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Can PDT Alter the Glycosylation of the Tumor Cell Membrane?

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Abstract

Photodynamic Therapy (PDT) is a cancer treatment that used the interaction of a photosensitizing drug and a light source. PDT can lead to changes in the expression of various cellular elements, compromising cell adhesion, and cytoskeleton integrity in cells undergoing treatment. However, the pathways of cellular alterations caused by this treatment are little known. Alterations in expression in surface glycoproteins and glycolipids are significant features in malignant tumor transformation and are strongly associated with tumor cell adhesion, invasion, and metastasis. This study evaluated photodynamic therapy effects on indirect distribution surface glycoproteins in human laryngeal carcinoma HEP-2 cell line surface, using Click-iT™ Metabolic Glycoprotein Labeling Reagent. Aluminum Phthalocyanine Tetrasulfonate (ALPcS4) was administrated at 5 $\mu\text{M}/\text{mL}$, followed by one hour of the incubation period for its accumulation in the tumor cells. After this time, cultures were irradiated with LED (light-emitting diode) dispositive (BioPdi/IRRAD-LED) $\lambda = 660 \text{ nm}$. Evaluation of glycoproteins was performed by flow cytometry. Knowledge of the cellular alterations caused by the treatment will allow obtaining tools for the potentiation or optimization and personalization of the anticancer treatment. This therapy has a low cost and better efficacy, when applied early, about radiotherapy chemotherapy.

Keywords: glycosylation, flow cytometry, cell culture, aluminum phthalocyanine tetrasulfonate

1. Introduction

Photodynamic therapy (PDT) is an oncology treatment based on photochemical reactions involved photosensitizer (PS) and light irradiation of an appropriate wavelength. The interaction of PS and light, producing reactive oxygen species (ROS), can directly induce cellular damage to organelles and cell membranes. PDT's action on cellular organelles, mainly in the endoplasmic reticulum (ER) and Golgi, interferes with several metabolic pathways [1]. The glycosylation process is a post-translational modification that occurs in the ER [2]. It results in the addition of carbohydrate motifs—glycans to proteins that are, in most cases, destined for the cell surface. The resultant glycoprotein structures at the cell surface form a carbohydrate-rich layer, which presents an essential role in the cell's interaction with its surrounding environment. Glycosylation of a given protein is a process catalyzed by glycosyltransferases, localized in the Golgi, leading to the formation of

protein-bound glycans with specific and diverse biological functions [3]. These carbohydrate side chains can modulate the protein's interaction with its environment, influencing key factors such as protein half-life, solubility, binding activity, and specificity. Two significant types of glycosylation occur on proteins. (1) O-linked glycosylation refers to the addition of N-acetyl-galactosamine to serine or threonine residues by the enzyme UDP-N-acetyl-D-galactosamine transferase, followed by the addition of other carbohydrates, such as galactose, N-acetyl-D-glucosamine, or sialic acid; (2) N-linked glycosylation occurs in the ER [4, 5]. It refers to the insertion of an oligosaccharide chain enzymatically attached to the amide group of asparagine in the consensus sequence Asn-X-Ser/Thr (where X represents any residue except proline). Alterations in glycosylation of malignant cells can take a variety of forms, including changes in the amount, linkage, and acetylation of sialic acids; changes in the branching of N-glycans mediated by glycosyltransferases; alterations in the expression of glycosaminoglycans such as heparan sulfate; and altered glycosylation of mucins, which are heavily glycosylated epithelial-derived proteins known to be implicated in certain cancers [5, 6].

Changes in glycoprotein glycans are significant in malignant tumor transformation and are closely associated with tumor cell adhesion, invasion, and metastasis [7]. Christiansen et al., 2014 [8] demonstrated changes in cell surface glycosylation in five different types of cancer: breast, colon, liver, skin (melanoma), and ovarian, and how these changes may be associated with carcinogenesis. Synthesis and expression of cell-surface carbohydrates is a highly regulated process that affects several cell-cell interactions. The presence of specific oligosaccharides in highly malignant cells is essential for expressing the metastatic phenotype [9]. Elevation of glycoproteins above normal levels reflects local tissue destruction processes with the release of preformed tissue proteins or becomes a local synthesis and releases glycoproteins through tumor cells [10].

Waiting for glycans and glycoconjugates to play a relevant role in various tumor progression stages, biosynthetic cell machinery involved in glycan biosynthesis and modification is a promising target for cancer treatment. Treatments that act on such targets should be researched to act or stimulate specific glycosylation inhibitors that may target a pro-metastatic biological product or interfere with the immune response's modulation.

2. Methods

2.1 Cell culture

The HEp-2 cell line (human laryngeal carcinoma) and MCF7 cell line (human mammary carcinoma) were obtained from the Bank of Cells of Rio de Janeiro (BCRJ). Cells were cultured in 25 cm² culture flask (Greiner), routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Gibco-Life-Technology), at 37°C, an atmosphere containing 5% CO₂ (Forma Scientific CO₂, MODEL 3110).

2.2 Photosensitizer

Aluminum phthalocyanine tetrasulfonate (AlPcS4-Frontier Scientific) at a 5 µM/mL concentration is diluted in phosphate-buffered saline (PBS).

Experimental groups HEp-2 cells were divided into four groups: control, a photosensitizer (cells incubated with AlPcS4), laser (irradiated cells only), and photodynamic therapy (PDT) (cells incubated with AlPcS4 and irradiated).

2.3 Photodynamic therapy

Cells were cultivated in 6-well plates at a density of 1×10^6 cells/well, at 37°C in a 5% CO₂ atmosphere, and incubated for 18 hours for cell adhesion. After plating, cells were incubated with AlPcS4 for 1 hour at 37°C in an atmosphere containing 5% CO₂. Then, they were washed with PBS to remove the photosensitizer not absorbed by the cells. Irradiation was performed by using a LED dispositive (Biopdi/IRRAD-LED) $\lambda = 660$ nm. Each well was exposed to 25 mW, an energy density of 5 J/cm². Immediately after treatment, all groups were incubated with 25 μ M Click-iT™ Metabolic Glycoprotein Labeling (according to the manufacturer's instructions, Thermo Fisher Scientific™- **Table 1**) for 24 and 48 hours to evaluate the changes in the protein glycosylation process by the Golgi complex (**Figure 1**). At the end of the incubation periods, the cells were scraped, added into 5 ml tubes, and centrifuged at 5259 g at 4°C for 5 minutes for cell sedimentation. After this, cells were resuspended and washed in PBS 2 times, fixed with 4% paraformaldehyde in PBS for 15 minutes, washed two more times with PBS, and permeabilized with 0.25% Triton x-100 in PBS for 15 minutes, washed with 3% BSA in PBS twice, and then incubated with an FTIC-conjugated antibody for one hour while diluted 1: 1000 in a Click-iT reaction buffer for one hour. At the end of the incubation period, it was washed

Markers	Target	Incubation time
Click-iT® GalNAz	O-linked glycans	24–72 hours
Click-iT® ManNAz	Sialic acid-modified glycoproteins	24–72 hours
Click-iT® GlcNAz	O-GlcNAz-modified glycoproteins	24–72 hours

Table 1.
The markers used to evaluate glycoproteins.

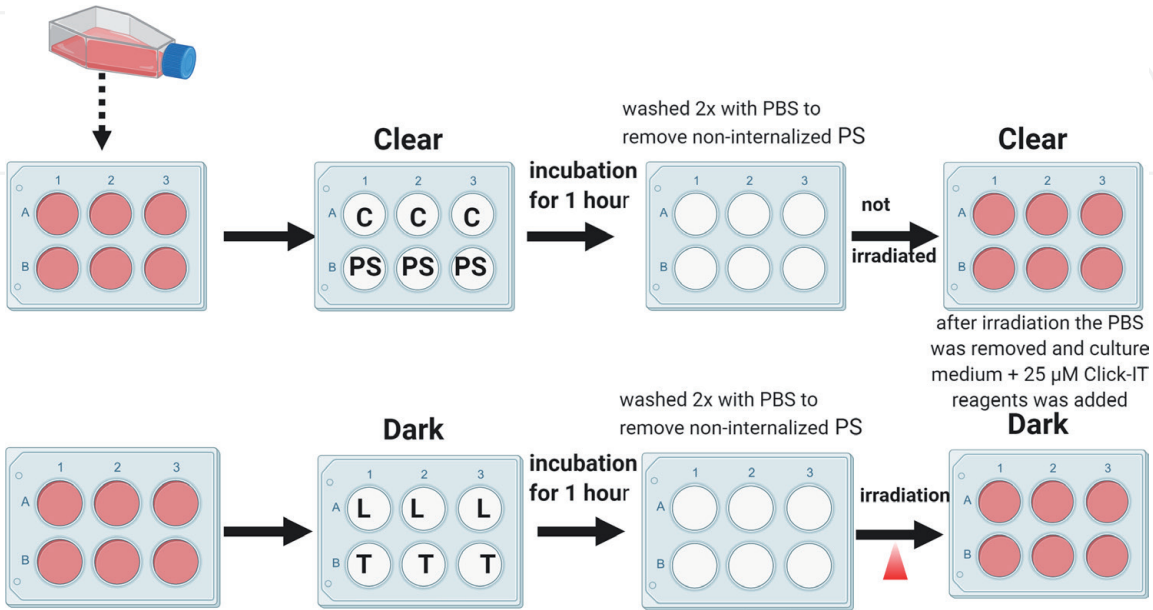


Figure 1.
Scheme of the experimental procedure. C – Control; PS – Photosensitizer; L – Led only; T – Treatment = PS + Led. Created with BioRender.com.

with PBS, resuspended in the Click-iT reaction buffer, and read by BD AccuriC6 Plus flow cytometer. All experiments were performed in duplicate (n = 6).

2.4 Statistical analysis

The data presented are in the form of mean and standard deviation, compared by the two-way ANOVA test and confirmed by the Tukey test. Statistical significance was admitted with $P < 0.05$ with * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ being considered significant. Experiments were performed in three independent replications with n = 8. GraphPad Prism 6® software was used to per-form statistical and graphical analyses.

3. Results

The evaluation of the glycoprotein synthesis by Flow Cytometry demonstrates that the modified sialic acid glycoproteins (Click-iT® ManNAz - **Figure 2**) in the treatment group present a higher fluorescence intensity in 24 hours, concerning the O-GlcNAz-modified glycoproteins and the O- glycans linked. The HEp-2 strain presents the synthesis of sialic acid-modified glycoproteins and O-GlcNAz-modified effectively in the first 24 hours; after 48 hours, a decrease in the synthesis of these glycoproteins is observed (**Figures 3 and 4**). They were probably modified due to the action of glycosidases and glycosyltransferases, changing their structures. O-linked glycans are less fluorescent in the first 24 hours; however, in the group treated within 48 hours, an increase in the synthesis of these glycoproteins is observed, which can be considered a possible target for photodynamic treatment.

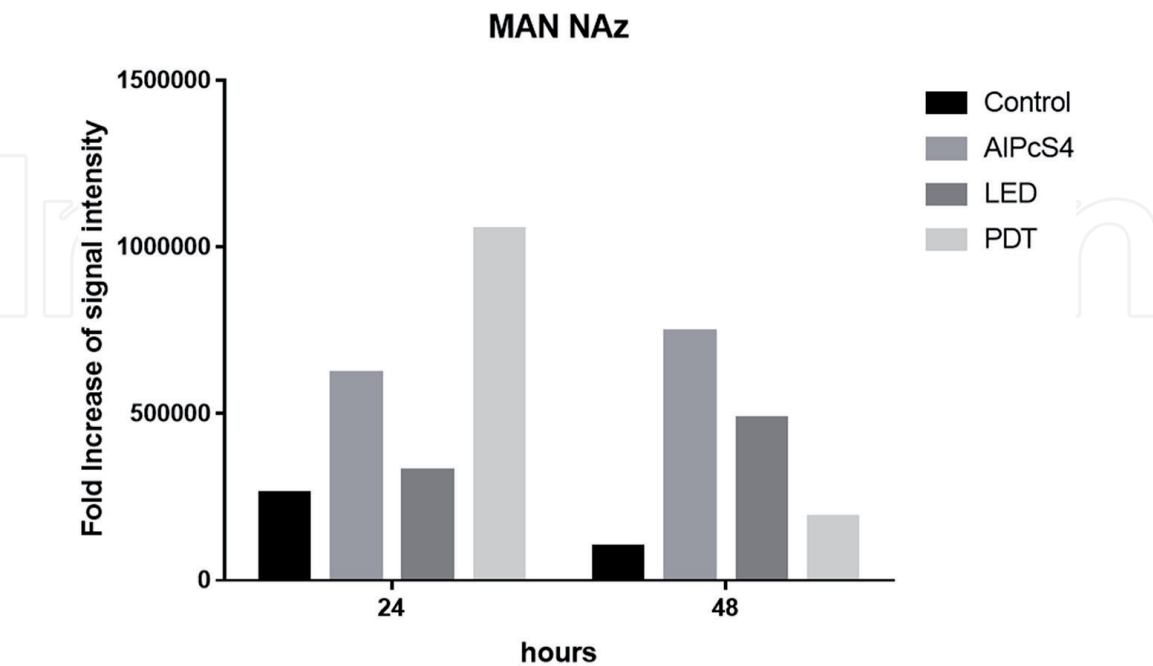


Figure 2. Analysis of cells treated with Click-iT®-ManNAz, the graph shows the fluorescence intensity of the cells of the control, LED, and treatment groups in the periods of 24 and 48 hours. The treatment group showed a high fluorescence intensity within 24 hours, with a severe fluorescence reduction occurring in all groups within 48 hours.

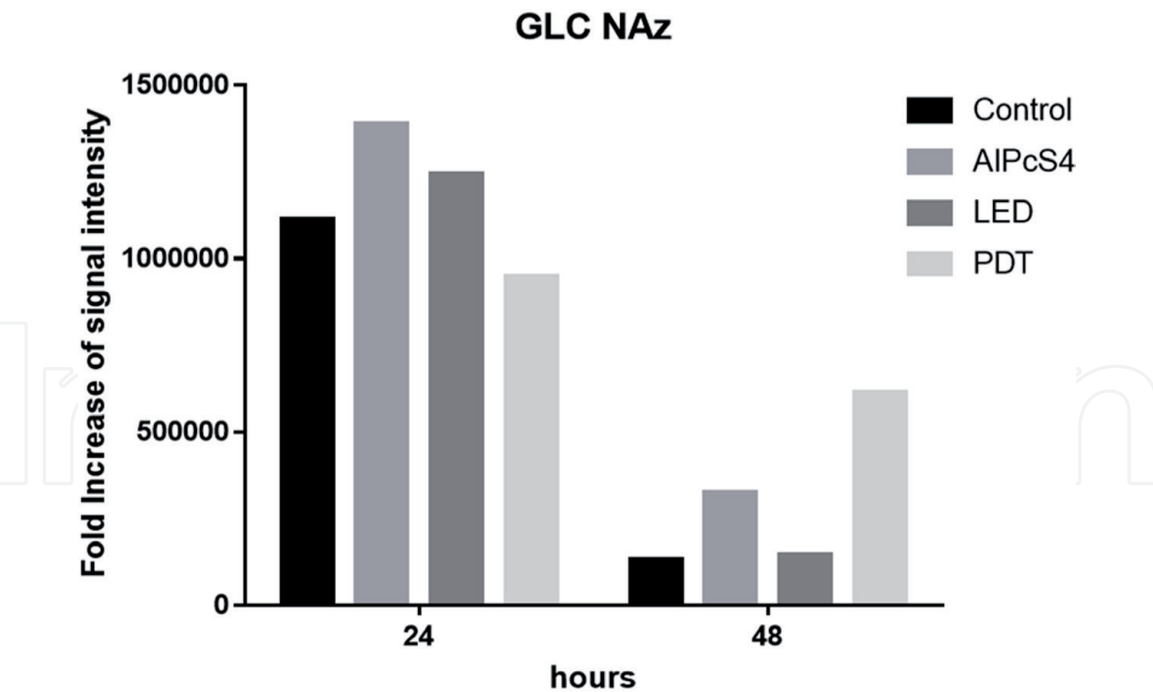


Figure 3.
Analysis of cells treated with Click-iT®-GlcNAz, the graph shows the fluorescence intensity of the cells of the control, led, and treatment groups in the periods of 24 and 48 hours. The fluorescence intensity in all groups in the 24 hours is the same for all. Within 48 hours it is possible to observe an increase in fluorescence intensity in the treatment group compared the control and LED groups.

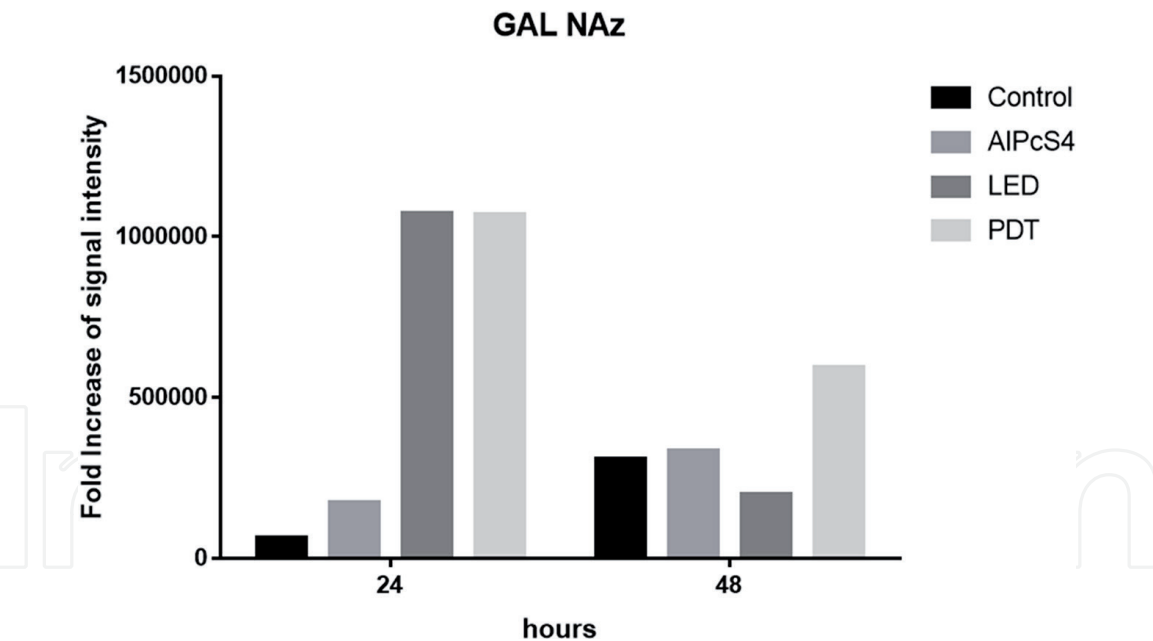


Figure 4.
Analysis of cells treated with Click-iT®-GalNAz, the graph shows the fluorescence intensity of the cells of the control, LED, and treatment groups in the periods of 24 and 48 hours. The treatment group, compared to the other groups, has a high fluorescence intensity.

4. Discussion

The use of the technique called metabolic oligosaccharide engineering (metabolic oligosaccharide engineering) allows for the labeling of glycans with probes for visualization in cells by enriching specific types of glycoconjugates for proteomic analysis. This methodology promotes the metabolic labeling of glycans with a specific reactive functional group, the azide. Azide-labeled carbohydrates are endocytosed

by cells and integrated with glycan biosynthesis in various glycoconjugates. The cells are incubated for periods of 24 to 72 hours to allow the synthesis of surface glycoproteins to be monitored [11, 12].

According to prior research [13, 14], glycosylation markers can assist in cancer detection and monitoring since the malignant transformation of cancer cells associated with changes in cell glycosylation are associated with tumor progression and, finally, metastasis. The schemes shown above demonstrate the biosynthesis of glycoproteins in a normal cell (**Figure 5**). The results obtained demonstrate indirectly that the photodynamic treatment altered the glycosylation of proteins in the lattice, consequently compromising the glycosylation in the Golgi and the insertion of glycoproteins in the plasma membrane (**Figure 6**). The statement concerning the reticule is based on previous data obtained by our group, which demonstrated changes in the reticular tubular network and the presence of surface glycoproteins N-acetyl glucosamine terminals [15, 16].

The glycoproteins, when sent via vesicle traffic to the Golgi complex, change with the removal of mannose residues, the addition of N-acetyl glucosamine, galactose, and sialic acid. The addition of carbohydrates is associated with the function that the glycoprotein will play on the cell surface.

The glycosylation markers can be used for cancer detection and monitoring, since changes in cell glycosylation are associated with the transformation of cancer cells into glycosylation, tumor progression, and, finally, metastasis [13, 14]. The schemes shown above demonstrate the biosynthesis of glycoproteins in a normal cell (**Figures 7 and 8**). The results obtained indirectly demonstrate that the photodynamic treatment altered the glycosylation of proteins in the lattice, consequently compromising the glycosylation in the Golgi and the insertion of glycoproteins in the plasma membrane. The reticule statement is based on previous data obtained by our group [15], which demonstrated changes in the reticular tubular network.

The photodynamic treatment action on surface glycans has a significant impact on cell signaling and the regulation of cell-tumor cell adhesion and cell-matrix

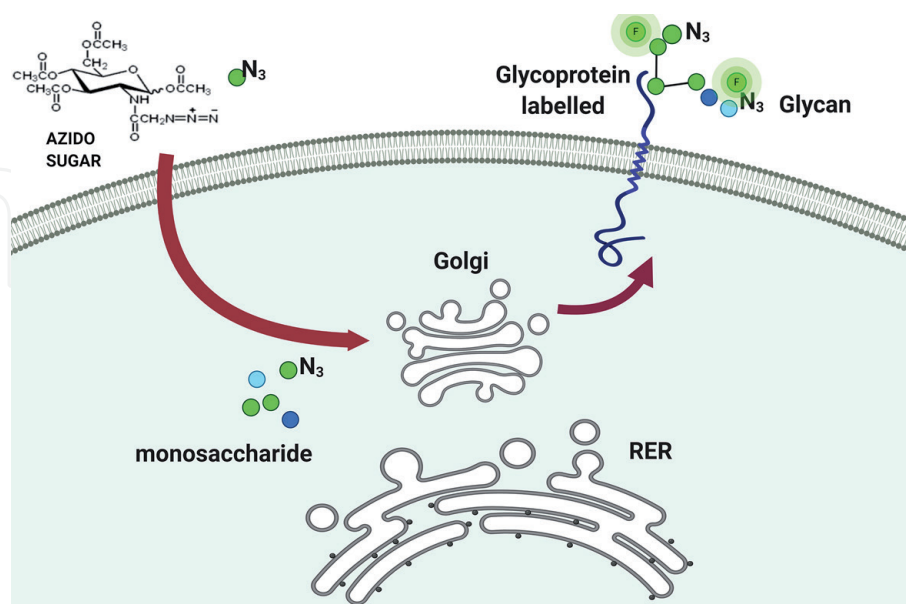


Figure 5. Membrane glycoprotein biosynthesis scheme. The monosaccharide complexed with azide (Click-iT™ Metabolic Glycoprotein Labeling Reagent), crosses the plasma membrane, becoming available in the cytoplasm. Golgi, responsible for the glycosylation process, captures the monosaccharide, which will be used in the processing of membrane protein, coming from the rough endoplasmic reticulum. After the incorporation of the labeled monosaccharide into the protein, the vesicle is released and fused to the plasma membrane, exposing the glycoprotein, allowing its detection by microscopy or cytometry. Created with BioRender.com.

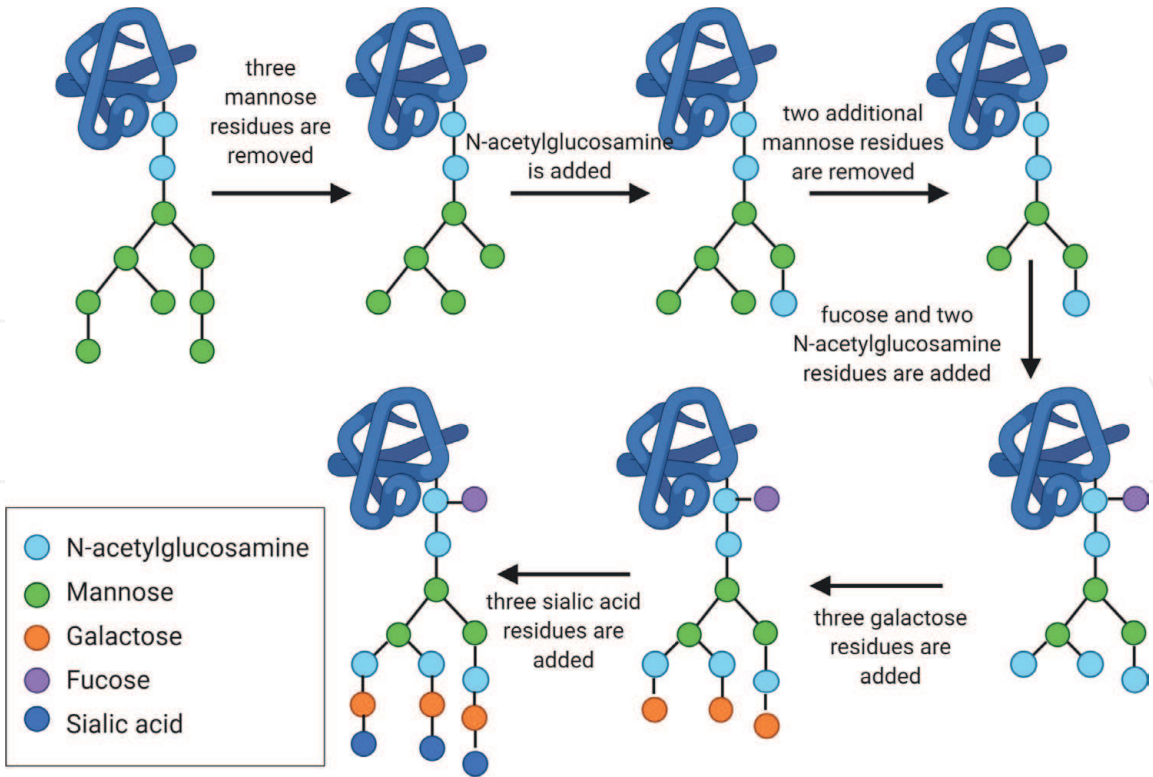


Figure 8.
Glycosylation scheme in the Golgi apparatus. Created with BioRender.com.

the action of light, the cell restores its synthesis process. Reinforcing the information that light and separate photosensitizers cannot cause damage to cells.

5. Conclusion

The action of photodynamic treatment on surface glycans has a significant impact on cell signaling and the regulation of cell-tumor, cell-adhesion, and cell-matrix interactions, compromising the interaction between cancer cells and the tumor microenvironment. The involvement of the glycosylation process in the Golgi apparatus prejudice the survival of tumor cells and can be exploited to develop strategies for immune system activation.

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Conflict of interest

The authors declare no conflict of interest.

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