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N-Glycomic Changes in Human Breast Carcinoma MCF-7 and T-Lymphoblastoid Cells After Treatment with Herceptin and Herceptin/Lipoplex

Erika Lattová,^{*,†,‡} Boguslaw Tomanek,^{\$,||} Dorota Bartusik,^{\$,⊥} and Hélène Perreault^{*,†}

Department of Chemistry, University of Manitoba, 144 Dysart Road, Winnipeg, Manitoba R3T 2N2, Canada,
The Institute of Chemistry, Centre for Glycomics, Slovak Academy of Sciences, 842 38 Bratislava, Slovakia,
Institute for Biodiagnostics (West), National Research Council Canada, 3330 Hospital Drive NW, Calgary,
Alberta T2N 4N1, Canada, Oncology Department, University of Alberta, 11560 University Avenue, Edmonton,
Alberta T6G 1Z2, Canada, and Medical Physics Department, Cross Cancer Institute, 11560 University Avenue,
Edmonton, Alberta T6G 1Z2, Canada

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The humanized monoclonal antibody IgG1 in combination with chemotherapy has been demonstrated to enhance survival benefit in cancer treatment. Despite positive outcomes, some cancer cells develop multidrug resistance. Numerous mechanisms in cancers can be involved in the process of treatment therapy and most of them are not still well understood. To address how the carbohydrate moieties of cells are affected during treatment, the glycan profiles from the two most common cancer cell lines — human breast MCF-7 carcinoma and T-lymphoblastoid CEM cells — were studied here and compared with profiles after treatment with Herceptin alone or in combination with Lipofectamine mixed with plasmid DNA to form Lipoplex. N-Glycans were released from total cells by digestion with PNGaseF and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). In summary, both original cell lines showed a dominant occurrence of high-mannose glycans. After treatment, these structures were suppressed and biantennary core-fucosylated glycans originating from IgG1 were the major carbohydrate products identified in cells. The high incidence of additional fucosylated or nonfucosylated galactosylated oligosaccharides, which were not detected in original cells or Herceptin, varied with conditions and time of exposure of cells to the antibody. The results presented in this study provide strong evidence for a role of glycosylation during antibody treatment.

Keywords: N-Glycans • MCF-7 cells • breast carcinoma • CEM cells • mass spectrometry

Introduction

Protein glycosylation is one of the major types of post-translational modifications (PTMs) with profound biological implications.^{1,2} Glycosylation of proteins has been shown to change during malignant transformation; oligosaccharide expression is highly relevant to many diseases and the alteration of PTMs in proteins is one of the common features of cancer.^{3,4} Among those, the most significant changes in carbohydrates have been described in terms of increased *N*-acetylglucosamine β -1-6 branching in oligosaccharides,⁵ inappropriate expression of polylactosamine extension,⁶ or sialylation of the MUC1 mucin molecule resulting in the presence of truncated O-linked oligosaccharides chains accompanying breast cancer.^{7,8} Aggressive cancers have also been reported to induce elevated

levels of mono- and trisialylated oligosaccharides, found only in trace levels in nonaggressive cancers.⁹

Current cancer therapy uses several approaches including chemotherapy and drugs based on monoclonal antibodies.^{10–13} The activation of the effector mechanism is dependent on structural characteristics of the antibody molecule that results from post-translational modifications, in particular, glycosylation.¹⁴ The therapeutic humanized immunoglobulin IgG1 known as Herceptin (Trastuzumab) is a glycoprotein bearing N-linked biantennary complex-type oligosaccharides, a majority of which are fucosylated.^{15,16} This monoclonal antibody is specifically directed against the human epidermal growth factor receptor 2 (HER2/neu) and is often used simultaneously with chemotherapy in the treatment of positive breast cancers.^{17,18} Even though this combined therapy has extended disease-free survival in a significant number of patients, not all HER2 overexpressing tumors have responded to this treatment, and some patients have developed resistance after an initial response.^{19,20} Despite the considerable research efforts carried out, the mechanisms for cellular and molecular responses that confer efficacy of antibodies remain mostly unclear. Thus, *in vitro* and *in vivo* preclinical studies are still needed. Moreover,

* To whom correspondence should be addressed. Erika Lattová, e-mail lattovae@cc.umanitoba.ca or Hélène Perreault, e-mail perreau@cc.umanitoba.ca.

[†] University of Manitoba.

[‡] Slovak Academy of Sciences.

§ National Research Council Canada.

¹¹ University of Alberta.

[†] Cross Cancer Institute.

the production of human therapeutic antibodies with a consistent glycoform profile remains a considerable challenge to the adjuvant treatment for HER2.

Over the past decade, extensive studies have followed the evaluation of glycan changes in proteins during disease from a variety of biological samples using different proteomic approaches, including high sensitive mass spectrometry.^{21–33} However, less attention has been paid to the study of glycosylation during and after treatment.^{34–36} In this regard, our present work is aimed to MS investigation of N-glycans in human breast MCF-7 carcinoma and T-lymphoblastoid CEM cells vs oligosaccharide profiles obtained from cells treated by *ex-vivo* assay with Herceptin alone or combined with Lipofectamine (LipA) and plasmid DNA. It is known that the functional lipid-DNA complex can be formed by simple mixing of cationic liposomes and negatively charged DNA.³⁷ The resulting complex has a net positive charge that facilitates transfection of the cells with plasmid DNA.³⁸

As Herceptin is a N-glycosylated protein, to validate the observed varieties in glycan profiles, oligosaccharides were also detached from Herceptin used for treatment and analyzed under the same conditions as those from original cells and from cells after exposure to the antibody.

Materials and Methods

Materials and Reagents. Herceptin was purchased from Genentech Inc. (San Francisco, CA). Lipofectamine2000 (LipA) and Plasmid DNA were obtained from InnoVita Inc. (Gaithersburg, MD). Trypsin, chymotrypsin, Pronase, phenylhydrazine (PHN), phenylhydrazine hydrochloride (PHN·HCl), 2,5-dihydroxy-benzoic acid (DHB) and 2-aza-2-thiothymine (ATT) were purchased from Sigma (St. Louis, MO). Peptide-N-glycosidase F (PNGase F; supplied glycerol free) was from Roche (Mannheim, Germany). STRATA-X-C cartridges were from Phenomenex (Torrance, CA). Solvents (acetonitrile, methanol) were HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water was obtained with a Milli-Q plus TOC water purification system (Millipore, Bedford, MA).

Three-Dimensional Cell Culture. The human breast carcinoma MCF-7 cell line (American Type Cells Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin, 50 µg/mL gentamycin, 15 g/L sodium bicarbonate, 10 mM sodium pyruvate and 2 mM L-glutamine. The human T-lymphoblastic (CEM) cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin solution. Both cell lines were introduced into a Hollow Fiber Bioreactor (HFB) device (FiberCell Systems, Frederick, MD) and cultured according to a protocol described in detail previously.³⁹ Briefly, cells were grown at 37 °C under 5% CO₂ /air. During the 4 weeks of culturing, the media were replaced each week. The flow rate was maintained at 14 mL/min and controlled by a peristaltic pump. The cells were harvested from the HFB, seeded in 6 well microplates and their viability was determined using trypan blue exclusion. Cell numbers were determined manually with a hemacytometer chamber (Hausser Scientific, Horsham, PA). The untreated cells (1 × 10⁹ cells) were washed with phosphate buffered saline (PBS) and collected by centrifugation.

Cells were treated with Herceptin alone (0.01 µg/mL) or transfected with Lipoplex in the HFB. LipA was mixed with the Herceptin to obtain the final concentration ratio of 3:1. Plasmid

DNA was diluted in serum free media and added to the Herceptin/LipA mixture to obtain a DNA plasmid to lipid ratio 1:9. The solution was then mixed with medium for 30 min to produce the Herceptin/Lipoplex complex. The transfection of 10⁹ cells/mL was performed with a 3 mL volume of Herceptin/Lipoplex. Cell viability was determined by trypan blue exclusion assay at 0, 6, 24, and 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 instruction (enzymatic kit, Promega) after 72 h. To avoid the presence of nonreacted 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 (BSA) in 3.5 mL of PBS and suspended in 100 µL of 1% BSA for centrifugation. Collected cells were washed with PBS and collected by centrifugation. To avoid cell contamination, the cell morphology was assessed using an inverted microscope (Micro Cap Version 1.0) with ESPA Systems Co. Ltd. software. The statistical evaluation of cell viability in group was done using Student's *t* test.³⁹ Cells were lysed by applying the traditional freeze/thaw technique⁴⁰ and underwent the procedures described next.

Release of N-Linked Oligosaccharides from Total Cells.

Dried cells (~500 µg) were first digested with trypsin (20 µg) and chymotrypsin (20 µg) dissolved in 25 mM ammonium bicarbonate (50 µL) at 37 °C for 18 h. The digest was left in a water bath (85 °C, 5 min) and after cooling N-linked oligosaccharides were released from peptides by treatment with PNGaseF enzyme (3 µL; 6U) at 37 °C (18 h) followed by Pronase digestion (20 µg) at 37 °C (8 h). During the incubation time, the reaction sample was mixed occasionally.

RP-HPLC Fractionation of the Digested Cells. Digested cells 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 cm × 25 cm (Separation Group, Hesperia, CA, USA) was used. The chromatograph was equipped with a Rheodyne injector (5 µL loop). The samples (5 µL) were eluted with 5% ACN in water as solvent A, and 90% ACN in 0.1% TFA as solvent B at a flow rate of 0.5 mL/min. An elution gradient was applied from 5 to 70% ACN over 60 min. UV detection was performed at 245 nm. All fractions were collected manually and concentrated *in vacuo*.

Solid Phase Extractions on STRATA-XC.³¹ Prior to extraction, the STRATA-X-C column (60 mg/3 mL) was washed with 100%, 50%, 0% ACN in water (10 mL of each). The PNGaseF digest (50 µL) was applied onto the column with wet carrier and left to permeate. The tube was rinsed twice with 50 µL of deionized water and again loaded directly onto the cartridge. From this time the eluate was immediately collected. The column was then washed five times with 200 µL of deionized water and the eluate containing glycans was collected into separate tubes without using any pressure. All collected fractions were evaporated and labeled with PHN as described in the following section.

Phenylhydrazine (PHN) Derivatization. The labeling of oligosaccharides was performed according to the adopted method reported previously.^{41,42} Fractions obtained from purified digested cells were reconstituted in deionized water (5 µL) and 1.5 µL of diluted sample was added to a tube containing 0.7–1 µL of derivatization reagent (10 µL of pure PHN dissolved in 10 µL deionized water and 10 µL ACN). The reaction mixture

was mixed by flushing the pipet tip several times and then loaded onto the spot with matrix (DHB or ATT/PHN.HCl in molar ratio 2:1⁴³). The target was left at room temperature until the spots had dried.

MALDI-MS Analysis. The released glycans were analyzed by MALDI-MS in positive ion mode on the Manitoba/Sciex prototype quadrupole–quadrupole-TOF (QqTOF) mass spectrometer.³¹ The collisional energy for each precursor ion m/z value was determined by applying a well-defined accelerating voltage at the entrance of the Q2 and values were around 50 V per 1000 Da. The resulting product ions were measured in the TOF analyzer. Orthogonal injection of ions from the quadrupole into the TOF section routinely produces a mass resolving power of $\sim 10,000$ FWHM, and accuracy 10 ppm, in both MS and MS/MS modes. All MS spectra were recorded by summing of 300 shots for each spectrum. MS/MS spectra of oligosaccharides were interpreted manually. For the assignment of all oligosaccharide fragment ions, the general nomenclature established by Domon and Costello was followed.⁴⁴ The proposed structures of N-glycans were deduced from the fragmentation patterns obtained for single precursor ions. For discrimination of isomeric structures, general rules described previously were applied.⁴⁵

Results

All cell samples used for our study were cultured and exposed to the treatment in the HFB device.³⁹ This technique enables the formation of a unique three-dimensional organization to induce *ex-vivo* of multicellular tissue and mimics *in vivo*-tumor conditions. During experiments, the high density of 3-D cell aggregation was monitored by noninvasive proton magnetic resonance imaging. It was reported that administration of Trastuzumab alone did not inhibit cell growth by more than 30–50% in relation to doses of 10–1000 $\mu\text{g/mL}$ over 72 h.⁴⁶ Furthermore when antibody is combined with other cytotoxic agents, it is difficult to evaluate the combined effect. Cytotoxic mediator not only affects the viability of cancer cells, but unfortunately the antibody is destroyed too. In an effort to improve the efficiency of the antibody and coherently avoid unwanted toxicity of cytotoxins, the combined treatment of Herceptin/Lipoflex was employed here. The collected viability of data indicated changes in viable cell numbers after 6, 24, and 72 h treatment with different drug concentrations (Supporting Figure 1, Supporting Information). The 72 h of exposure to Herceptin treatment showed to be the optimal time regarding decreased viability of cancer cells. Another decrease in the viability of cancer cells under the same conditions was enhanced when cells were treated with Herceptin transfected with LipA. It can be supposed that this biocompatible agent plays a direct role in plasmid DNA fusion and delivery to cell cytoplasm as well as membrane destabilization to allow more efficient surface targeting when using Herceptin. Moreover, Lipoflex contains plasmid DNA with the commonly used β -gal reporter and thus enhances direct growth inhibition. It should be pointed out that MCF-7 and CEM cells which were exposed to LipA without antibody for 72 h, did not show any decrease in their growth. The evaluation of experiments was discussed in detail in our last report.³⁹ The results of HPLC and MALDI-MS analyses indicated drug-induced early proteomic changes in the samples, supporting the results published by Reyzer *et al.*,⁴⁷ that antibody initiated changes in protein molecule as early as 16 h after treatment.

In the present study, we demonstrate detailed analysis of N-glycans obtained from original human MCF-7 breast and T-lymphoblastic cells, and from these types of cells exposed to the treatment with Herceptin alone or in combination with Lipoflex. Cell samples were first digested with trypsin/chymotrypsin and oligosaccharides were released from peptides by PNGaseF. The digested mixtures were fractionated on HPLC or STRATA-XC ion exchange/reverse phase cartridges. Two complementary purification procedures were used with the purpose of verifying glycans profiles from single lines. MALDI-MS analysis was carried out on PHN-labeled oligosaccharides. Both types of neutral and acidic glycans produced single $[M + \text{Na}]^+$ ions. Generally, neutral oligosaccharides were dominant in all cells analyzed; sialylated glycans were observed to contain NeuAc residue/s and were detected at lower abundances. The total glycans identified from MCF-7 and CEM cells before and after treatment with Herceptin under different conditions are listed in Table 1.

N-Glycans of Herceptin. The main oligosaccharides derived from this antibody were observed in PHN-derivatized samples at m/z 1575.5, 1737.6, and 1899.7 and were consistent with biantennary core-fucosylated structures (Figure 1). It was reported previously that small amounts of oligosaccharides (less than 3%) observed in Trastuzumab showed structures lacking a GlcNAc residue in oligosaccharide **1**, and that glycans **2** and **3** had NeuAc residues.¹⁵ These oligosaccharides were also observed in our study as minor peaks at m/z 1372.5, 2028.7, and 2190.8. An additional minor peak detected at m/z 1347.5 was consistent with a high-mannose structure and glycans detected at m/z 1429.5, 1591.5, and 1753.6 had no fucose residue at their reducing termini.

MALDI-MS Analysis of N-Glycans from MCF-7 and CEM Cells. Although there were obvious differences regarding the intensities of some peaks in the spectra recorded from pools of MCF-7 and CEM samples, generally the main N-glycans analyzed in both original cell lines were detected at m/z 1347.5, 1509.5, 1671.6, 1833.6, and 1995.7 (Figures 2a and 3a). MS/MS analysis clearly showed that these oligosaccharides had only hexose residues on both antennae. B- and C-fragment ions resulted from the loss of chitobiose core and were consistent with compositions of single isomers of high-mannose glycans for peaks detected at m/z 1347.5, 1509.6 (Table 1) and at m/z 1995.6 (Supporting Figure 2b, Supporting Information). The loss of 3-positioned hexose(s) from those B, C fragment ions resulted in abundant ions at m/z 671.2 ($\text{B}_3/\text{Y}_{3\beta}$). These ions underwent the characteristic subsequent loss of water as detected at m/z 653.2.⁴⁵ Glycans observed at m/z 1671.6 and at 1833.7 appeared as mixtures of two isomers (Supporting Figure 2a, Supporting Information, and Table 1). The high incidence of oligomannose structures was shown to be associated with human ovarian marker and human follicular lymphoma cells^{48,49} and increased levels of high-mannose oligosaccharides were as well observed in sera of animals affected with cancer.^{26,31}

The peaks corresponding to compositions of hybrid, complex and tri- or tetraantennary sialylated oligosaccharides including their fucosylated analogs, were detected in both cell lines (Figures 2a and 3a). However, in contrast to the high-mannose glycans, these structures were present at low abundances <25% in both lines examined. Very similar pattern and significant decrease in sialylation and fucosylation in breast cancer cell lines was recently reported by Goetz *et al.*⁵⁰ In our study, the largest glycans was observed at m/z 3997.4 and its composition

Table 1. N-Glycans Analyzed in MCF-7 and CEM Cancer Cell Lines by MALDI-MS^a

Observed <i>m/z</i> ^b [M+Na] ⁺	Structure	MCF-7				CEM			
		A	B	C	D	A	B	C	D
1023.3		(+)	no	no	(+)	(+)	no	no	(+)
1185.4		no	no	(+)	no	no	no	(+)	no
1226.4		no	(+)	(+)	(+)	no	(+)	(+)	(+)
1314.5		no	no	no	no	(++)	no	no	(+)
1347.5		(++)	(+)	(+)	(+)	(++)	(+)	(+)	(+)
1372.5		no	(++)	(+)	(+)	(+)	(++)	(+)	(+)
1388.5		no	(+)	(++)	no	no	(+)	(++)	(+)
1429.5		no	(+)	(+)	(+)	no	(+)	(+)	(+)
1509.5		(++)	(+)	no	(+)	(++)	(+)	no	(+)
1534.5		no	(++)	(+)	(+)	no	(++)	(+)	(+)
1550.5		no	no	(+)	no	no	no	(+)	no
1575.5		no	(++)	(++)	(++)	no	(++)	(++)	(++)
1591.6		no	(+)	(++)	(+)	no	(+)	(++)	(+)
1671.6		(++)	(+)	no	(+)	(++)	no	no	(+)
1679.6		no	no	no	no	no	no	(+)	no
1737.6		no	(++)	(++)	(++)	no	(++)	(++)	(++)
1753.6		no	(+)	(++)	(+)	no	(+)	(++)	(+)
1825.6		no	(+)	(+)	(+)	no	(+)	(+)	(+)
1833.7		(++)	no	no	(+)	(++)	no	no	(+)
1841.7		no	no	(+)	no	no	no	(+)	no
1899.7		(+)	(++)	(++)	(++)	(+)	(++)	(++)	(++)
1956.7		no	no	(++)	no	no	no	(++)	no
1995.7		(++)	no	no	(+)	(++)	no	no	(+)
2044.8		(+)	(+)	(+)	(+)	(+)	no	(+)	no
2118.8		no	no	(++)	no	no	no	(++)	no
2132.8		no	no	no	no	(+)	no	no	no
2190.8		(+)	no	(+)	(+)	(+)	no	(+)	(+)
2206.7		no	no	(+)	no	no	no	(+)	no

Observed <i>m/z</i> ^b [M+Na] ⁺	Structure	MCF-7				CEM			
		A	B	C	D	A	B	C	D
2264.8		(+)	no	no	no	(+)	no	no	no
2335.8		(+)	no	(+)	(+)	(+)	no	(+)	(+)
2409.8		(+)	no	(+)	no	no	no	no	no
2481.9		(+)	no	no	(+)	(+)	no	(+)	(+)
2555.9		(+)	no	no	no	(+)	no	no	no
2629.9		(+)	no	no	no	no	no	no	no
2701.0		(+)	(+)	(+)	no	(+)	no	no	no
2775.0		(+)	no	no	no	(+)	no	no	no
2789.0	Hex ₃ GlcNAc ₂ NeuAc ₃	no	no	no	no	(+)	no	no	no
2992.1		(+)	no	(+)	(+)	(+)	no	no	no
3068.1		(+)	no	no	no	no	no	no	no
3138.2		(+)	no	no	no	(+)	no	no	no
3214.1		(+)	no	no	no	no	no	no	no
3283.2		(+)	no	(+)	no	no	no	no	no
3357.2		(+)	no	no	no	no	no	no	no
3429.2		(+)	no	no	no	no	no	no	no
3503.2		(+)	no	no	no	no	no	no	no
3520.2	Hex ₃ GlcNAc ₂ NeuAc ₂ Fuc ₁	(+)	no	no	no	no	no	no	no
3574.2		(+)	no	no	no	no	no	no	no
3591.3	Hex ₃ GlcNAc ₂ NeuAc ₂ Fuc	(+)	no	no	no	no	no	no	no
3648.3		(+)	no	no	no	no	no	no	no
3997.4		(+)	no	no	no	no	no	no	no

^a Symbols: ▲Fuc; ●Gal; ○Man; ■GlcNAc; ◇NeuAc; ◆NeuGc. A - N-glycans detected from original cell lines. B - N-glycans detected from cell lines after treatment with herceptin for 6 h. C - N-glycans detected from cell lines after treatment with herceptin for 72 h. D - N-glycans detected from cell lines after exposure to herceptin/LipA for 72 h. (+) present under 5% from total glycans in MS spectra; (++) glycans detected with abundances higher than 5% in MS spectra. ^b N-glycans are labeled with PHN on the reducing end (+90 increasing mass units).

corresponded to a tetra-antennary core fucosylated structure with bisecting moiety and NeuAc residues on each antenna (Figure 2a, Table 1). Due to low intensity, CID spectra of sialylated glycans were noisier than others discussed above, however the signal was sufficient for structural investigation. Triantennary sialylated oligosaccharides generally in all samples produced a fragmentation pattern for dominant isomer with a branched 3-antenna (B/Y₃–H₂O detected at *m/z* 694). A second isomer with 6-branching (B/Y₃–H₂O ions at *m/z* 1059) was observed only in triantennary oligosaccharides with 6-fucose substitution on the reducing termini.

Sialylated oligosaccharides in CEM cells showed lower heterogeneity vs those found in breast cancer cells, and new structures were identified (Figure 3a). The major peak for a sialylated glycan was observed at *m/z* 1314.5 and corresponded

to composition Hex₃GlcNAc₂NeuAc with a PHN-labeled GlcNAc terminus whose structure is proposed in Figure 4. This glycan was previously observed in gastric adenocarcinoma cells, and combined studies using MS/MS and sequential digestions with specific glycosidases confirmed that the Man 1–6 arm was removed to form a characteristic biantennary glycan.⁵¹ Non-typical oligosaccharide peaks which were detected at *m/z* 1103.4 and 1265.4 (Figure 3a) produced MS/MS fragmentation patterns consistent with Hex₆PHN and Hex₇PHN compositions. These neutral oligosaccharides were found in all CEM cell line samples and their presence is suspected to be as a result of culturing cells in medium rather than expression in cancer cells.

MALDI-MS Analysis of N-Glycans in Treated MCF-7 and CEM Cells. (a) Treatment with Herceptin. The N-glycans detected in Herceptin-treated cells showed remarkably different

Figure 1. MALDI-QqTOF mass spectrum of Herceptin N-glycans. Oligosaccharides were released with PNGaseF following a digestion with trypsin/chymotrypsin and labeled with PHN from 0.5 mg of pure Herceptin. Data were acquired in the positive ion mode. All peaks are $[M + Na]^+$. Symbols: ▲ Fuc; ● Gal; ○ Man; ■ GlcNAc; ◇ NeuAc; ◆ NeuGc.

Figure 2. Representative MALDI-QqTOF mass spectra of glycans obtained from breast carcinoma MCF-7 cells. Oligosaccharides were released with PNGaseF following a digest with trypsin/chymotrypsin and labeled with PHN: (a) Glycans from original untreated cells and purified by HPLC. (Inset) Expansion of the spectrum of the fraction eluted prior to that shown here in regular size; (b) Glycans obtained from cells after treatment with Herceptin for 6 h and purified on STRATA-XC cartridges; (c) Glycans obtained from cells after treatment with Herceptin for 72 h, purified on STRATA-XC cartridges. All peaks are $[M + Na]^+$. For details, see Table 1.

profiles vs those of untreated cells (Figures 2b,c and 3b,c). The most abundant glycans detected at m/z 1575.6 and 1737.6 corresponded to biantennary complex core fucosylated structures. These glycans and those detected at m/z 1429.5 and 1899.7 were also found in the Herceptin antibody itself. However, cells which were treated with antibody only for 6 h showed higher incidence of additional significant peaks at m/z 1372.5 and 1534.6 (marked with blue arrows in Figures 2b and

Figure 3. Representative MALDI-QqTOF mass spectra of glycans obtained from lymphoblastoid (CEM) cells. Oligosaccharides were released with PNGaseF following a digestion with trypsin/chymotrypsin and labeled with PHN: (a) Glycans from original untreated cells, purified on STRATA-XC cartridges; (b) Glycans obtained from cells after treatment with Herceptin for 6 h and purified by HPLC; (c) Glycans obtained from cells after treatment with Herceptin for 72 h, purified on STRATA-XC cartridges. All peaks are $[M + Na]^+$. For details, see Table 1. Symbols are the same as in Figure 1.

Figure 4. MALDI-MS/MS spectra of sialylated glycan detected in CEM cells for precursor ions at m/z 1314.4. Fragment ions are sodiated. Symbols are the same as in Figure 1.

3b; MS/MS spectra are presented in Supporting Figure 3, Supporting Information). Surprisingly, these two core-fucosylated hybrid oligosaccharides were suppressed in the samples analyzed after 72 h of exposure to antibody and *vice versa* glycan observed at m/z 1388.5, 1591.6, and 1753.6 clearly showed significant increase (marked with red arrows in Figures 2c and 3c). MS/MS data recorded for these peaks confirmed nonfucosylated structures with hexose (Gal) substitution on GlcNAc of an antenna (Table 1). The large peaks at m/z 1956.7 and 2118.8 were exclusively detected only in samples treated for longer times (marked with red arrows in Figures 2c and 3c). CID-MS/MS data for glycans with precursor ions at m/z 2118.8 were consistent with neutral complex triantennary structure bearing galactosyl residues on each antenna (Figure 5a). Characteristic B_4/Y_{3a} fragment ions at m/z 712.2 ac-

Figure 5. MALDI-MS/MS spectra of PHN-glycans with $[M+Na]^+$ precursor ions recorded from cells after treatment with Herceptin for 72 h: (a) at m/z 2118.7; (b) m/z 2992.1; and (c) m/z 3283.1. Symbols are the same as in Figure 1.

accompanied by the following loss of water at m/z 694.2⁴⁵ could be assigned to the isomer with a branched antenna on 3-linked core mannose (1).

Sialylated glycans were minor in all treated cells. However, small trends were observed in the alterations of these oligosaccharides as well. The complex sialylated tetra-antennary and triantennary fucosylated glycans detected in original breast cancer cells were not found in treated cells. The most significant peaks corresponding to sialylated structures were observed at m/z 2992.1 and 3283.1 in the spectrum recorded from MCF-7 cells after 72 h treatment with Herceptin (Figure 2c). Fragmentation patterns of these glycans were consistent with triantennary structures and NeuAc residues on each antenna (Figures 5b) or with a structure comprising four sialic acid residues (Figure 5c). Fragment ions could be assigned one of two isomers for this composition. However, peaks at m/z 712.2 accompanied by characteristic ions at m/z 694.2 ($B_5/Y_{3a}-H_2O$) were again in favor of the structure with branching on the 3-linked core mannose. These sialylated glycans were not observed in CEM cells treated under the same conditions. Otherwise the peak at m/z 2206.8, which was consistent with biantennary fucosylated structure with NeuGc residue (Table 1), was detected in higher occurrence in treated CEM cells (Figure 3c). The peak with m/z 2206.8 was also observed in MCF-7 samples after exposure to Herceptin under the same conditions; however the MS/MS pattern indicated the presence of two isomers—a biantennary fucosylated structure with a

Figure 6. Representative MALDI-QqTOF mass spectra of glycans obtained from cells after combined treatment with Herceptin transfected with LipA for 72 h: (a) breast carcinoma MCF-7 cells and (b) CEM cells. Oligosaccharides were released with PNGaseF following a digest with trypsin/chymotrypsin and labeled with PHN. Samples were purified on STRATA-XC cartridges. All peaks are $[M+Na]^+$. Peaks labeled with stars are unidentified oligosaccharide structures.

NeuGc residue and a hybrid glycan with a NeuAc residue on the antenna.³¹

(b) Combined Treatment with Herceptin/Lipoplex. N-glycans obtained from cell lines which were exposed to the combined treatment of Herceptin in the presence of LipA for 72 h showed very close glycan profiles to those of control Herceptin, as only small differences were observed (Figure 6). High-mannose glycans (m/z at 1347, 1509, 1671, 1833, and 1995) were observed in higher occurrence than in the original antibody and a linear sialylated glycan at m/z 1314 found in the original CEM cells was still abundant enough to be analyzed (Figure 6b). Fucosylated glycans with one unprocessed arm or galactosylated oligosaccharides associated with samples treated with Herceptin (6–72 h) alone were not observed when the Herceptin/Lipoplex complex was employed. The charts in Figure 7 illustrate the occurrence of N-linked oligosaccharide types in analyzed samples.

Discussion

In the present study, we investigated human breast carcinoma MCF-7 and CEM cell lines to monitor glycan profiles before and after treatment with Herceptin alone or in combination with Lipoplex. Oligosaccharides from all cell samples were enzymatically released and labeled with PHN. The resulting glycans pools were analyzed by using the high sensitive MALDI-MS, which allows the characterization of different N-glycan classes. In addition, the abundance ratios of oligosaccharides can be obtained from a comparison of relative ion intensities corresponding to individual glycans.

A major population of N-glycans detected in original MCF-7 and CEM cells corresponded to high-mannose structures (Figures 2a and 3a). The cells exposed to treatment with Herceptin only, revealed dramatic changes in glycans. Major peaks corresponded to biantennary core-fucosylated oligosaccharides originating from IgG1, and incidence of other glycans varied with time of treatment. Samples examined after the 6 h exposure of cells to antibody were associated with a remarkable increase of fucosylated structures having one of core-linked

Figure 7. Charts illustrating the occurrence of N-glycans in: (a) MCF-7 cells, (b) CEM cells and (c) Herceptin. Samples analyzed before treatment - 1; after treatment with Herceptin for 6 h - 2; after treatment with Herceptin for 72 h - 3; after combined treatment with Herceptin/Lipoplex for 72 h - 4. The proportions of the different types of N-glycans (shown by colors) were obtained from average abundances measured over three sets of experiments.

mannose unprocessed (m/z 1372.5 and 1534.6; Figures 2b and 3b). However, after 72 h of treatment these oligosaccharides were suppressed and neutral nonfucosylated complex biantennary glycans showed a significant increase (m/z 1388.5, 1591.6, 1753.7). Moreover, new galactosylated triantennary structures were observed in relatively high proportions (m/z at 1956.7 and 2118.8; Figures 2c and 3c). Although sialylated oligosaccharides were generally present at lower abundances in all examined samples, the alterations were notable at this level too. Fucosylated tri and tetra-antennary sialylated oligosaccharides present in the original cells were not observed after treatment and the most abundant acidic glycan corresponded to a triantennary structure with a NeuAc residue on each arm (m/z 2292.1). Perhaps, it is worthwhile mentioning that biantennary fucosylated glycan with a NeuGc residue at m/z 2206 was observed only in samples after 72 h of exposure to Herceptin. This undesirable nonhuman N-glycolylneuraminic acid (NeuGc) has been shown to be potentially immunogenic and unacceptable as therapeutic agent.¹⁴

The glycans profiles obtained from the cancer cells after exposure to Herceptin transfected with LipA were very similar to those obtained from the Herceptin antibody, with a higher proportion of oligo-mannose structures and some sialylated glycans (Figure 7).

Overcoming resistance to Herceptin represents an important challenge to improve outcomes for patients with HER2 metastatic breast cancer. Achieving a synergistic interaction by optimizing treatment conditions could considerably improve the therapeutic response.¹⁹ Neutral galactosylated triantennary oligosaccharides observed in our study at m/z 1956.7 and 2118.8 in samples treated with Herceptin alone (Figures 2c and 3c), showed a dominant preference for the isomer with 3-branched antenna when analyzed by MS/MS. The biosynthesis of branched triantennary oligosaccharides is generated by glycosyltransferase GnT-IV in living systems and the change

in activity of this enzyme has been described in connection with some cancers.^{52,53} In a previous study of N-glycan alterations associated with drug resistance in human hepatocellular carcinoma, glycosyltransferase GnT-IV was reported to be either down- or upregulated in resistant cells depending on the drug used.³⁶ The evaluation of cell viability under the conditions described in this study, and the results presented in our previous report showed that lipoplex targeted samples resulted in more efficient delivery of the antibody into cancer cells and produced a higher growth-inhibitory effect than the monoclonal antibody alone.³⁹ To translate this observation into a comparison of glycosylation profiles obtained under different treatment conditions, the presence of galactosylated glycans, or biantennary fucosylated glycans with NeuGc residue analyzed in cells exposed to antibody without Lipoplex, may be a significant change associated with the development of drug resistance in cancer cells.

Although further investigation is necessary to address the mechanism by which antibody causes the putative glycomic changes, nonetheless the results reported here demonstrate new evidence on glycan regulation during treatment with antibody. We hope that dramatic alterations described here can contribute to the understanding the role of glycoforms in drug resistance and may assist in developing diagnostic and therapeutic approaches.

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Supporting Information Available: Supporting Figures 1–3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357–2364.
- (2) Solis, D.; Jiménez-Barbero, J.; Kaltner, H.; Romero, A.; Siebert, H. C.; von der Lieth, C. W.; Gabius, H. J. *Cells Tissues Organs* **2001**, *168*, 5–23.
- (3) Ravindranath, M. H.; Yesowitch, P.; Sumobay, C.; Morton, D. L. *Future Oncol.* **2007**, *3*, 201–214.
- (4) Kobata, A.; Amano, J. *Immunol. Cell Biol.* **2005**, *83*, 429–439.
- (5) Fernandes, B.; Sagman, U.; Auger, M.; Demetrio, M.; Dennis, J. W. *Cancer Res.* **1991**, *51*, 718–723.
- (6) Hiraizumi, S.; Takasaki, S.; Ohuchi, N.; Harada, Y.; Nose, M.; Mori, S.; Kobata, A. *Jpn. J. Cancer Res.* **1992**, *83*, 1063–1072.
- (7) Brockhausen, I.; Yang, J. M.; Burchell, J.; Whitehouse, C.; Taylor-Papadimitriou, J. *Eur. J. Biochem.* **1995**, *233*, 607–617.
- (8) Freire, T.; Bay, S.; von Mensdorff-Pouilly, S.; Osinaga, E. *Cancer Res.* **2005**, *65*, 7880–7887.
- (9) Dwek, M. V.; Lacey, H. A.; Leatham, A. J. *Clin. Chim. Acta* **1998**, *271*, 191–202.
- (10) Carter, P.; Presta, L.; Gorman, C. M.; Ridgway, J. B.; Henner, D.; Wong, W. L.; Rowland, A. M.; Kotts, C.; Carver, M. E.; Shepard, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4285–4289.
- (11) Slamon, D. J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; Baselga, J.; Norton, L. *N. Engl. J. Med.* **2001**, *344*, 783–792.
- (12) De Lorenzo, C.; Cozzolino, R.; Carpentieri, A.; Pucci, P.; Laccetti, P.; D'Alessio, G. *Carcinogenesis* **2005**, *26*, 1890–1895.
- (13) Lohrisch, C.; Piccart, M. *Clin. Breast Cancer* **2001**, *2*, 129–135, and discussion 136–1377.

- (14) Jefferis, R. *Biotechnol. Prog.* **2005**, *21*, 11–16.
- (15) Kamoda, S.; Nomura, C.; Kinoshita, M.; Nishiura, S.; Ishikawa, R.; Kakehi, K.; Kawasaki, N.; Hayakawa, T. *J. Chromatogr., A* **2004**, *1050*, 211–216.
- (16) Jefferis, R. *Methods Mol. Biol.* **2009**, *483*, 223–238.
- (17) Jones, A. *Ann. Oncol.* **2003**, *14*, 1697–1704.
- (18) Romond, E. H.; Perez, E. A.; Bryant, J.; Suman, V. J.; Geyer, C. E. J.; Davidson, N. E.; Tan-Chiu, E.; Martino, S.; Paik, S.; Kaufman, P. A.; Swain, S. M.; Pisansky, T. M.; Fehrenbacher, L.; Kutteh, L. A.; Vogel, V. G.; Visscher, D. W.; Yothers, G.; Jenkins, R. B.; Brown, A. M.; Dakhil, S. R.; Mamounas, E. P.; Lingle, W. L.; Klein, P. M.; Ingle, J. N.; Wolmark, N. N. *Engl. J. Med.* **2005**, *353*, 1673–1684.
- (19) Gennari, R.; Menard, S.; Fagnoni, F.; Ponchio, L.; Scelsi, M.; Tagliabue, E.; Castiglioni, F.; Villani, L.; Magalotti, C.; Gibelli, N.; Oliviero, B.; Ballardini, B.; Da Prada, G.; Zambelli, A.; Costa, A. *Clin. Cancer Res.* **2004**, *10*, 5650–5655.
- (20) Arnould, L.; Gelly, M.; Penault-Llorca, F.; Benoit, L.; Bonnetain, F.; Migeon, C.; Cabaret, V.; Fermeaux, V.; Bertheau, P.; Garnier, J.; Jeannin, J.; Coudert, B. *Br. J. Cancer* **2006**, *94*, 259–267.
- (21) Baumann, N. A.; Vidugiriene, J.; Machamer, C. E.; Menon, A. K. *J. Biol. Chem.* **2000**, *275*, 7378–7389.
- (22) Butler, M.; Quelhas, D.; Critchley, A. J.; Carchon, H.; Hebestreit, H. F.; Hibbert, R. G.; Vilarinho, L.; Teles, E.; Matthijs, G.; Schollen, E.; Argibay, P.; Harvey, D. J.; Dwek, R. A.; Jaeken, J.; Rudd, P. M. *Glycobiology* **2003**, *13*, 601–622.
- (23) Zhao, J.; Qiu, W.; Simeone, D. M.; Lubman, D. M. *J. Proteome Res.* **2007**, *6*, 1126–1138.
- (24) Saldova, R.; Royle, L.; Radcliffe, C. M.; Abd Hamid, U. M.; Evans, R.; Arnold, J. N.; Banks, R. E.; Hutson, R.; Harvey, D. J.; Antrobus, R.; Petrescu, S. M.; Dwek, R. A.; Rudd, P. M. *Glycobiology* **2007**, *17*, 1344–1356.
- (25) Peracaula, R.; Barrabés, S.; Sarrats, A.; Rudd, P. M.; de Llorens, R. *Dis. Markers* **2008**, *25*, 207–218.
- (26) Lattová, E.; Varma, S.; Bezabeh, T.; Petrus, L.; Perreault, H. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 671–685.
- (27) Sturiale, L.; Barone, R.; Fiumara, A.; Perez, M.; Zaffanello, M.; Sorge, G.; Pavone, L.; Tortorelli, S.; O'Brien, J. F.; Jaeken, J.; Garozzo, D. *Glycobiology* **2005**, *15*, 1268–1276.
- (28) Wuhler, M.; Porcelijn, L.; Kapur, R.; Koeleman, C. A. M.; Deelder, A.; de Haas, M.; Vidarsson, G. *J. Proteome Res.* **2009**, *8*, 450–456.
- (29) Irungu, J.; Go, E. P.; Zhang, Y.; Dalpathado, D. S.; Liao, H.; Haynes, B. F.; Desaire, H. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1209–1220.
- (30) Lee, C.; Pang, P.; Yeung, W. S. B.; Tissot, B.; Panico, M.; Lao, T. T. H.; Chu, I. K.; Lee, K.; Chung, M.; Lam, K. K. W.; Koistinen, R.; Koistinen, H.; Seppälä, M.; Morris, H. R.; Dell, A.; Chiu, P. C. N. *J. Biol. Chem.* **2009**, *284*, 15084–15096.
- (31) Lattová, E.; McKenzie, E. J.; Gruwel, M. L. H.; Spicer, V.; Goldman, R.; Perreault, H. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2983–2995.
- (32) Distler, U.; Hülsewig, M.; Souady, J.; Dreisewerd, K.; Haier, J.; Senninger, N.; Friedrich, A. W.; Karch, H.; Hillenkamp, F.; Berkenkamp, S.; Peter-Katalinić, J.; Müthing, J. *Anal. Chem.* **2008**, *80*, 1835–1846.
- (33) Montesino, R.; Toledo, J. R.; Sanchez, O.; Zamora, Y.; Barrera, M.; Royle, L.; Rudd, P. M.; Dwek, R. A.; Harvey, D. J.; Cremata, J. A. *J. Proteome Res.* **2009**, *8*, 546–555.
- (34) Kramer, R.; Weber, T. K.; Arcenci, R.; Ramchurren, N.; Kastrinakis, W. V.; Steele, G. J.; Summerhayes, I. C. *Br. J. Cancer* **1995**, *71*, 670–675.
- (35) Dall'Olio, F.; Malagolini, N.; Guerrini, S.; Serafini-Cessi, F. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 714–720.
- (36) Kudo, T.; Nakagawa, H.; Takahashi, M.; Hamaguchi, J.; Kamiyama, N.; Yokoo, H.; Nakanishi, K.; Nakagawa, T.; Kamiyama, T.; Deguchi, K.; Nishimura, S.; Todo, S. *Mol. Cancer* **2007**, *6*, 32.
- (37) Felgner, P. L.; Tsai, Y. J.; Sukhu, L.; Wheeler, C. J.; Manthorpe, M.; Marshall, J.; Cheng, S. H. *Ann. N.Y. Acad. Sci.* **1995**, *772*, 126–139.
- (38) Pirolo, K. F.; Xu, L.; Chang, E. H. *Curr. Opin. Mol. Ther.* **2000**, *2*, 168–175.
- (39) Bartusik, D.; Tomanek, B.; Lattová, E.; Perreault, H.; Fallone, G. *J. Pharm. Biomed. Anal.* **2010**, *51*, 192–201.
- (40) Turk, T. M.; Rees, M. A.; Pietrow, P.; Myers, C. E.; Mills, S. E.; Gillenwater, J. Y. *Prostate* **1999**, *38*, 137–143.
- (41) Lattová, E.; Perreault, H. *J. Chromatogr., A* **2003**, *1016*, 71–87.
- (42) Lattová, E.; Perreault, H. *Methods Mol. Biol.* **2009**, *534*, 65–77.
- (43) Lattová, E.; Chen, V. C.; Varma, S.; Bezabeh, T.; Perreault, H. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1644–1650.
- (44) Domon, B.; Costello, C. *Glycoconj. J.* **1988**, *5*, 397–409.
- (45) Lattová, E.; Perreault, H.; Krokhin, O. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 725–735.
- (46) Kauraniemi, P.; Hautaniemi, S.; Autio, R.; Astola, J.; Monni, O.; Elkhouloun, A.; Kallioniemi, A. *Oncogene* **2004**, *23*, 1010–1013.
- (47) Reyzer, M. L.; Caldwell, R. L.; Dugger, T. C.; Forbes, J. T.; Ritter, C. A.; Guix, M.; Arteaga, C. L.; Caprioli, R. M. *Cancer Res.* **2004**, *64*, 9093–9100.
- (48) Kui Wong, N.; Easton, R. L.; Panico, M.; Sutton-Smith, M.; Morrison, J. C.; Lattanzio, F. A.; Morris, H. R.; Clark, G. F.; Dell, A.; Patankar, M. S. *J. Biol. Chem.* **2003**, *278*, 28619–28634.
- (49) Radcliffe, C. M.; Arnold, J. N.; Suter, D. M.; Wormald, M. R.; Harvey, D. J.; Royle, L.; Mimura, Y.; Kimura, Y.; Sim, R. B.; Inogès, S.; Rodríguez-Calvillo, M.; Zabalegui, N.; de Cerio, A. L.; Potter, K. N.; Mockridge, C. I.; Dwek, R. A.; Bendandi, M.; Rudd, P. M.; Stevenson, F. K. *J. Biol. Chem.* **2007**, *282*, 7405–7415.
- (50) Goetz, J. A.; Mechref, Y.; Kang, P.; Jeng, M.; Novotny, M. V. *Glycoconj. J.* **2009**, *26*, 117–131.
- (51) Ishizuka, A.; Hashimoto, Y.; Naka, R.; Kinoshita, M.; Kakehi, K.; Seino, J.; Funakoshi, Y.; Suzuki, T.; Kameyama, A.; Narimatsu, H. *Biochem. J.* **2008**, *413*, 227–237.
- (52) Zhu, T. Y.; Chen, H. L.; Gu, J. X.; Zhang, Y. F.; Zhang, Y. K.; Zhang, R. A. *J. Cancer Res. Clin. Oncol.* **1997**, *123*, 296–299.
- (53) D'Arrigo, A.; Belluco, C.; Ambrosi, A.; Digito, M.; Esposito, G.; Bertola, A.; Fabris, M.; Nofrate, V.; Mammano, E.; Leon, A.; Nitti, D.; Lise, M. *Int. J. Cancer* **2005**, *115*, 256–262.

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