Inhibition of the key enzyme of sialic acid biosynthesis by C6-Se modified N-acetylmannosamine analogs†

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Synthetically accessible C6-analogs of N-acetylmannosamine (ManNAc) were tested as potential inhibitors of the bifunctional UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE/MNK), the key enzyme of sialic acid biosynthesis. Enzymatic experiments revealed that the modification introduced at the C6 saccharide position strongly influences the inhibitory potency. A C6-ManNAc diselenide dimer showed the strongest kinase inhibition in the low μM range among all the substrates tested and successfully reduced cell surface sialylation in Jurkat cells.

Introduction

Sialic acid is an essential component of the periphery of the glyocalyx. Sialylated glycoconjugates are involved in a broad range of cell functions and biological interactions including recognition of pathogens and immune regulation, among others.1–3 For instance, it is known that the influenza virus invades host cells through sialic acid recognition.4 Many tumor cells show an altered glycosylation pattern with increased expression of sialic acids on their surface.5,6 Assuming this oversialylation may act as a shield to protect these tumor cells from immune recognition.7,8 Nowadays, the elucidation of the precise role of sialic acids in glycan-mediated cellular interactions is a field of growing interest. For studying cell-surface glycocalyx metabolism oligosaccharide-engineering (MOE) is a well-established tool to modify and visualize sialic acids on the cell membrane even in living animals.9 By this strategy, unnatural N-acetylmannosamine (ManNAc) analogs can be efficiently incorporated into membrane glycoconjugates utilizing the sialic acid biosynthetic pathway, which can be subsequently labeled with bioorthogonal reactions.9–12 Regarding sialic acid expression, inhibitors of sialyltransferases, a group of enzymes that facilitate the transfer of sialic acid onto glycoconjugates in the Golgi lumen, were reported to efficiently reduce cell surface sialylation in vitro13,14 and in vivo.15 Another promising approach for the study of sialic acid related biological processes is the development of inhibitors targeting the de novo biosynthesis of this sugar. The key enzyme of sialic acid biosynthesis is the bifunctional UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE/MNK), which consequently serves as an important regulator of cell surface sialylation in mammals.16 Specifically, the GNE domain of the enzyme first isomerizes UDP-N-acetylglucosamine (UDP-GlcNAc) into ManNAc, which is then phosphorylated at the 6-hydroxy position under consumption of adenosine triphosphate (ATP) by the MNK domain (Scheme 1).

To date, known GNE/MNK inhibitors were synthesized based on unnatural monosaccharides, including N-acetylglucosamine (GlcNAc), UDP-GlcNAc and ManNAc analogs (Fig. 1).17–23 Among all reported inhibitors, only 3-O-methyl-N-acetylglucosamine (1) and the methylated ManNAc analog 2 were tested in eukaryotic cells. For instance, the methylated GlcNAc analog 1 at 1 mM final concentration reduced the incorporation rate of N-GlcNAc and N-ManNAc into glycoproteins by 70% in HepG2 cells.24 More recently, the methylated ManNAc analog 2 showed reduction of cell surface sialylation to 20% using 500 μM final concentration of the inhibitor in culture medium.25 Other reported inhibitors, including 6-O-Ac-ManNAc (3), iminosugars 4–6 and 2',3'-dialdehyde-UDP-N-acetylglucosamine (7) were tested with either the purified ManNAc kinase (3 at 5 mM final concentration) or UDP-GlcNAc-2-epimerase/MNK kinase (4–6 at 1 mM; 7 at 300 μM) and showed an inhibition of the corresponding enzyme by 60%, 70% and 93%, respectively.19,20,23 Despite these studies, the need of high inhibitor concentrations for an efficient reduction of cell surface sialylation limits the applicability for further studies, especially for in
moiety at the C6 position of ManNAc is sterically accessible for substrate modifications. For this purpose, we planned to access C6-modified ManNAc analogs, in which larger selenium or sulfur atoms replaced the oxygen at position C6, presumably leading to a better blockage of the binding pocket. Furthermore, we reasoned that these C6-modifications would be promising for inhibitory studies in living cells in contrast to compound 3, in which the acetylated C6-hydroxyl group is cleaved by endogenous esterases.

**Results and discussion**

The synthetic strategy was designed to be versatile and accessible from previously described tosylated ManNAc 8 (Scheme 2), which can react with appropriate S- or Se-nucleophiles. Considering the S-derivatives, the peracetylated thiol derivative 9 was obtained from 8 by nucleophilic substitution employing potassium thioacetate. In order to obtain water-soluble compounds suitable for enzymatic in vitro assays, simultaneous deprotection of S- and O-acetyl groups was carried out using standard basic conditions; however, to avoid epimerization at position C2 it was crucial to limit the time of basic deprotection with sodium methoxide to 10 min. We observed the formation of thiols 11 and the disulfide 12 based on the $^1$H-NMR shift of the protons at position C6, which were both isolated and employed in further enzymatic MNK-assays. To prepare the corresponding C6-Se-ManNAc-analogues, we employed the same electrophilic building block 8 from above. After reaction with nucleophilic potassium selenocyanate as the Se-source, compound 10 was obtained in 91% yield after purification. The chemoselective reduction of selenocyanate in presence of O-acetyl groups was ensured by using sodium borohydride, followed by acidification and stirring under inert atmosphere. Although the selenol could be observed after the acidification as a minor peak by UPLC-MS analysis, the high oxidation potential of the selenolate prevented the isolation of the corresponding selenol. Instead, the diselenide dimer 14a was obtained in 83% yield and analyzed by $^{77}$Se NMR, displaying the characteristic shift for diselenides at 319–323 ppm. The treatment with sodium methoxide in methanol and subsequent neutralization afforded the unprotected seleno sugar-dimer 14b in a moderate yield of 47%, most likely due to the cleavage of the labile C-Se bond as previously reported. With compounds 11, 12 and 14b in hand we tested the inhibitory effect in an MNK activity assay. Human MNK was expressed in BL21-CodonPlus (DE3)-RIL Escherichia coli (Stragene) and purified according to an established method. The half maximal inhibitory concentrations (IC$_{50}$) of modified ManNAc analogs for MNK activity have been measured in vitro using a coupled optical assay based on consumption of nicotinamide adenine dinucleotide (NADH, details in ESI†). Herein, purified enzyme was incubated with ManNAc (125 μM), the natural substrate for the kinase, ATP and varying concentrations of the previously synthesized C6-modified ManNAc analogs 11, 12 and 14b. With an IC$_{50}$ value of 8.5 μM, the diselenide 14b showed the strongest inhibition of enzyme activity among all tested substances (Table 1, entries 1–3 and Fig. 2A). Encouraged by these results, we also prepared

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**Scheme 1** The bifunctional UDP-$N$-acetylglucosamine-2-epimerase/ $N$-acetylmannosamine kinase (GNE/MNK) catalyzes the first two steps in the de novo synthesis of sialic acids in cells. UDP-GlcNAc is converted by GNE/MNK to ManNAc-6-phosphate, which is then condensed with phosphoenolpyruvate (PEP) to $N$-acetyleneuraminic acid-9-phosphate (Neu5Ac-9-P). The latter substrate is further dephosphorylated, activated to CMP-sialic acid and used for the synthesis of sialylated glycans.

In this manuscript, we report the synthesis and evaluation of sulfur and selenium-based C6-derivatives of $N$-acetylmannosamine as potential inhibitors of the human MNK. We hypothesized that C6-ManNAc derivatives cannot be phosphorylated by MNK, making them competitive inhibitors for this enzyme. Our concept was supported by investigating the crystal structure of human MNK in complex with its natural substrate, ManNAc. The geometry of the active site revealed that only the hydroxyl

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**Fig. 1** Some unnatural modified monosaccharides reported as inhibitors of GNE/MNK kinase.
the selenoethers 16b and 18b, to further probe the influence of the second selenium atom as well as the second sugar moiety on enzyme activity. Thereby, we took advantage of the nucleophilic properties of the selenolate anion precursor 13 to access the selenoethers. Seleno cyanate 10 was reductively cleaved with sodium borohydride for 5–10 min (monitored by TLC) and the high reactive selenolate anion 13 was caught in situ by Ac3-6-Br-ManNAc (15) or 1-iodopentane (17) (Scheme 2). An excess of the electrophiles was used to decrease the formation of the diselenide 14a. The desired seleno compounds 16a and 18a were isolated in 69% and 65%, respectively. The treatment with sodium methoxide delivered unprotected seleno sugars 16b and 18b as described for 14b before. MNK activity studies with selenoethers revealed significantly lower inhibition in comparison to the diselenide 14b (Table 1, entry 4 and 5).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>IC50 (mM)</th>
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<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>&gt;10</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>4.2 (±0.7)</td>
</tr>
<tr>
<td>3</td>
<td>14b</td>
<td>8.5 × 10⁻³ (±1.9 × 10⁻³)</td>
</tr>
<tr>
<td>4</td>
<td>16b</td>
<td>3.0 (±0.7)</td>
</tr>
<tr>
<td>5</td>
<td>18b</td>
<td>1.9 (±0.5)</td>
</tr>
</tbody>
</table>

The performed MNK activity studies for the sulfur and seleno analogs 11, 12, 14b, 16b and 18b indicated that a combination of a diselenide bond together with an additional ManNAc unit is highly beneficial for the enzyme inhibition. In the homodimeric ManNAc derivatives the backbone structure of ManNAc appears twice, giving two potential binding partners for the ManNAc-binding site of MNK. Consequently, this leads to a stoichiometric advantage for dimeric inhibitors, and therefore increases their inhibitory potential compared to monosaccharide ManNAc analogs that have been previously reported as MNK inhibitors. Comparing the results of the enzymatic studies with 14b and 16b, the length and type of the selenide-linker is apparently of great importance for MNK inhibition. The C-Se-Se-C linker in 14b is longer, but also could be more flexible than the C-Se-C linker in 16b. This feature as well as the length and the angle of the C-Se bond could be essential for successful positioning of the inhibitor into the active binding pocket of the MNK. In addition, different van der Waals interactions and H-bonding properties of selenium and sulfur atoms could play a role for protein binding. Even though they share a high structural similarity, compound 12 had an approximately 500-fold higher IC50 in comparison to 14b.

With the potent diselenide compound 14b in hand, we further studied the binding properties of this potent MNK inhibitor, which is to our knowledge the strongest MNK inhibitor reported to date. Diselenide 14b inhibited MNK activity in a dose dependent manner (Fig. 2A). Additionally, we performed the enzymatic assay in presence of 14b (25 µM) and different concentrations of ManNAc and confirmed a competitive inhibition of 14b for ManNAc. The inhibitor constant (Kᵢ) value obtained was 15.7 µM (Fig. 2B). Additionally, we tested if diselenide 14b influenced the GNE activity of the bifunctional GNE/MNK. Analysis of GNE/MNK in mouse liver cytosol revealed an IC50 of 14b for inhibiting the GNE activity in the range of 200 µM (details in ESI†). This result indicates that the main inhibitory effect of 14b on sialic acid biosynthesis is due to
MNs inhibition, although the GNE activity appears to be influenced by the diselenide 14b, likely due to an allosteric effect of the binding of the inhibitor to the MNK domain. To evaluate the specificity of the inhibitor towards other sugar kinases, we performed further inhibition studies using human GlcNAc kinase (GNK) and hexokinase (HK) from yeast (details in ESI†). The IC50 of 14b for GNK activity was about 1.7 mM, and higher than 5 mM for HK activity, respectively, indicating that the effects of 14b on these other sugar kinases are non-specific and negligible.

At this stage, we also wanted to evaluate the stability of the diselenide 14b in presence of thiol-containing reducing agents like dithiothreitol (DTT) or glutathione (GSH) to address the application of our compounds in living cells. Therefore, the possible reduction of the diselenide bond was monitored by 77Se NMR using a solution of 60 mM concentration of 14b in an aqueous saturated DTT solution at room temperature. Immediately after the addition of 14b to the DTT solution, a characteristic signal for the selenol compound at −48.1 ppm was observed and a major peak at 294 ppm related to the diselenide. Concretely, the integration of diselenide/selenol peaks gave a 90 : 10 ratio (details in ESI†). Due to the most stable oxidation state, in the time period between 15 min and 5 h, only signals from the diselenide were detected. In the case of saturated GSH solution, using the same procedure as before, only diselenide 14b was detected after incubation at 60 mM.

Encouraged by the strong in vitro inhibition of 14b and its high stability in the presence of reducing agents, we evaluated the capability of this substance to reduce cell surface sialylation. Due to the fact that peracetylated ManNAc derivatives are known to have better membrane permeability,29 the corresponding peracetylated diselenide 14a was used as a prodrug like precursor of 14b for cell experiments. Additionally, selenide 16a was chosen as a control because it shares high structural similarity to 14a, but had only weak effects on the enzyme activity of MNK (Table 1). Jurkat cells were treated for 72 h with different concentrations of peracetylated diselenide 14a and selenide 16a. First, the cytotoxicity of 14a as well as 16a was evaluated using the AlamarBlue® cell viability assay. Up to a concentration of 50 µM in culture medium, diselenide 14a exposed negligible cytotoxicity (details in ESI†). However, at a final concentration of 100 µM cell viability was reduced by approximately 70%. Therefore, the following experiments on cell surface sialylation were limited to an inhibitor concentration of 50 µM in culture medium. In the same concentration range, selenide 16a reduced cell viability up to by 25%. Cell surface sialylation was evaluated by measuring the expression of 6′-sialyl-N-acetyllactosamine (6′-sialyl-LacNAc) via flow cytometry using fluorescein isothiocyanate-conjugated Porcine squamosus lectin (FITC–PSL).22,30 Only treatment with diselenide 14a led to a significant reduction of sialic acid expression in tested cells. At a final concentration of 50 µM in cell culture medium, the 6′-sialyl-LacNAc entity was reduced by approximately 85% (Fig. 3).

As expected, selenide 16a did not alter cell surface sialylation at tested conditions. Total inhibition of cell surface 6′-sialyl-LacNAc expression was not observed. This is most likely due to uptake and reutilization of sialic acids from the serum

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**Fig. 2** (A) ManNAc diselenide dimer 14b inhibited the MNK in dose dependent manner. (B) Lineweaver–Burk plot showed a competitive inhibition of 14b and ManNAc, the natural substrate of MNK. Sigmoidal (A) and linear (B) approximation curves were used to determine IC50 and Kᵢ values. Data shown represent the means ± SEM from five independent experiments.

**Fig. 3** Diselenide 14a reduced 6′-sialyl-LacNAc expression in Jurkat cells. Selenide 16a had no visible effect on cell surface sialylation. Data shown represent the means ± SEM obtained in a triplicate.
supplement and its conversion to CMP-sialic acid independent of GNE/MNK activity. A slow turnover of sialylated glycoproteins could additionally contribute to this finding.

Conclusions

In summary, the performed enzymatic assays showed that diselenide 14b is a potent MNK inhibitor both in vitro and in cellulo with an IC_{50} value of 8.5 μM, representing the strongest inhibition of all previously reported MNK inhibitors. The homodimeric structure including a diselenide linker introduced at position C6 proved to be highly beneficial for the inhibitory activity of the compound. Finally, we demonstrated that the peracetylated diselenide 14a was successfully capable of reducing cell surface sialylation in Jurkat cells. With the C6-modified ManNac analogs presented in this work new insight could be attained into inhibition of the key enzyme of sialic acid biosynthesis, the GNE/MNK. We hope that strong GNE/MNK inhibitors like the described 14a/14b, pave the way for future studies elucidating the effects of GNE/MNK inhibition in vivo.

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