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A Novel Approach to Decrease Sialic Acid Expression in Cells by a C-3-modified *N*-Acetylmannosamine*[S]

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Background: Inhibitors of cellular sialic acid expression offer substantial therapeutic promise for diseases associated with oversialylation.
Results: 2-Acetylamino-2-deoxy-3-*O*-methyl-D-mannose reduces the sialic acid concentration in cells and inhibits the UDP-GlcNAc-2-epimerase/ManNAc kinase.
Conclusion: Inhibition of the key enzyme of sialic acid biosynthesis by a ManNAc analog decreases cellular sialic acid expression.
Significance: ManNAc analogs represent a new class of sialic acid inhibitors.

Due to its position at the outermost of glycans, sialic acid is involved in a myriad of physiological and pathophysiological cell functions such as host-pathogen interactions, immune regulation, and tumor evasion. Inhibitors of cell surface sialylation could be a useful tool in cancer, immune, antibiotic, or antiviral therapy. In this work, four different C-3 modified *N*-acetylmannosamine analogs were tested as potential inhibitors of cell surface sialylation. Peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose decreases cell surface sialylation in Jurkat cells in a dose-dependent manner up to 80%, quantified by flow cytometry and enzyme-linked lectin assays. High-performance liquid chromatography experiments revealed that not only the concentration of membrane bound but also of cytosolic sialic acid is reduced in treated cells. We have strong evidence that the observed reduction of sialic acid expression in cells is caused by the inhibition of the bifunctional enzyme UDP-GlcNAc-2-epimerase/ManNAc kinase. 2-Acetylamino-2-deoxy-3-*O*-methyl-D-mannose inhibits the human ManNAc kinase domain of the UDP-GlcNAc-2-epimerase/ManNAc kinase. Binding kinetics of the inhibitor and human *N*-acetylmannosamine kinase were evaluated using surface plasmon resonance. Specificity studies with human *N*-acetylglucosamine kinase and hexokinase IV indicated a high specificity of 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose for MNK. This substance represents a novel class of inhibitors of sialic acid expression in cells, targeting the key enzyme of sialic acid *de novo* biosynthesis.

Sialic acid is an essential constituent of the glycocalyx. Due to its chemical characteristics, sialic acid interacts with the environment of the cell. Its terminal position in glycoconjugates enables this amino sugar to disguise or create recognition sites for receptors or ligands and thus modulates biological cell functions (1). Sialic acid is a ligand for the selectin and the siglec family of adhesion molecules, mediating immune regulation and leukocyte rolling (2–5). Various pathogenic microorganisms, similar to the influenza virus with hemagglutinin, use sialylated glycans to recognize and bind host cells (6). Most tumor cells demonstrate hypersialylation and an alteration of their sialic acid pattern, allegedly to protect themselves from immune surveillance (7, 8). There is evidence that oversialylated tumor cells are more resistant to anti-cancer drugs (9).

The bifunctional enzyme UDP-GlcNAc-2-epimerase/ManNAc[4] kinase is the major determinant of cell surface sialylation (10). It catalyzes the first two steps in the *de novo* biosynthesis of sialic acid, namely the epimerization of UDP-GlcNAc to ManNAc and the phosphorylation of ManNAc to ManNAc-6-phosphate. Former studies succeeded in synthesizing inhibitors of the UDP-GlcNAc-2-epimerase/ManNAc kinase (11–14). However, these inhibitors were membrane-impermeable and could not be used in cell-based assays. Consequently, we focused on developing membrane-permeable analogs of *N*-acetylmannosamine that inhibit sialic acid expression in intact cells.

Non-natural ManNAc analogs are well established precursors of modified sialic acid on the cell surface. They, for exam-

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[4] The abbreviations used are: ManNAc, *N*-acetylmannosamine; DMB, 1,2-diamino-4,5-methylenedioxybenzene; GlcNAc, *N*-acetyl-glucosamine; GNE, human *N*-acetyl-glucosamine-2-epimerase; MNK, human *N*-acetylmannosamine kinase; Neu5Ac, *N*-acetyl-neuraminic acid.

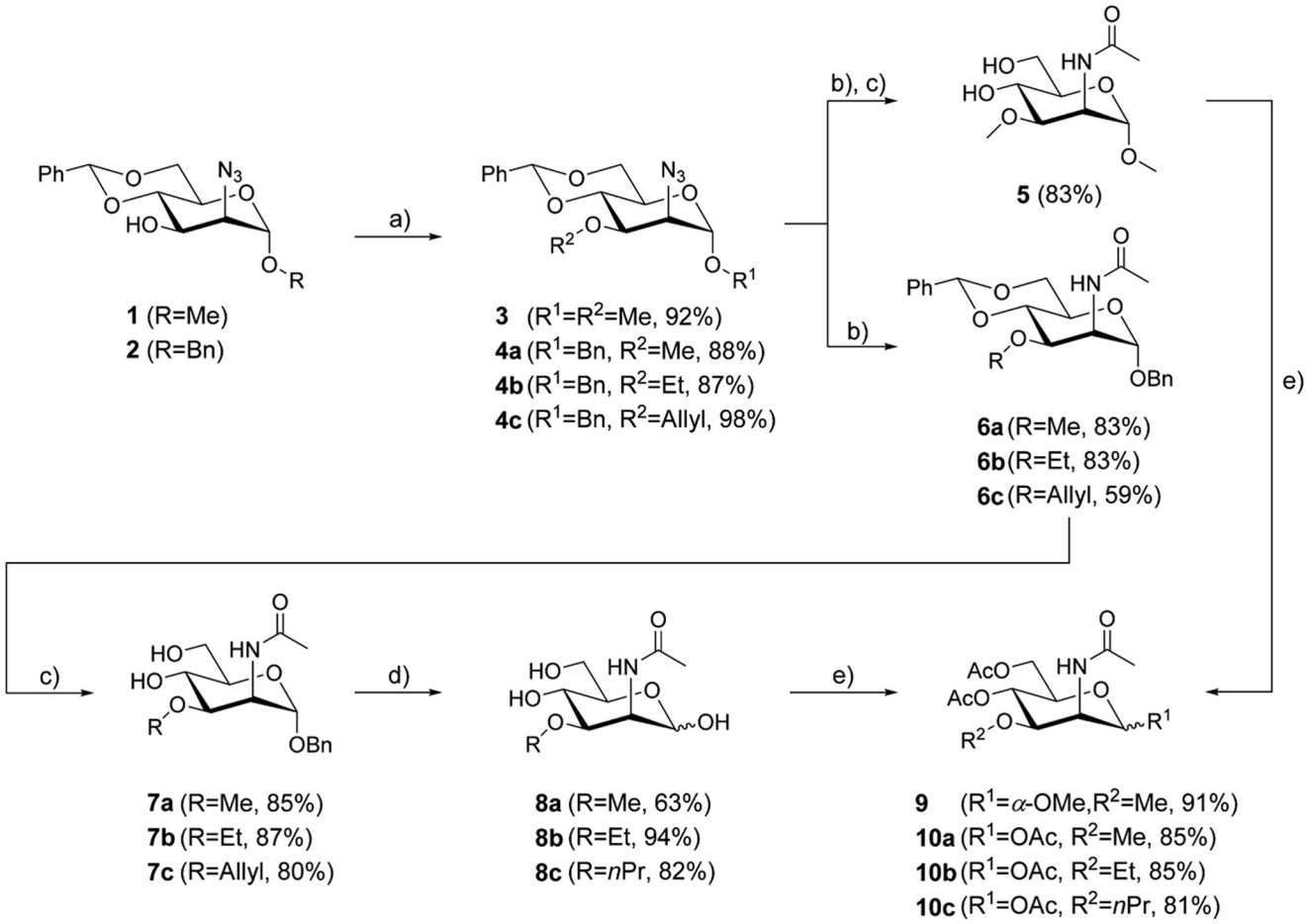

FIGURE 1. **Synthesis of C-3-modified mannosamine analogs.** 2-Acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**8a**), methyl 2-acetylamino-2-deoxy-3-*O*-methyl-α-D-mannopyranoside (**5**), 2-acetylamino-2-deoxy-3-*O*-ethyl-D-mannose (**8b**), and 2-acetylamino-2-deoxy-3-*O*-propyl-D-mannose (**8c**) were prepared in three to four steps. For cell experiments, the given ManNAc analogs have been peracetylated (**9**, **10a**, **10b**, and **10c**, respectively) to increase their permeability for the plasma membrane of the cells: (*a*) NaH, alkyl halide (MeI, EtI, and allyl bromide), 0 °C → room temperature, dimethylformamide; (*b*) 1) H_2 (10% v/v), Pd/C, MeOH (for reduction of **4c**; PPh_3, THF/H_2O); 2) AcCl, NEt_3, DMAP, DCM; (*c*) AcOH (80%), 80 °C; (*d*) H_2 (11 bar), Pd/C, MeOH; and (*e*) Ac_2O, pyridine.

ple carrying a keto or an azido group, can be utilized to visualize sialic acid in cells (15–17). These analogs are either modified at the C-2 or the C-4 position of ManNAc (18, 19).

In our study, we introduced C-3-modified ManNAc analogs and tested their effect on cell surface sialylation. C-3 derivatives of ManNAc are not likely to be metabolized by cells into the respected sialic acids (20). Inhibition studies for the ManNAc kinase domain of the UDP-GlcNAc-2-epimerase/ManNAc kinase were performed.

EXPERIMENTAL PROCEDURES

Preparation of ManNAc Analogs

Chemical synthesis of C-3-modified *N*-acetyl-D-mannosamines (Fig. 1) started from azido mannopyranosides **1** and **2**, respectively (21, 22). Attachment of the alkoxy residue at C-3 was achieved by Williamson ether synthesis to give the alkylated derivatives **3** and **4a**–**c** in good to excellent yields. Reduction of the azido function with H_2-Pd/C or through Staudinger reaction and subsequent acylation under standard conditions yielded the *N*-acetylated derivatives. In the case of methyl glycoside **3** cleavage of the 4,6-*O*-benzylidene group by treatment with acetic acid at elevated temperature provided ManNAc analog **5**. In addition benzyl glycosides **6a**–**c** were also synthesized and then subjected to acidic hydrolysis to afford derivatives **7a**–**c**. Cleavage of the benzyl group through palladium-catalyzed hydrogenolysis furnished the free *N*-acetyl-3-*O*-alkylmannosamine derivatives **8a**–**c**.

To increase their membrane permeability, peracetylated ManNAc analogs have been synthesized for cell experiments. In the cytoplasm, the *O*-acetyl groups of these molecules are removed by cytoplasmic esterases releasing the active monosaccharides (23–25). Peracetylation of **5** and **8a**–**c** with acetic anhydride in pyridine afforded compounds **9** and **10a**–**c**.

Details are included in the supplemental data. Identity and purity of all intermediates and final compounds were verified by spectroscopic and spectrometric methods (data not shown).

Determination of Cell Surface Sialylation

Cell Preparation—Approximately 20,000 Jurkat cells (ATCC) in 100 μl of RPMI 1640 culture medium (PAN Biotech) with 10% FBS (PAN Biotech) and 2 mM L-glutamine, containing peracetylated ManNAc analogs in varying concentrations, were cultured for 72 h.

After incubation, cells were washed with PBS containing 0.5% bovine serum albumin (PBS + 0.5% BSA) and labeled with various lectins. Labeling was carried out for 1 h at 4 °C and

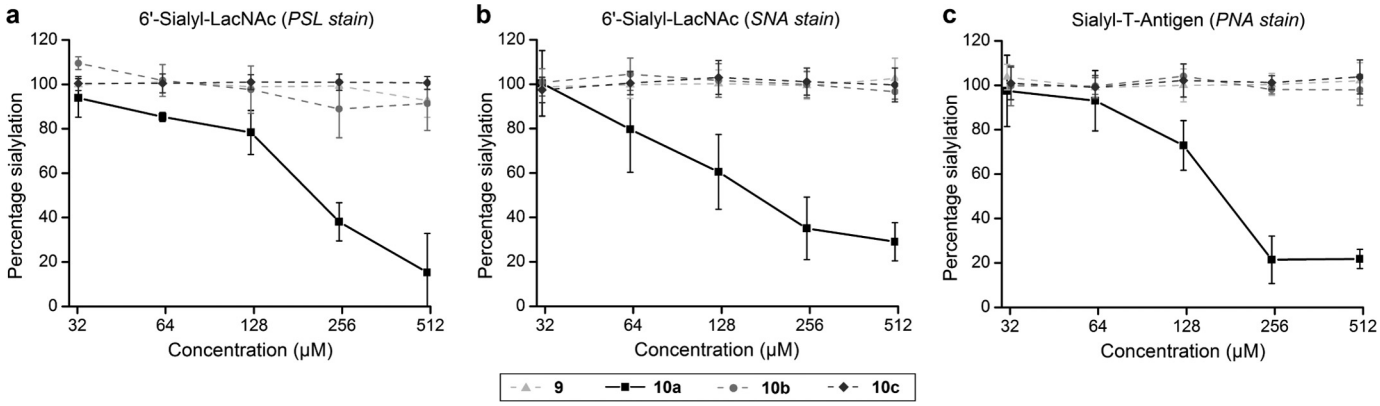

FIGURE 2. **Peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (10a) is an inhibitor of cell surface sialylation.** Jurkat cells were treated with varying concentrations of peracetylated ManNAc analogs for 72 h. Sialylated epitopes were analyzed with glycan specific lectins via flow cytometry (*a*) and in cell enzyme-linked lectin assay (*b* and *c*). Obtained data were normalized to untreated cells (100%) and cells treated for 1 h with 0.2 units/ml sialidase (0%). Data shown represent the mean values and S.D. of three independent experiments ($n = 3$). Only **10a** shows inhibition of cell surface sialylation. The peracetylated forms of methyl 2-acetylamino-2-deoxy-3-*O*-methyl-α-D-mannopyranoside (**9**), 2-acetylamino-2-deoxy-3-*O*-ethyl-D-mannose (**10b**), and 2-acetylamino-2-deoxy-3-*O*-propyl-D-mannose (**10c**) had no effects on cell surface sialylation. *PSL*, *P. squamosus* lectin; *SNA, S. nigra* agglutinin; *PNA, A. hypogaea* agglutinin.

completed by three washing steps with PBS + 0.5% BSA. Obtained data were normalized to untreated cells, and cells were treated for 1 h with 0.2 units/ml sialidase from *Clostridium perfringens* (Sigma Aldrich). All experiments were performed in triplicate.

In Cell Enzyme-linked Lectin Assay—Alkaline phosphatase-conjugated *Arachis hypogaea* agglutinin (2 μg/ml, EY Laboratories) and alkaline phosphatase-conjugated *Sambucus nigra* agglutinin (3 μg/ml, EY Laboratories) were used in an in cell enzyme-linked lectin assay. 200 μl of *p*-nitrophenyl phosphate solution (Sigma-Aldrich) was added to start the reaction. Alkaline phosphatase activity was recorded at a wavelength of 405 nm.

Flow Cytometry—Flow cytometry experiments were conducted using FITC-conjugated *Polyporus squamosus* lectin (0.1 μg/ml, a gift from Prof. H.-J. Gabius, Technische Universität München).

Cytotoxicity Assays

The cell proliferation assay AlamarBlue© (AbD Serotec) was used to determine the cytotoxicity of given peracetylated ManNAc analogs. Samples of 100-μl cell suspension in RPMI 1640 + 10% FBS and 2 mM L-glutamine (20,000 Jurkat cells per well), containing different concentrations of peracetylated ManNAc analogs, were prepared. Cells were cultured for 72 h. Then, 10 μl of AlamarBlue© solution was added. After 4 h of incubation, samples were analyzed at wavelengths of 570 and 620 nm. Experiments were performed in triplicate and normalized to untreated control cells.

Quantification of Cytosolic and Membrane-associated Sialic Acid

Concentrations of free, CMP-conjugated and membrane-bound sialic acid were measured by DMB HPLC (26). Jurkat cells, cultured 72 h in 5 ml of RPMI 1640 (10% FBS, 2 mM L-glutamine) containing 500 μM **10a**, were harvested and homogenized by sonication in ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, 5 mM EDTA, 1 mM PMSF, 40 μM leupeptin, 1.5 μM aprotinin, pH 8.0). Cytosolic fractions were separated by centrifugation at 21,000 × *g* and 4 °C for 2 h. Subsequently, a chloroform-methanol precipitation was performed to isolate the glycan moiety. The aqueous phase was filtered via a 3-kDa size exclusion filter to remove residual macromolecules. Half of the samples were subjected to sodium borohydride reduction (200 mM sodium borohydride in 200 mM sodium borate buffer, pH 8.0) for 12 h to reduce non-CMP-conjugated sialic acid.

All membrane and the cytosolic fractions were hydrolyzed with 1 M trifluoroacetic acid for 4 h at 80 °C. Hydrolyzed samples were dissolved in 2 M acetic acid. Subsequently, samples were labeled with 50 μl of ice-cold DMB solution (6.9 mM DMB, 0.67 mM β-mercaptoethanol, 0.19% sodium bisulfide).

Labeled samples were analyzed on a Dionex Ultimate 3000 HPLC System (Thermo Scientific) using a Gemini-NX C18 column (110 Å, 3 μM particle size, 4.6 mm × 150 mm, Phenomenex). Probes were separated at 1 ml/min flow rate with methanol/acetonitrile/water (6:8:86) as eluent. The detector was configured with 373 nm for excitation and 448 nm for emission.

Cloning and Protein Purification

Cloning and protein purification of the MNK were performed according to an established method (27). His-tagged MNK was expressed in BL21-CodonPlus (DE3)-RIL *Escherichia coli* (Stragene), followed by nickel-nitrilotriacetic acid affinity chromatography and gel filtration with a Superdex^{TM} HighLoad 16/600 column (GE Healthcare) (28). Human *N*-acetyl-glucosamine kinase was purified as described previously (29). GST-tagged *N*-acetyl-glucosamine kinase was expressed in *E. coli* followed by glutathione affinity chromatography, gel filtration, and GST tag cleavage by thrombin. Human hexokinase IV was purchased from Sigma-Aldrich.

Spectrophotometric Assays (Enzyme Activity, Kinetics, and Inhibition)

Enzyme activity, kinetics, and inhibition were assessed via a coupled optical assay using purified MNK. 120 μl of reaction mixture contained the following buffers and reagents: 66.5 μl of buffer A (65 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.1), 10.0 μl of ATP (varying concentrations), 7.5 μl of NADH (15 mM), 5.0 μl

of phosphoenolpyruvate (100 mM), 2 units of pyruvate-kinase, 2 units of lactate dehydrogenase, 10 μl of ManNAc of varying concentrations, and 10 μl of ManNAc analogs of varying concentrations. The reaction was started by adding 80 μl of MNK (1.25 μM) in buffer B (10 mM Tris-HCl, 150 mM NaCl, pH 8.0).

All spectrophotometric experiments were performed at 37 °C, and the results obtained were normalized to blanks, consisting of 120 μl of reaction mixture and 80 μl of buffer B without MNK. For hexokinase IV and *N*-acetyl-glucosamine kinase, the activity assay was adapted using glucose or *N*-acetyl-glucosamine as educts, respectively.

Dynamic Light Scattering Analysis—Dynamic light scattering experiments have been performed using a Laser Spector Scatter 201 (RiNA). Cuvettes were prepared with 30 μl of purified MNK (10 μM) in buffer B containing ManNAc or **8a** at concentrations of 5 mM. All measurements were performed at 21 °C. Results were compared with negative controls containing untreated MNK only and positive controls containing MNK incubated for 10 min at 45 °C to induce protein aggregation.

Surface Plasmon Resonance—Experiments were conducted using a Biacore T100 biosensor instrument (GE Healthcare) and a nitrilotriacetic acid sensor chip (GE Healthcare). The following buffers were used: (*a*) running buffer (10 mM HEPES, 150 mM NaCl, 0.05 mM EDTA, 0.005% Tween, pH 7.4) and (*b*) protein regeneration solution (10 mM taurodeoxycholic acid, 100 mM Tris-HCl, pH 9.0).

His-tagged MNK was covalently immobilized to a level of ~12,000 resonance units (1 resonance unit = 1 pg/mm^2). The immobilization was carried out according to an established procedure with slight modifications, using a Biacore amine coupling kit (GE Healthcare) (30). Prior to the amine coupling procedure, the nitrilotriacetic acid sensor chip was activated with 0.5 mM $NiCl_2$. After covalent attachment of the protein to the surface of the sensor chip, the coating procedure was completed by injecting 350 mM EDTA in running buffer. A flow cell without immobilized protein was used as reference.

All binding experiments were performed at 25 °C and a flow rate of 20 μl/min. Analytes of different concentrations were injected for 180 s followed by a 1000-s dissociation. In case of ManNAc and its analogs, regeneration was carried out by two injections of protein regeneration solution (10 μl/min, 60 s). With nucleosides 10 mM ManNAc in running buffer and 100 mM EDTA were injected (each 10 μl/min, 60 s) to assure regeneration. Each cycle was followed by a stabilization period of 1000 s. Binding data were evaluated with BIAevaluation (version 4.1, GE Healthcare), using 1:1 Langmuir binding model after reference cell and buffer injection subtraction.

RESULTS

Inhibition of Sialic Acid on the Cell Surface—**10a** decreases cell surface sialylation in a dose-dependent manner. Treatment of Jurkat cells for 72 h with **10a** led to a substantial reduction of sialic acid expression on the cell surface. The 6′-sialyl-LacNAc entity was reduced by nearly 80%, determined by *Polyporus squamosus* lectin binding (Figs. 2*a* and 3) and by ~70%, detected using *Sambucus nigra* agglutinin binding (Fig. 2*b*). The exposure of non-sialylated T antigen (Galβ1–3GalNAcα-Ser/Thr) was increased under treatment with **10a**, verified by *Arachis hypogaea* agglutinin binding, which equals a reduction of the sialylated epitope of ~80% (Fig. 2*c*). An IC_{50} for **10a** of 176 μM in medium was determined. Other tested ManNAc derivatives (**9**, **10b**–**c**) did not influence the sialic acid expression on the cell surface, even at final concentrations of 500 μM (Fig. 2).

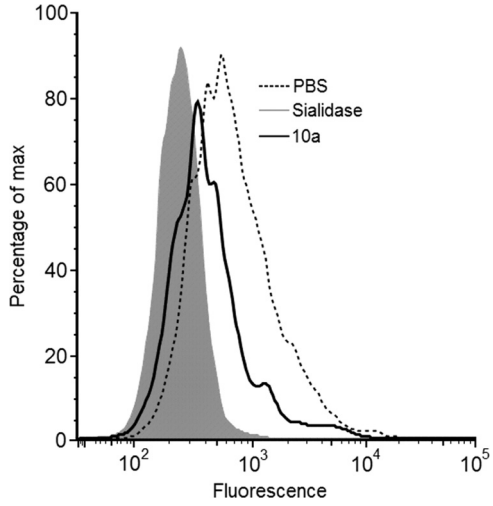

FIGURE 3. **Flow cytometry analysis of Jurkat cells with FITC-conjugated *P. squamosus* lectin.** Jurkat cells were treated for 72 h with peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**10a**) in a concentration of 125 μM. To evaluate their 6′-sialyl-LacNAc content, cells were labeled with *Polyporus squamosus* lectin (*PSL*). Results obtained were compared with untreated cells (PBS, *dashed line*) and cells treated for 1 h with 0.2 units/ml sialidase (*gray shading*).

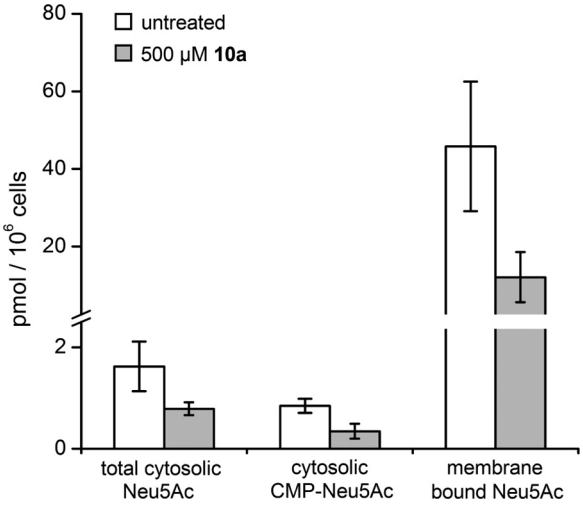

FIGURE 4. **Analysis of cytosolic and membrane bound sialic acid in Jurkat cell lysates.** Jurkat cells were treated for 72 h with 500 μM peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**10a**). Concentrations of Neu5Ac were measured by DMB-HPLC. CMP-activated Neu5Ac was determined after sodium borohydride reduction of the cytosolic fraction. Data shown represent the mean values and S.D. of three independent experiments ($n = 3$). **10a** inhibits the expression of cytosolic and membrane-bound Neu5Ac.

Quantification of Sialic Acid in Cell Lysates—Cell lysates of Jurkat cells treated for 72 h with 500 μM **10a** showed decreased concentrations of free, CMP-conjugated and membrane-bound *N*-acetyl-neuraminic acid (Neu5Ac, Fig. 4). Comparing obtained HPLC chromatograms of treated cells and control cells, no additional peaks appeared, showing that **10a** was not metabolized into a modified sialic acid (Fig. 5).

Cytotoxicity of Peracetylated ManNAc analogs—The effect of tested peracetylated ManNAc analogs on cell viability was neg-

ligible under given conditions. The proliferation rate of Jurkat cells after 72 h of treatment with the peracetylated sugars at final concentrations of 1 mM was not significantly compromised. It is known that other peracetylated ManNAc derivatives such as peracetylated *N*-propionylmannosamine show comparable low cytotoxicity (31).

Inhibition of MNK Enzyme Activity—In spectrophotometric assays with purified MNK, the K_m values of the enzyme have been measured to be 54 μM for ManNAc (specific activity, 0.77 units/mg) and 782 μM for ATP (specific activity, 0.70 units/mg), respectively.

8a inhibits purified MNK in a dose-dependent manner with an IC_{50} value of 1.29 mM (Fig. 6*a*). Other ManNAc derivatives tested (**5**, **8b**–**c**) revealed no inhibition of enzyme activity, even at maximum concentrations of 10 mM. Dynamic light scattering experiments of purified MNK with **8a** demonstrated that the enzyme inhibition of this substance is not caused by unspecific protein aggregation (Fig. 7).

To evaluate the selectivity of **8a**, its inhibition was assessed for human *N*-acetyl-glucosamine kinase and hexokinase IV (29). For these two related sugar kinases, inhibition of enzyme activity could be observed only at significantly higher concentrations ($IC_{50} > 10$ mM, data not shown).

Linear curve approximation, using the Lineweaver-Burk plot for MNK activity, shows that **8a** is non-competitive for ManNAc, but competitive for ATP (Fig. 6, *b* and *c*). The K_i value of **8a** for the interaction with ATP is 649 μM. Spectrophotometric assays revealed that **5** and **8a**–**c** were not metabolized by the following enzymes: MNK, *N*-acetyl-glucosamine kinase, and hexokinase IV.

Binding Affinity to MNK—The binding affinity between **8a** and MNK was determined using surface plasmon resonance. The immobilization of MNK to a level of 12,000 resonance units resulted in expected maximal response of ~120 resonance units after injection of **8a** (Fig. 8*a* and Table 1). Assuming a 1:1 interaction, a dissociation constant (K_D) of 755 μM was calculated for the interaction between MNK and **8a**. Under the given experimental conditions, no binding of ManNAc to MNK could be detected. ATP and ADP bound MNK with K_D values of 429 and 306 μM, respectively (Fig. 8*b* and Table 1). The slightly lower K_D value of ADP is due to its higher off rate (k_d). UDP and **5**, which were used as controls, did not bind to MNK.

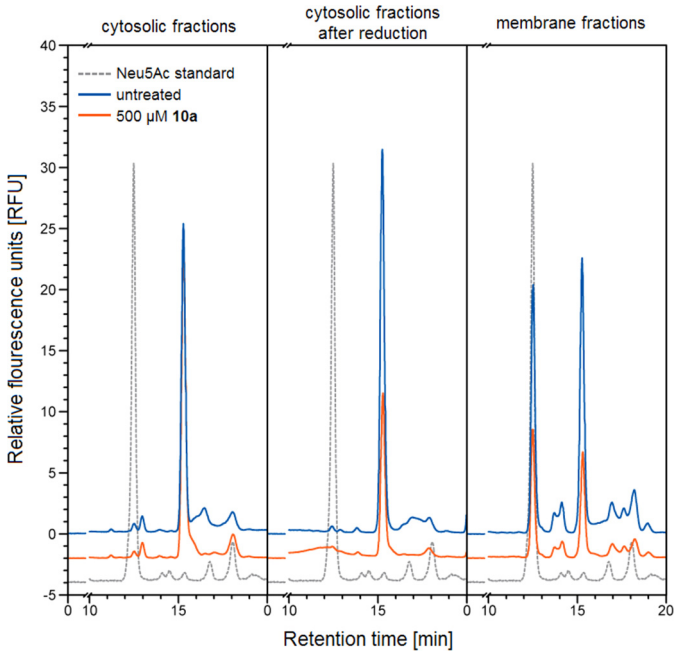

FIGURE 5. **Representative chromatograms of DMB-labeled sialic acid residues from Jurkat cell lysates.** Jurkat cells were treated for 72 h with 500 μM peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**10a**). Contents of CMP-activated Neu5Ac were determined after sodium borohydride reduction of the cytosolic fraction. Chromatograms of treated cells (*orange*) and untreated cells (*blue*) are plotted against a standard of 14 ng of Neu5Ac. Comparing treated and untreated cells no additional fluorescence peak appeared, indicating that **10a** is not metabolized into a modified sialic acid.

DISCUSSION

Cell surface sialic acid expression is involved in key processes between cells and their environment. Thus, having simple tools

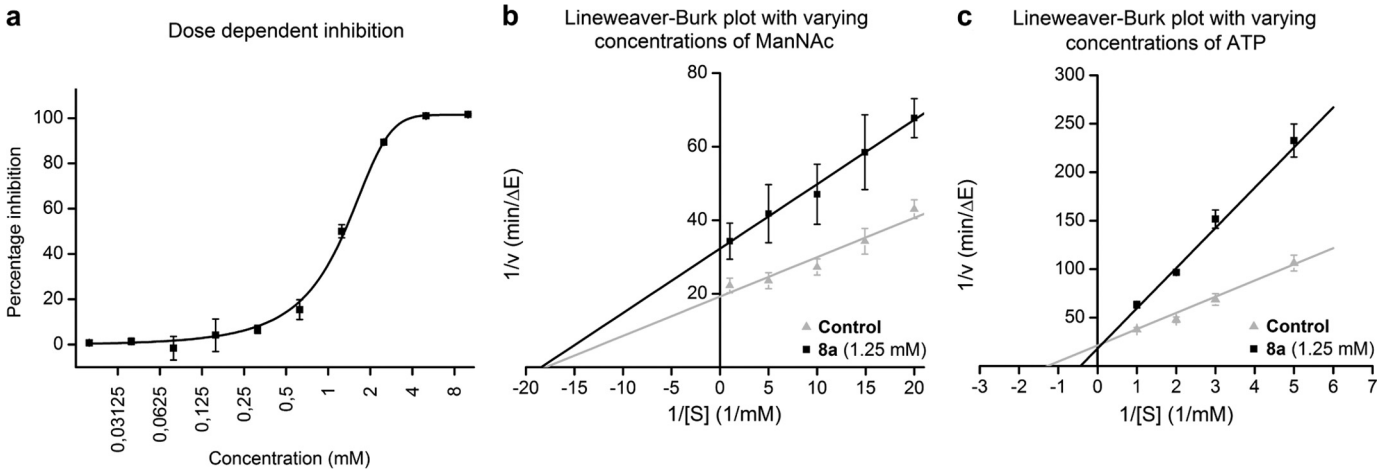

FIGURE 6. **2-Acetylamino-2-deoxy-3-*O*-methyl-D-mannose (8a) inhibits purified MNK.** A coupled optical assay using enzymatic consumption of NADH was performed to show the effect of **8a** on MNK activity. In *a*, purified MNK was incubated with ManNAc (125 μM), ATP (2 mM), and different concentrations of **8a**. Obtained activity data were normalized to controls without inhibitor (0%). In *b* and *c*, MNK was tested with **8a** (1.25 mM) and varying concentrations of ManNAc (*b*) or ATP (*c*), respectively. Measured data were compared with controls without inhibitor (*gray triangles*). Data shown represent the mean values and S.D. obtained in three independent experiments ($n = 3$) for *a* and *b* and five independent experiments ($n = 5$) in *c*. Linear (*b* and *c*) and sigmoidal dose response (*a*) curve approximations were performed to determine K_m, K_i, and IC_{50} values.

to decrease cell surface sialylation will greatly benefit research in finding roles of this sugar in biological systems and diseases. Although traditional methods are available to decrease sialic acid expression on the cell surface, *e.g.* sialyltransferase inhibition or UDP-GlcNAc-2-epimerase/ManNAc kinase-deficient cells (10, 32–34), a systemic inhibitor for the *de novo* biosynthesis of sialic acid is still not available.

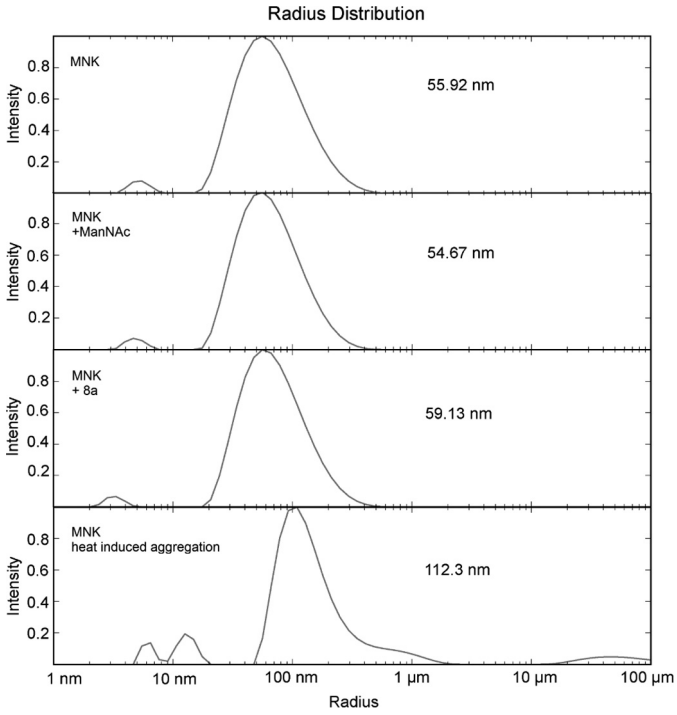

FIGURE 7. **Density light-scattering experiments.** Cuvettes were prepared with purified MNK (10 μM) and ManNAc or 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**8a**) at concentrations of 5 mM. All measurements have been performed at 21 °C. As a positive control, MNK was preincubated for 10 min at 45 °C to induce protein aggregation. The radius of the highest peak for each graph is shown. Data depicted represent the mean values obtained in three independent experiments ($n = 3$). **8a** does not cause any protein aggregation under given experimental conditions.

Within this study, we presented a small molecule inhibitor of sialic acid expression, namely 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**8a**). Applying its peracetylated form (**10a**), cell surface sialic acid concentration in Jurkat cells was decreased in a dose-dependent manner (Fig. 2). At final concentrations of 500 μM in culture medium, the sialic acid concentration was reduced significantly to ~20%. Total inhibition of cell surface sialylation could not be observed, most likely due to uptake and reutilization of sialic acid from the serum supplement (35). In addition, a slow turnover of sialylated glycoproteins could also contribute to this finding (36). The IC_{50} of the peracetylated analog (**10a**) is 176 μM. With a molecular weight of 235 g/mol, **8a** has the potential for further chemical modification to improve binding affinity.

Performing DMB high-performance liquid chromatography on Jurkat cells treated with **10a** revealed that this ManNAc analog causes a substantial reduction of Neu5Ac both on the cell surface and in the cytosol. Cytosolic concentrations of free and CMP-conjugated Neu5Ac were decreased. **10a** was not metabolized into a modified sialic acid in cells (Fig. 5). These results indicate that **10a** inhibits sialic acid expression at the beginning of the *de novo* biosynthesis.

Other tested C-3-modified ManNAc analogs (**9**, **10b–c**) did not alter cell surface sialylation in treated cells. The low cytotoxicity of **10a** underlines the fact that inhibition of sialic acid biosynthesis does not cause cell viability defects (10, 32).

We suggest that the reduction of sialic acid expression achieved by **10a** is a consequence of MNK inhibition. The enzyme activity of MNK was inhibited by **8a**. The IC_{50} for the interaction is 1.29 mM. Dynamic light scattering experiments verified that the inhibition of **8a** is not due to protein aggregation. Related substances **5**, **8b,** and **8c**, which in their peracetylated forms (**9**, **10b–c**) did not alter cell surface sialylation, also showed no inhibitory effects on the MNK.

The mode of inhibition for **8a** is non-competitive for ManNAc, but competitive for ATP (K_i, 649 μM). Apparently, this

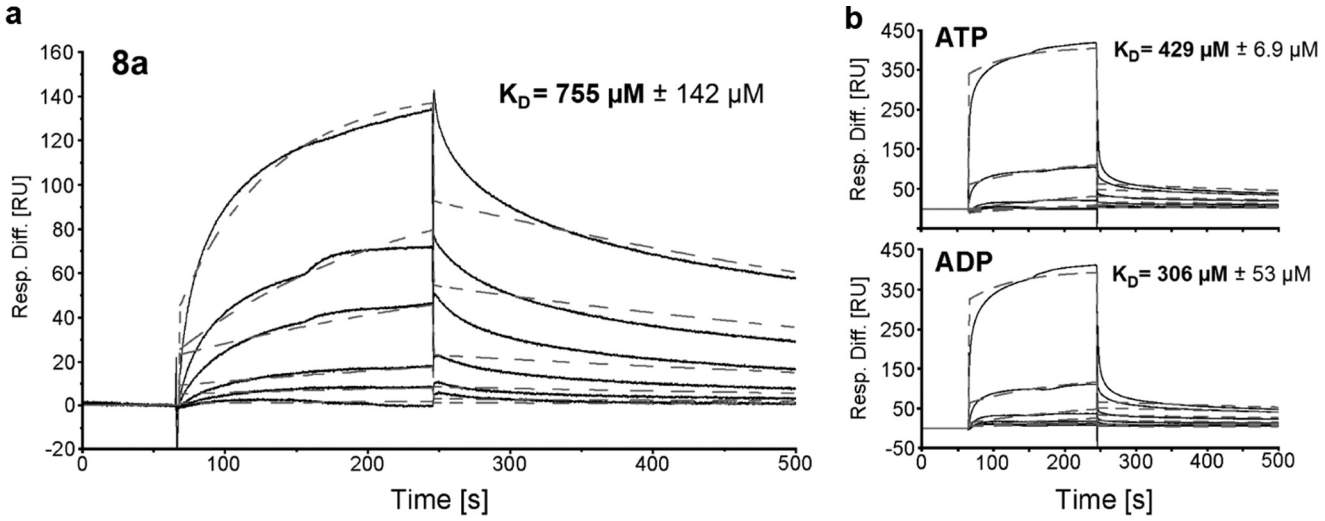

FIGURE 8. **2-Acetylamino-2-deoxy-3-*O*-methyl-D-mannose (8a), ATP, and ADP binding to MNK.** In *a*, a representative sensorgram of **8a** binding to MNK is depicted. The analyte was injected in a 3-fold dilution series starting from 5 mM. Representative sensorgrams of ATP and ADP binding to MNK are shown in *b*. The analytes were injected in a 2-fold dilution series starting from 6.25 mM. Obtained data (*solid lines*) was fitted using a 1:1 Langmuir binding model (*dashed lines*) and was calculated. Depicted K_D values represent means and S.D. of three independent experiments ($n = 3$). *Resp. Diff.,* response difference; *RU,* response units.

TABLE 1

Comparison of rate constants for 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (8a) binding to covalently immobilized, His-tagged MNK

SPR-binding sensorgrams were fitted using a 1:1 Langmuir binding model. Data was obtained in three independent experiments ($n = 3$) at a temperature of 25 °C. For ManNAc and methyl 2-acetylamino-2-deoxy-3-*O*-methyl-α-D-mannopyranoside (**5**), no binding could have been observed under given experimental conditions (NA, data not available). k_a, on rate; k_d, off rate; K_D, dissociation constant.

	ManNAc	8a	5	ATP	ADP	UDP
k_a (M^{-1} s^{-1})	NA	2.92	NA	2.48	2.76	NA
k_d (s^{-1})	NA	2.25×10^{-3}	NA	1.06×10^{-3}	8.40×10^{-3}	NA
K_D (μM)	**NA**	**755**	**NA**	**429**	**306**	**NA**
X^2	NA	7.65	NA	19.20	18.87	NA

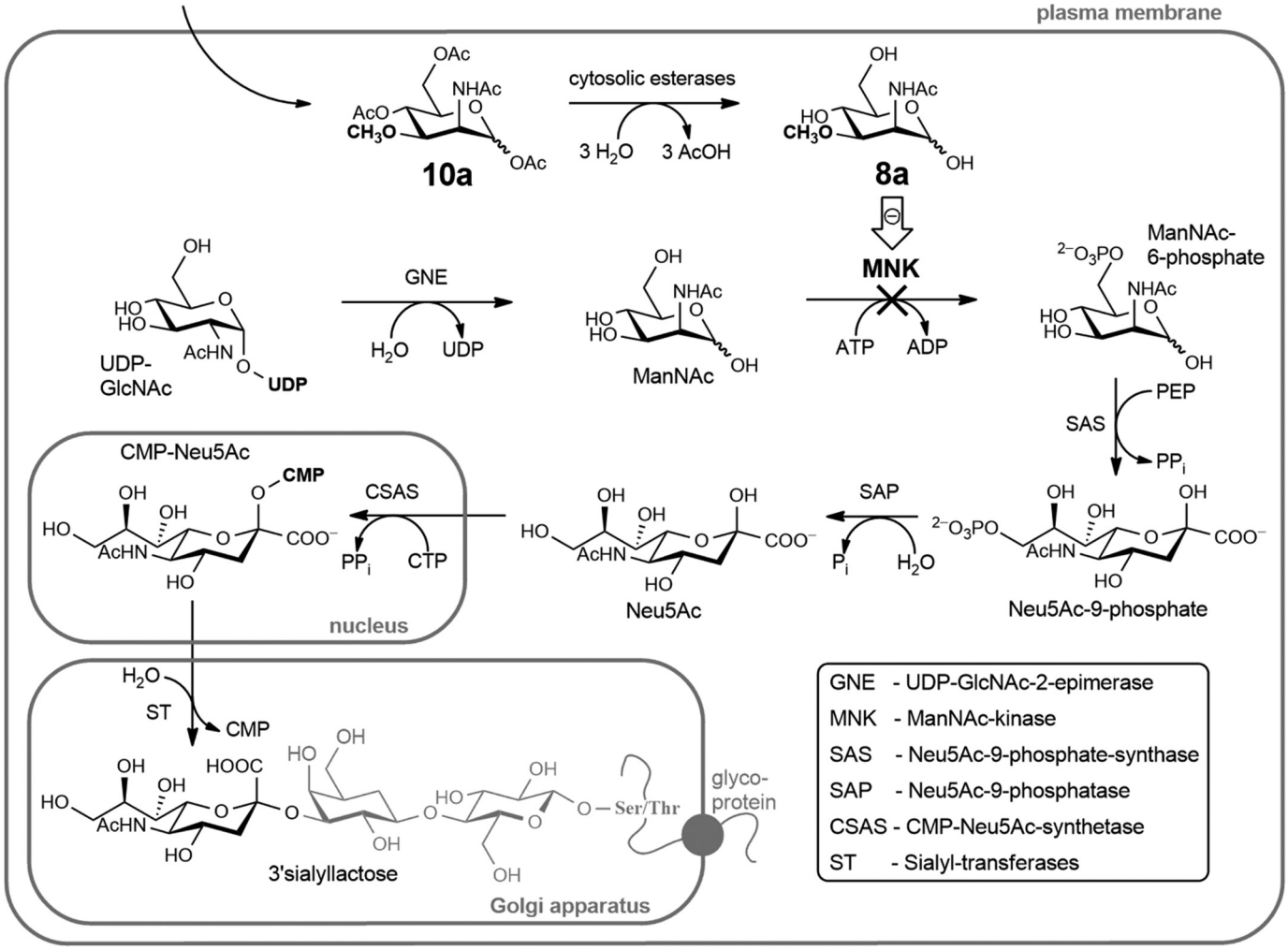

FIGURE 9. **Proposed mechanism of inhibition for peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (10a). 10a** is taken up by cells and converted to the corresponding non-peracetylated sugar (**8a**) by unspecific esterases. **8a** inhibits the kinase domain of the bifunctional UDP-GlcNAc-2-epimerase/ManNAc kinase (*GNE/MNK*). Inhibition of the MNK lowers the *de novo* biosynthesis of sialic acid. The depicted glycan (3′-sialyllactose) is representative for sialylated glycans in general.

substance has its binding pocket in the ATP-binding site of MNK.

Surface plasmon resonance confirms binding of **8a** to MNK with a K_D of 755 μM. While covalently immobilized to the nitrilotriacetic acid sensor chip, MNK does not bind ManNAc. This may be caused by conformational changes at the ManNAc binding site due to the immobilization procedure, or because of the technical set-up of the experiment. The selective binding of ADP, ATP and **8a** but not ManNAc also indicates that the inhibitor has the same binding site as ADP/ATP. The fact that **5** as well as UDP did not bind to MNK shows that the ATP-binding pocket of the enzyme was still intact.

The selectivity of **8a** for MNK was affirmed by evaluating its inhibitory capacity on other pyranose kinase enzymes (*N*-acetyl-glucosamine kinase, hexokinase IV). **8a** shows significantly weaker inhibitory effects on these enzymes ($IC_{50} > 10$ mM). MNK, *N*-acetyl-glucosamine kinase, and hexokinase IV did not metabolize any of the tested ManNAc analogs (**5** and **8a–c**).

Based on our results, we conclude that **10a** decreases sialic acid expression in cells by inhibiting cytosolic MNK. Being the key enzyme of sialic acid biosynthesis, its inhibition ultimately leads to the observed reduction of cell surface sialylation (10). The proposed model of sialic acid reduction in cells caused by **10a** is depicted in Fig. 9.

Peracetylated 2-acetylamino-2-deoxy-3-*O*-methyl-D-mannose (**10a**) is the first membrane permeable inhibitor of the UDP-GlcNAc-2-epimerase/ManNAc kinase described. With an inhibitor of the *de novo* biosynthesis of sialic acid, access to biological effects of global cell surface sialic acid reduction is facilitated. It paves the way for deeper understanding on the role of sialic acid in health and disease.

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