjugates (Fig. 3, A). Whilst the carbamoylated mannosides 23–24 and glucose conjugate 21 showed no increase in uptake upon increasing their concentration, mannose conjugate 22 did show a clear concentration dependent increase in fluorescence. Uptake of mannose conjugates 22 was temperature dependent in A549 and THP-1 cell-lines hinting at an active uptake process. To evaluate the extent of NDB mannose uptake, we compared the uptake of 22 to NBD-glucosamine (NBDG) a frequently used NBD conjugated probe to measure glucose uptake [20]. In A549 cells, both mannose conjugate 22 and glucosamine conjugate 25 performed equally well showing that the uptake of 22 is significant. In THP-1 cells, mannose conjugates 22 outperformed NBDG and was taken up to a much larger extend, which may be related to the increased expression of the human passive glucose transporter GLUT3 in monocytes [21,22]. Overall these results indicate that large fluorophores such as Cy5GE and AF 647 may influence the properties of the small molecules attached, whereas small fluorophores such as NBD are more likely to show a substrate dependent uptake.

3. Conclusion

In conclusion, we developed a robust synthetic route towards carbamoyl functionalized mannosides for conjugate synthesis. The mannosides were conjugated to Cy5GE, AF 647 and NBD and their temperature dependent uptake was studied by FACS in two cancerous cell lines (A549 and THP-1). In contrast to earlier studies, the uptake was not related to carbamoylation of the conjugates but rather to the cyanine dye attached. The NBD-conjugates however, gave evidence of a temperature and concentration dependent uptake in case of mannose conjugates. These results suggest a profound impact of the dye which should be taken into consideration when studying the uptake of small molecules by dye conjugation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2019.06.008.

Conflict of interest

The authors declare that they have no conflict of interest.

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