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Alterations in Glycopeptides Associated with Herceptin Treatment of Human Breast Carcinoma MCF-7 and T-Lymphoblastoid Cells*

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The therapeutic humanized monoclonal antibody IgG1 known as Herceptin® has shown remarkable antitumor effects. Although this type of therapy has increased the cancer-free survival of patients, not all tumors respond to this treatment and cancers often develop resistance to the antibody. Despite the fact that Herceptin function has been extensively studied, the precise mechanism underlying its antitumor activity still remains incompletely defined. We previously demonstrated on human breast MCF-7 carcinoma and T-lymphoblastoid CEM cells that monoclonal antibody in combination with Lipoplex consisting of Lipofectamine mixed with plasmid DNA showed a more profound effect on cancer cell viability than antibody alone. The analyses of N-glycans isolated from cancer cells showed dramatic differences in profiles when cells were exposed to Herceptin. Moreover, the investigation of glycosylated peptides from the same cancer cell models after treatment revealed further alterations in the post-translational modifications. Tandem mass spectra obtained from the samples treated confirmed the presence of a series of glycopeptides bearing characteristic oligosaccharides as described in IgG1. However some of them differed by mass differences that corresponded to peptide backbones not described previously and more of them were detected from Herceptin treated samples than from cells transfected with Heceptin/Lipoplex. The results indicate that the presence of Lipoplex prevents antibody transformation and elongates its proper function. The better understanding of the multipart changes described in the glycoconjugates could provide new insights into the mechanism by which antibody induces regression in cancers.

Glycosylation of proteins is a ubiquitous type of post-translational modification in living systems. Variations in oligosaccharide structures are associated with many normal and pathological events such as cellular growth, host-pathogen interaction, differentiation, migration, cell trafficking, or tumor invasion (1, 2). Targeted glycosylation research has become important in the area of developing novel therapeutic approaches (3–5). The structures of asparagine-linked oligosaccharides in the conserved C2 domain of human immunoglobulin-γ (IgG1) have been shown to affect the pharmacokinetics, antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (6, 7). In the last decade, many recombinant antibody molecules have been licensed for the treatment of a variety of cancers and chronic diseases (8). Herceptin, also known as Trastuzumab, marketed by Genentech Inc. is one example of therapeutic IgG1 antibody. It is produced from mammalian cell culture using Chinese hamster ovary cells (9). The main oligosaccharide forms found in this polypeptide chain in the Fc domain at asparagine 297 are biantennary core-fucosylated complex type structures with variable terminal galactosylation (zero, one, or two galactose residues) on their nonreducing termini (10, 11). This humanized monoclonal antibody is known to effectively target breast cancer cells overexpressing the human epidermal growth factor receptor HER2/neu (12). HER2 is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation. It can be found overexpressed in a variety tumors’ cells of epithelial origin and hematological malignancies, including acute lymphoblastic leukemia (13). When antibody binds to defective HER2 protein, this protein no longer causes cells to reproduce uncontrollably. This increases the survival of people with cancer. However, cancers usually develop resistance to trastuzumab. Unfortunately, only 25–30% of patients with HER2/neu positive breast cancer respond to this antibody (14–17). Therefore search for the potential biomarkers that could predict the efficacy of clinical outcomes is needed. More precise inves-
tigation on cellular and molecular level might provide many exciting insights in understanding of mechanism resistance cancer cells to the antibody, so that antibody-based therapies can be optimized more individually (18).

We recently demonstrated how the carbohydrate moieties of two cancer cell models were affected during treatment with antibody (19). The detailed glycans profiles studied by means of mass spectrometry (MS) from the two most common cancer cell lines—human breast MCF-7 carcinoma and T-lymphoblastoid CEM cells before and after treatment with Herceptin showed significant differences. Dominant high-mannose structures analyzed in both original cancer cells were suppressed after treatment and instead, complex bi- and triantennary glycans were the major structures found in the treated samples. Their ratio or occurrence varied with conditions and time of exposure of the cancer cells to the antibody. The results provided very good evidence for involvement of glycosylation during treatment. In this regard, continuous work presented here on this subject has been aimed at the MS investigation of glycosylated peptides generated by proteolytic digestions of the cancer cells before and after exposure to Herceptin or Herceptin/Lipoplex. Direct analysis of glycopeptides by tandem MS has been shown as one of the most sensitive and fast methods for a site-specific characterization of glycosylation. It can provide information on glycan composition, glycan attachment site with determination of peptide sequence (20–28), and may offer more specific biomarkers to monitor changes in the post-translational modification at the onset, during cancer progression or during treatment.

EXPERIMENTAL PROCEDURES

Cell Lines and Three-Dimensional Culture Conditions—The human breast carcinoma MCF-7 cell lines were obtained from the American Type Culture Collection, Manassas, VA. T-lymphoblastoid (CEM) cells were purchased from the American Type Culture Collection, Rockville, MD. Both cell samples were cultured in a hollow fiber bioreactor device (HFB, FiberCell Systems, Frederick, MD) as previously described (29, 30). Briefly, cells were grown at 37 °C under 5% of carbon dioxide and air. The culture conditions for MCF-7 cells consisted of Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin, 50 μg/ml gentamicin, 15 g/L sodium bicarbonate, 10 mM sodium pyruvate, and 2 mM L-glutamine. In case of CEM cells, the RPMI 1640 culture medium was supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution. During 4 weeks of cultivation, cell cultures were supplied with fresh media. The cells were harvested from the HFB, seeded in 6-well microplates and their viability was determined using the trypan blue exclusion method. Cell numbers were determined manually with a hemocytometer chamber (Hauser Scientific, Horsham, PA).

Treatment/Transfection—When the cells reached a concentration of 10⁶ cells per ml, treatment was performed with Herceptin (Genentech Inc, San Francisco, CA) at concentration 0.01 μg/ml or transfected with Herceptin/Lipoplex in the HFB. The day before transfection, cells were maintained in serum-free growth media. Lipofectamine™2000 (LipA, InvivoGen Inc., Gaithersburg, MD) was mixed with Herceptin to obtain a final concentration ratio of 3:1. Plasmid DNA containing β-Gal (InvivoGen Inc., Gaithersburg, MD) was diluted in serum free media and added to the Herceptin/LipA mixture to obtain a DNA plasmid to lipid ratio of 1:9. The solution was then mixed with medium for 30 min to produce the Herceptin/Lipoplex complex. The transfection was performed with a 3 ml volume of Herceptin/Lipoplex. Cell viability was determined by trypan blue exclusion assay at 0–72 h after transfection. The efficiency of transfection with Herceptin/Lipoplex was determined by β-gal expression and a protein concentration assay kit according to the manufacturer’s instructions (enzymatic kit, Promega) after 72 h. To avoid the presence of free Herceptin, after treatment the media were pumped with a flow rate of 14 ml/min during 1 h at 37 °C, then washed twice with 1% bovine serum albumin in 3.5 ml of phosphate buffered saline and suspended in 100 μl of 1% bovine serum albumin for centrifugation. Collected cells were washed with phosphate-buffered saline and collected by centrifugation. Cells were lysed by applying the traditional freeze/thaw technique (31).

Trypsin Digestion—Lysed cells (200 μg) were dissolved in 25 mM ammonium bicarbonate (100 μl) and digested directly with trypsin (Sigma) at 37 °C for 18 h under nonreducing conditions or first incubated with 100 mM diithiothreitol (2.5 μl) in presence of 0.1% RapiGest for 30 min at 56 °C. Subsequently, 5 μl 50 mM iodoacetamide was added and the mixture was kept in the dark at room temperature for 30 min. Next, the reaction mixture was diluted with 25 mM ammonium bicarbonate (total volume 200 μl) and digestion was performed with trypsin at a substrate-to-enzyme ratio of 20:1 at 37 °C for 18 h. After the incubation the digest solution was evaporated and the volume was adjusted with deionized water (20 μl).

RP-HPLC Fractionation—Digested samples (5 μl) were fractionated on a System Gold HPLC chromatograph equipped with a System Gold 166 UV Detector and 32-Karat software (Beckman-Coulter, Canada, ON). For reversed-phase HPLC, a Vydac 218 TP54 Protein&Peptide C18 analytical column, 300-Å pore size, 0.46 × 25 cm (Separation Group, Hespgera, CA) was used. The chromatograph was equipped with a Rheodyne injector (5 μl loop). The samples (5 μl) were eluted with 5% acetonitrile in water as solvent A, and 90% acetonitrile (ACN) in 0.1% trifluoroacetic acid as solvent B at a flow rate of 0.5–1 ml/min. An elution gradient was applied from 5% to 70% ACN over 40–60 min. UV detection was performed at 245 nm. All fractions were collected manually, then completely dried and stored at −25 °C.

Solid phase extractions on STRATA-XC

a) Glycans—PNGaseF digest was purified on a STRATA-XC column (30 mg/g1 ml, Phenomenex, Torrance, CA) according to a procedure previously described (32). Briefly, The STRATA-X-C column was washed with 100%, 50%, 0% ACN in water (5 ml each). The PNGaseF digest (50 μl) was applied onto the column with wet carrier and left to permeate. The column was then washed five times with 200 μl of deionized water and the eluate containing glycans was collected.

1. The abbreviations used are: ACN, acetonitrile; AT2, 2-aza-2-thiothymine; BSA, bovine serum albumin; CDD, collision-induced dissociation; DHB, dihydroxy benzoic acid; Her2/neu, human epidermal growth factor receptor; HPLC, high performance liquid chromatography; IgG, immunoglobulin-γ; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio; LipA, lipofectamine (the polycationic lipid 2,3-dioleolyl-N-[2(sperminecarboxymido)-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and neutral; lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water; PNGaseF, peptide N-glycosidaseF; pDNA, plasmid DNA; PHN, phenylhydrazine; PTV, postranslational modification; QqTOF MS, quadrupole-quadrupole time-of-flight mass spectrometer.
collected into separate tubes without using any pressure. All fractions were collected, evaporated and analyzed after PHN labeling.

b) Glycopeptides—Before extraction, the column was washed with 0%, 50%, 100%, ACN in water (3 ml of each). Tryptic digest (50 µl) was applied onto the column with a wet carrier and left to permeate (~15 min). The tube was rinsed twice with 50 µl of 100% ACN and the solvent was again loaded directly onto the cartridge. The column was then washed five times with 500 µl of 100% ACN, then with 500 µl of 70% ACN. Glycopeptides were eluted from the column three times with 500 µl of 50% ACN in deionized water and three times with 500 µl of deionized water. Fractions were collected into separate tubes without using any pressure, then evaporated and completely dried.

Mass Spectrometric Analysis—Fractionated samples were reconstituted in 7 µl of deionized water and 1 µl was spotted onto partially dried matrix of 2,5-dihydroxybenzoic acid (Sigma) or onto a mixture of 2-aza-2-thiohymine and phenylhydrazine hydrochloride (2:1w/w) predeposited on the surface of a MALDI target, and were air-dried. MALDI-Qq (TOF)-MS and tandem (MS/MS) experiments were carried out on the Manitoba/Sciex prototype quadrupole-quadrupole-TOF mass spectrometer operated in the positive ion mode with a mass resolving power of ~10,000FWHM, and accuracy 10 ppm (33). The instrument was calibrated externally using angiotensin I, substance P and ACTH over a mass range of 500–5000 Da. Spectra were recorded by summing 300 laser shots for each spectrum. Individual parent ions were manually selected for MS/MS. The collision energy was set between 50–140 V as a function of the mass ion.

The peaklist was generated using MoverZ software (2001.02.13 freeware edition) available from Genomic Solutions (http://bioinformatics.genomicsolutions.com/MoverZD.htm). The peptide identities were first assigned manually using a partial de novo approach, and then verified using the online version of Mascot search engine (version 2.3.02; Matrix Science; www.matrixscience.com) with an error tolerance on the monoisotopic ions set to 0.8 Da. The data were searched against the databases NCBI (20110419; 236538 sequences) and SwissProt (2011_04; 20233 sequences) for Homo Sapiens (human) taxonomy. Database searches were conducted for trypsin as enzyme, with one missed cleavage permitted and variable modifications were set for carbamidomethyl, carboxymethyl, deamidation of glutamine and asparagine, oxidized methionine, pyroglutamic acid from N-terminal and glutamic acid. All identifications were manually verified. In addition BLASTp (version 2.2.25) searches for manually assigned peptide sequences were performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Collision-induced dissociation (CID) spectra of peptides in figures were annotated according nomenclature proposed by Roepstorff and Fohlman (34).

The assignment of oligosaccharide types attached to the peptides was performed on the fragmentation pattern produced under MS/MS conditions and verified after deglycosylation (PNGaseF) and detection of glycans with phenylhydrazine (21), (35). For discrimination of glycan structures, general rules described previously were applied (36).

RESULTS

Samples of whole cell lysates obtained before and after treatment/transfection were subjected to protease digestion, following HPLC fractionation or SPE on STRATA-Xc cartridges. Each fraction was then analyzed by MALDI-MS as described in the experimental section.

Although the glycosylation pattern of monoclonal IgG1 has been studied extensively and well characterized, nonetheless it has been reported that commercially available monoclonal antibodies can differ in their lot-to-lot heterogeneity and therefore detailed analysis of global glycoprofiles is always required (37). Thus, the same batch of Herceptin used for treatment in our study underwent a thorough analysis of glycans, as reported previously (19). Furthermore, a detailed analysis of glycopeptides obtained from the antibody is presented here along with the investigation of glycosylated peptides isolated from treated samples, to validate the profiles observed.

Analysis of Glycopeptides from Herceptin—Glycopeptides obtained from trypsin digested Herceptin were eluted on a RP-column at a flow rate of 0.5 ml/min, in the 5–15 min range. MS spectra recorded from these fractions are presented in Fig. 1. Glycosylated peptides analyzed in the first fraction produced peaks at m/z 3540.40 and 3249.31 (Fig. 1A) and according to CID spectra their composition corresponded to a peptide bearing biantennary fucosylated oligosaccharides with two and one NeuAc residue/s (supplemental Fig. S1). The latter glycopeptide (m/z 3249.31) was observed as the dominant peak in the next fraction, accompanied by a series of peaks, most of which corresponded to sialylated and neutral oligosaccharides having one unprocessed core-linked mannose (m/z 2884.13, 2738.04, and 2447.00; Fig. 1B). The dominant glycopeptides detected in the third fraction were bearing characteristic fucosylated neutral biantennary glycans associated with IgG1 molecule and were detected at m/z 2834.06, 2796.09, and 2958.15 (Fig. 1C). Additional glycopeptides identified in the last fraction were observed at m/z 2487.99, 2405.93, 2284.87, 2246.94, and 2084.92, and their composi- tion corresponded to high-mannose (Man6GlcNAc2) and complex afucosylated structures with no galactose at the nonreducing ends (Fig. 1D).

Most glycopeptides analyzed in Herceptin were consistent with a peptide of composition EEQYNSTYR (m/z 1189.51), a characteristic amino acid sequence with glycosylation site at asparagine 297. Deglycosylation by PNGaseF, modifying the peptide backbone from asparagine to aspartic acid was confirmed by MS/MS, as fragment ions corresponded to high-mannose (Man6GlcNAc2) and complex afucosylated structures with no galactose at the nonreducing ends (Fig. 1D).

Minor peaks (~3%) detected at m/z 2618.05 and 2780.11 (Table I). These glycopeptides produced fragmentation pattern with peptide backbone detected at m/z 1173.5. In our previous study during analysis of polyclonal IgG, the glycopeptide types producing peptide with m/z 1173.50 of composition EEQFNSTYR was confirmed (21). However, because of too low intensities and a lack of the fragment ions, the MS/MS spectra of these glycopeptides detected here from Herceptin were not sufficient for complete amino acids assignment.

The compositions of N-glycans analyzed here through tryptic glycopeptides are in good accordance with the oligosaccharide profile obtained after PNGaseF release from intact IgG1. Both approaches showed more heterogeneity in glycans than usually described in association with IgG1. Although the preparations of monoclonal antibodies are generally aimed to be devoid of sialic acids (38), our data indicate
Fig. 1. MALDI-QqTOF mass spectra of glycopeptides analyzed in Herceptin after digestion with trypsin under nonreducing conditions and fractionated on RP-HPLC at a flow rate 0.5 ml/min. A, fraction with elution time 5 min; B, fraction with elution time 6 min; C, fraction with elution time 12.2 min; D, fraction with elution time 15 min. Symbols: ▲Fuc; ●Gal; ○Man; ■GlcNAc; ◦NeuAc.
### Glycopeptides Associated with Herceptin Treatment of Cancer Cells

**Table I**

| Glycopeptide (observed m/z) | Oligosaccharide (composition) | Peptide (m/z) | CEM | MCF-7 |
|----------------------------|--------------------------------|---------------|-----|-------|
| 2268.87a                   | GlcNAc<sub>2</sub>,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2284.87a                   | GlcNAc<sub>2</sub>,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2405.93a                   | Man,GlcNAc<sub>2</sub>         | 1189.51       | ++  | ++    |
| 2430.96a                   | GlcNAc<sub>2</sub>,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2447.06a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2487.99a                   | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2593.03a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2634.06a                   | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2650.05a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2738.03a                   | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2796.09a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2884.13a                   | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2958.15a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 3087.20a                   | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 3249.31a                   | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 3540.40a                   | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1189.51       | ++  | ++    |
| 2618.09a                   | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1173.52       | ++  | ++    |
| 2780.11a                   | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1173.52       | ++  | ++    |
| 2816.06                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1171.53       | ++  | ++    |
| 2778.17                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1171.53       | ++  | ++    |
| 2940.19                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1171.53       | ++  | ++    |
| 2128.85                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2274.93                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2331.96                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2478.02                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2494.05                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2640.11                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2802.13                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1033.50       | --  | --    |
| 2449.88                    | Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2323.91                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2469.99                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2527.01                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2673.05                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2689.06                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2835.10                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2851.23                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2997.29                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 3288.30                    | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1228.54       | ++  | ++    |
| 2545.00                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 2691.04                    | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 2853.11                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 3015.20                    | Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 3145.28                    | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 3306.29                    | NeuAc,Gal,GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 1246.55       | ++  | ++    |
| 3742.92a                   | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 640.31        | ++  | ++    |
| 2246.94a                   | GlcNAc<sub>2</sub>,Man,GlcNAc<sub>2</sub> | 640.31        | ++  | ++    |

*a Originated from original antibody (Herceptin) used for the treatment.

considerable amounts of sialylated glycans (＞5%) in Herceptin, even though positive mode was used for MS detection.

Analysis of Glycopeptides Obtained from Cancer Cells after Treatment—In the MALDI-QqTOF mass spectra recorded from cancer cells treated with the antibody, peaks corresponding to glycopeptides were observed in a range of m/z 2000–3600, producing single [M+H]<sup>+</sup> ions with the characteristic mass differences of monosaccharide residues—146, 162, 203 and 291 Da. All glycopeptides identified from MCF-7 and CEM cells after treatment with Herceptin or transfection with Herceptin/LipA are listed in Table I. The selected MALDI-QqTOF spectra recorded from RP-HPLC fractions of CEM cell samples after Herceptin treatment are shown in Fig. 2A, 2B and compared versus a profile obtained from Herceptin digested and analyzed under the same conditions (Fig. 2C).

Generally, the most abundant glycopeptides were detected at m/z 2634.06 and 2796.09 in all samples. These peptides plus smaller peaks at m/z 2958.15, 2487.99, 2405.93, and 2284.87 (Fig. 2A, 2B), including peaks of sialylated glycopeptides eluted in previous fractions (m/z 3249.31 and 3540.40) were found in all treated cells. Under tandem MS conditions, these ions produced typical glycopeptide fragmentation patterns (Fig. 3A). The total loss of monosaccharide moieties appeared at m/z 1189.51. The fragment ions detected at lower m/z indicated the
presence of the amino acid sequence EEQYNSTYR, the same sequence as detected and analyzed in Herceptin (supplemental Table S1). The major oligosaccharides corresponded to neutral biantennary core-fucosylated structures.

In the same fractions from treated cells, the additional peaks corresponded to compositions of new glycosylated peptides, which were detected neither in the original cells nor in the antibody itself (Fig. 2A, 2B; the m/z values in colors). Small peaks observed at m/z 2616.06, 2778.17, and 2940.21 were detected after both types of treatment (m/z values in green in Figs. 2A, 2B; supplemental Fig. S3b) and were associated with a peptide of m/z 1171.53 (supplemental Fig. S4). However, because of too low intensity and lack of fragment ions in the CID-spectra of these glycopeptides or their peptide after deglycosylation (PNGaseF), it was not possible to determine the total composition of amino acid sequence. In the case of CEM cells transfected with Herceptin/Lipoplex and digested with trypsin under nonreducing conditions, other small peaks were observed at m/z 2128.85, 2274.93, 2331.96, 2478.02, 2494.05, 2640.11, and 2802.13 in the fraction eluted prior above discussed glycopeptides (supplemental Fig. S3a). These peaks were consistent with the mass of a peptide backbone at m/z 1033.50 and the same glycans types as found in Herceptin (Table I).

Peaks observed at m/z 2323.91, 2527.01, 2673.05, 2689.06, 2835.10, 2851.23, and 2997.25 were detected in
fractions from cells after both types of treatment (the \( m/z \) values are in violet in Figs. 2 A, 2 B). Under MALDI-MS/MS conditions, these glycopeptides lost the same glycans as seen for the antibody. Based on the glycan compositions and characteristic MS/MS pattern, the peak detected at \( m/z \) 1228.54 could be assigned to \( M^+H \) fragment ions corresponding to peptide backbone (Fig. 3 B) and supplemental Table S3. Fragment ions detected at \( m/z \) 175.12 (\( y_1 \)), 298.10 (\( b_3 \)), 388.22 (\( y_2 \)), 439.25 (\( y_3 \)), 526.26 (\( y_4 \)), 589.23 (\( b_5 \)), 640.27 (\( y_5 \)), 703.27 (\( b_6 \)), and 790.32 (\( b_7 \)) indicated a peptide of sequence PAEQYNS-TY-R or APEQYNS-TY-R or EPAQYNS-TY-R. The further ions observed at \( m/z \) 98.067 (\( b_1 \)), 804.35 (\( y_6 \)), and 932.40 (\( y_7 \)) in the CID spectrum recorded for \( [M+H]^{+} \) precursor ions with \( m/z \) 1229.54 observed after PNGase deglyco-

Fig. 3. Selected MALDI-MS/MS spectra of tryptic glycopeptides analyzed in cancer cells after treatment for \([M+H]^+\) precursor ions. A, \( m/z \) 2634.01 (CEM/Her-Lipoplex); B, \( m/z \) 2673.07 (MCF-7/Her-Lipoplex); C, \( m/z \) 2691.07 (CEM/Her).
Glycopeptides Associated with Herceptin Treatment of Cancer Cells

**Fig. 4.** The pie charts illustrating the occurrence of glycopeptides detected in: A, Herceptin; B, cancer cells after treatment with Herceptin for 72 h; and C, cancer cells after transfection with Herceptin/Lipoplex for 72 h. The proportions of the glycopeptides, differentiated according their m/z of peptide backbone, were obtained from average of normalized intensities over four sets of experiments. Glycopeptides with peptide of m/z 1033.5 were observed only from treated CEM cells digested under nonreducing conditions and were excluded from this evaluation.

sylation, provided more evidence in favor the amino acid sequence PAEQYDSTYR (supplemental Fig. S5).

In the spectra of cells treated with Herceptin only, additional significant peaks of glycoconjugates were detected at m/z 2545.00, 2691.06, 2853.11, and 3015.20 (Fig. 2A; m/z values in blue). The compositions of these glycopeptides were again consistent with the biantennary fucosylated glycans as detected in IgG1, and differed by amino acid residues. For example, Fig. 3C shows MS/MS spectrum recorded for [M+H]+ precursor ions at m/z 2691.07. After the initial loss of HexNAc (m/z 2488) and Hex (m/z 2326), the next cleavage was associated with the loss of HexNAc-Hex residue detected at m/z 1595. The subsequent loss of 146 units observed at m/z 1499 indicated linkage of Fuc at the GlcNAc residue attached to the asparagine. The final cleavage of 203 units produced a characteristic peak associated with a peptide at m/z 1246.55. The fragment ions observed next at m/z 175.12, 338.19, 439.24, 526.25, 640.30, 769.34, 897.40, 996.45, and 1143.54 could be assigned to y-type cleavages from amino acid sequence CFVQENSTYR. The existence of this peptide can be strongly supported by a number of b-type fragment ions accompanied with a characteristic loss of 28 u.

The new glycopeptides discussed above did not generate MASCOT scores and to our knowledge have not been described previously; their incidence was evidenced associated with the response of cancer cells to Herceptin treatment (Fig. 4).

**DISCUSSION**

In our work we used two cancer cell lines to study viability of cells before and after treatment with Herceptin on different conditions. Although this model of treatment does not fully resemble to real treatment in human, it can provide important information about molecular changes associated with treatment. In one set of experiments the cancer cells were treated with the antibody alone for 72 h. This time showed to be the optimal time regarding decreased viability. In the second set, the same cells were exposed to the antibody combined with Lipoplex and in this case for the same time cancer cells were eradicated even more effectively. It is believed that LipA plays a direct role in plasmid DNA delivery into cell cytoplasm as well as in destabilization of the plasma membrane (39) and can allow more effective antibody targeting. Both types of these experiments showed also differences at the proteome level. The monitoring of N-glycans in cancer cells before and after exposure to the antibody showed significant alterations in glycosylation (19). Dominant high-mannose glycans (Man3-GlcNAc2) accompanied by less abundant of fucosylated tri- and tetraantennary higher sialylated glycans were found in both original cancer cells and after treatment these structures were replaced by complex biantennary fucosylated oligosaccharides of the same compositions as found in IgG1. A number of additional significant oligosaccharide peaks, particularly corresponding to nonfucosylated triantennary galactosylated structures, which were present neither in the antibody nor in the original cancer cells, were found in cells treated only with Herceptin and were not detected when Herceptin was combined with LipA. Thus, the original goal of this work was focused on the study of glycosylated peptides isolated from cancer cells after treatment and confirming glycoprotein associated with the observed N-glycans changes.

It is expected that during treatment, antibody targeted cancer cells are driven into apoptosis, proteins/glycoproteins present in the original cancer cells can be expected to be suppressed and IgG1 molecules would be among major biomolecules present and analyzed in treated cells. Therefore, we expected major glycopeptides analyzed in treated samples to be of the same compositions as found in IgG1 (see m/z values in black in Figs. 2A–2C); their MS/MS fragmentation patterns confirmed a characteristic peptide peak at m/z 1189.51 with the amino acid sequence—EEQYKNSYR (Fig. 3A).

According glycopeptide analysis, the major oligosaccharides detected in all treated cells corresponded to biantennary fucosylated complex type, differing in the level of terminal galactose. Other minor forms of glycans as sialylated complex types, and other less mature structures such as high-mannose (Man3-GlcNAc2) or hybrid structure were found in all treated samples. The comparison of glycans profiles obtained from these samples through glycopeptide analysis versus that obtained by PNGaseF release from total cell lysates showed good similarity for both types of cancer cells which were transfected with Herceptin/Lipoplex (Fig. 5B, 5C). In the experiments of which cancer cells were exposed to Herceptin without LipA, the glycopeptides analysis confirmed the same glycans types as above mentioned (Fig. 5D, 5E, charts in.
black). However, none of the peptides bearing higher amount of nonfucosylated biantennary and triantennary galactosylated oligosaccharides, including non-fucosylated triantennary higher sialylated structures were detected as it was observed after direct PNGaseF release from the whole cell lysates (Fig. 5D, 5E, dotted charts). In our previous study on the investigation of oligosaccharides from glycopeptides was shown that the relative abundances of individual oligosaccharides were consistent with those of the glycopeptides (21). Therefore, according to glycan profiles obtained from cancer cells after treatment with Herceptin without Lipoplex, by two different approaches, the seemingly absence of glycosylated peptides bearing nonfucosylated galactosylated oligosaccharides could be explained by suppression of abundant nongly-
Glycopeptides Associated with Herceptin Treatment of Cancer Cells

cosylated peptides present in the fractions. It is also possible that masses of these glycopeptides were beyond the upper mass range set for the instrument (5000 Da). Although the identification of the glycoprotein associated with those non-fucosylated galactosylated glycans require another mass spectrometric approach or methodological procedure, evidently they are from other glycoprotein than IgG1, of which growth was a result of Herceptin treatment without the presence of LipA. It should be emphasized that none of glycopeptides or glycans analyzed in treated cells were detected in original MCF-7 and CEM cells.

It was surprising to learn that the MS/MS spectra acquired from new aberrant glycopeptides analyzed in all treated cells provided evidence only for the glycans of the same compositions as found in IgG1, however they differed in peptide sequences. Among them, the most significant peaks corresponded to glycoconjugates with peptides PAEQNSTYR and CVQGENSTYR (violet and blue m/z values in Figs. 2A, 2B). Low abundant glycopeptides detected at m/z 2802.13 and 2640.11 from CEM cells after transfection with Herceptin/LipA (supplemental Fig. S3a) have been previously observed in the study of Fc domain of immunoadhesine affected by drugs (40).

According to MS/MS fragmentation patterns all newly detected peptides, including those with not fully assigned amino acid sequences, showed NSTYR motif as a part of their sequence as found in IgG1 and moreover were bearing the same type glycans of the same profiles as were analyzed in the original antibody. These facts and the consistency of the data presented on two different cancer cell models suggest that all of these new glycosylated peptides described here are results of unknown transformation in the IgG1 molecule itself during treatment. As we have previously shown, transfection of cancer cells with Herceptin/Lipoplex decreased the viability of these cells to a significantly higher degree than treatment with Herceptin alone (29). Lipofectamine is known to alter the plasma membrane but it remains unclear how it enhances the effects of Herceptin.

Although further studies are needed to identify regulatory pathways that cause alterations in glycoconjugates, in the present study we have shown that more new aberrant glycopeptides were found in cancer cells treated with Herceptin alone, than in cells exposed to Herceptin/Lipoplex. The amino acid sequences of the analyzed peptides and the glycan structures indicate that these newly described glycopeptides might be the result of intracellular degradation of Herceptin. Therefore it is worthy to study in future, whether the cotreatment with Lipoplex results in altered uptake of Herceptin, localization of the Herceptin/Her2 complex to different plasma membrane domains or a change in the intracellular compartmentalization of Herceptin. Nonetheless, our results indicate that presence of LipA could play a role not only in better surface targeting, but also prevents antibody from degradation and elongates its proper function during treatment. In conclusion, the results discussed in this study indicate that treatment is more complex process and involves series pathways on different molecular levels. The precise evaluation and better understanding of changes in glycoconjugates presented here might provide new insight how to improve treatment involving monoclonal antibody.

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[5] This article contains supplemental Figs. S1 to S5 and Tables S1 to S5.

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