ANTICANCER RESEARCH *25*: 3689-3696 (2005)

Review

Gc Protein (Vitamin D-binding Protein): Gc Genotyping and GcMAF Precursor Activity

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Abstract. *The Gc protein (human group-specific component (Gc), a vitamin D-binding protein or Gc globulin), has important physiological functions that include involvement in vitamin D transport and storage, scavenging of extracellular G-actin, enhancement of the chemotactic activity of C5α for neutrophils in inflammation and macrophage activation (mediated by a GalNAc-modified Gc protein (GcMAF)). In this review, the structure and function of the Gc protein is focused on especially with regard to Gc genotyping and GcMAF precursor activity. A discussion of the research strategy "GcMAF as a target for drug discovery" is included, based on our own research.*

The Gc protein (human group-sepecific component (Gc)), a well-known vitamin D-binding protein (DBP) or Gc globulin, is a 55 kDa serum protein secreted by the liver and belonging to the albumin superfamily. The proposed secondary structure of the Gc protein (Gc1) is shown in Figure 1.

The physiological functions of the Gc protein include vitamin D transport and storage, scavenging of extracellular G-actin and enhancement of the chemotactic activity of C5a for neutrophils in inflammation and macrophage activation (1). This macrophage activation is mediated by a sugar-modified Gc protein designated as the Gc protein-derived macrophage activating factor (GcMAF) (2). This post-translational glycosylation, that converts the Gc protein to a macrophage activating factor (MAF precursor activity of Gc protein), is a very interesting process. We felt that a pleiotropic effect of Gc protein could be far-reaching and have initiated a drug discovery strategy based on the Gc protein as a lead. In this review, the structure and function of the Gc protein (vitamin D-binding protein) is focused on, especially with regard to Gc genotyping and GcMAF precursor activity. We also discuss our research project, "GcMAF as a target for drug discovery", which is based on results from our previous research (3-8).

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Key Words: Gc protein, Gc protein-derived macrophage activating factor (GcMAF), alpha-*N*-acetylgalactosaminidase, review.

Gc protein (vitamin D-binding protein)

Molecular structure of Gc protein. The molecular structure of the Gc protein (vitamin D-binding protein) bound to actin was reported independently by three groups in 2002 and 2003 (9-11). The X-ray structural analysis of the Gc protein itself has also been reported (12). These results provide the molecular structure with the complete amino acid sequence. Unfortunately, no complete structure of the Gc protein has included its sugar moiety, a structural feature in which we are very interested. Nonetheless, the structural data are very important for studies on the structure-function relationship of domain I as a vitamin D-binding site and domain III containing the six amino acid sequences of its C-terminal actin-binding site.

Genotype of Gc protein. The Gc protein gene is encoded on human chromosome 4, sublocalized to bands 4q11-q13.

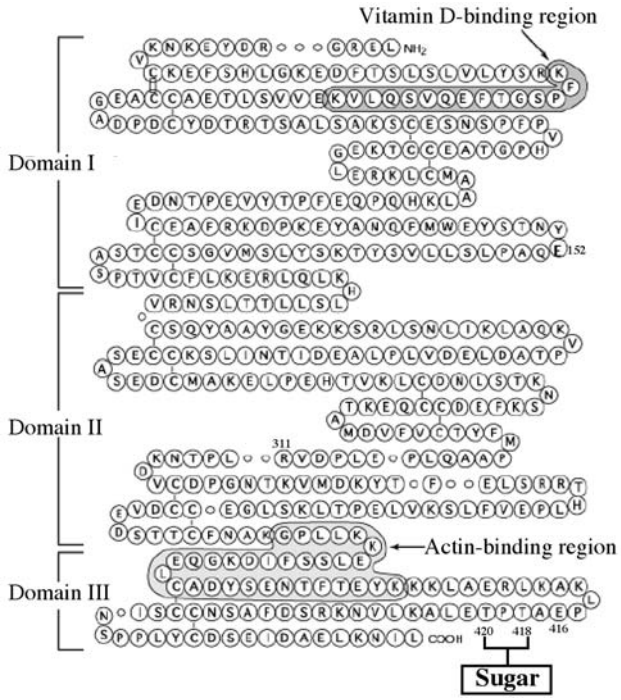

Figure 1. *Gc protein (Gc1): amino acid sequence and proposed secondary structure.*

Table I. *Gc: genotype and phenotype.*

Allele	Amino acid position			
	152	311	416	420
Gc*1F	GAA	AGA	GAT	ACG
Gc*1S	GAA	AGA	GAG	ACG
Gc*2	GGA	GAG	GAT	AAG
Phenotype	**Amino acid position**			
	152	311	416	420
Gc1F	Glu	Arg	Asp	Thr
Gc1S	Glu	Arg	Glu	Thr
Gc2	Gly	Glu	Asp	Lys

Table II. *Gc protein: Gc phenotypes and their carbohydrate structures.*

Gc Type	Gc1F	Gc1S	Gc2
Oligosaccharide	418 or 420 Thr Gal-GalNAc SA	418 or 420 Thr Gal-GalNAc α-Man	418 Thr Gal-GalNAc

Gal: galactose; GalNAc: N-acetylgalactosamine; SA: sialic acid; α-Man: α-mannose

There are three common alleles (Gc*2, Gc*1F and Gc*1S) and more than 120 variants of the Gc system in the human population (13). These are divided into six main genotypes, which include three homozygotes, Gc1F-1F, Gc1S-1S and Gc2-2, and heterozygotes made up of Gc1F-1S, Gc2-1F and Gc2-1S. The primary division of the human Gc phenotype contain two types of Gc1 and Gc2, as shown in Table I (prepared based on ref. 14-18), which differ in only four amino acids (152, 311, 416 and 420).

Gc1 is further divided into Gc1S and Gc1F, which differ in only one amino acid (16). In the Gc genotype, there is a one-base difference between Gc1S and Gc1F and a six-base difference between Gc1S and Gc2. There are three major sugar moieties in human Gc proteins. As shown in Table II, Gc1F contains a branched trisaccharide with N-acetylgalactosamine (GalNAc) attached to the core protein, a galactose moiety, and a sialic acid (in Gc1F) to a mannose moiety (in Gc1S). Gc2 has a simple glycosylation pattern with a core GalNAc linked to a terminal galactose moiety (18).

Note, however, that in human Gc protein more than 90% of Gc2 is likely to be in the non-glycosylated form (19). Recently, polymorphism of the Gc protein has attracted attention as a genetic marker for risk of chronic obstructive pulmonary disease (COPD) (20-22). Thus, Schellenberg *et al.* suggested that Gc1F-1F may indicate a hereditary risk of COPD and, conversely, that Gc2-2 may have a protective effect against COPD. They based this suggestion of the fact that Gc1F-1F is more active than Gc2-2 with respect to GcMAF precursor activities (potency of macrophage activation in the inflammation site) and the relationship between Gc gene polymorphism and the sensitivity of COPD (22). The relationship between the Gc gene polymorphism and GcMAF precursor activity of Gc should also be investigated.

GcMAF(Gc protein-derived macrophage activating factor)

Generation of GcMAF in inflammation. As shown in Figure 1, Yamamoto proposed an inflammation-initiated macrophage-activation cascade where the Gc protein participates as a precursor of GcMAF (Gc protein-derived macrophage activating factor, macrophage activation factor, DBP-*maf*) (2, 18, 23-25). Thus, it was demonstrated that GcMAF was derived from the Gc protein through the stepwise modification of its sugar moiety. As shown in Figure 1, an initial removal of a galactose moiety, mediated by a membrane-bound beta-galactosidase induced in inflammation by lyso-PC on B cells, is followed by removal of a sialic acid residue by membrane-bound sialidase on T cells. It is noteworthy that an activation time of only 3 hours is required to produce fully active ingestion function and

cytotoxicity (25). The macrophage activation process by GcMAF is thought to be controlled by a mechanism different from that by lipopolysaccharide (LPS, endotoxin).

Activation of osteoclasts by GcMAF. Swamy *et al.* (26) reported the dose-dependent osteoclast-activating property of GcMAF and evaluated the essential role of glycosylation in this process. Thus, the binding of 25-hydroxyvitamin-D_3 to GcMAF had no effect on the osteoclast-activating ability of GcMAF. The activated form of a full length, but non-glycosylated, recombinant DBP, expressed in *E. coli*, showed no activity in the *in vitro* assay. Contrary to this finding, baculovirus-expressed recombinant GcMAF demonstrated significant osteoclast-activating activity. These data support the essential role of the core GalNAc (N-acetyl-galactosamine) moiety in GcMAF in the activation of osteoclasts.

Adjuvant effect of GcMAF for photodynamic therapy (PDT). Korbelik *et al.* examined the effect of Photofrin-based photodynamic therapy (PDT) and adjuvant treatment with GcMAF using a mouse SCCVII tumor model (squamous cell carcinoma) (27) and found that GcMAF can markedly enhance the curative effect of PDT. The most effective GcMAF therapy consisted of a combination of intraperitoneal and peritumoral injections (50 and 0.5 ng/kg, respectively) administered on days 0, 4, 8 and 12 after PDT. PDT treatment alone gave a curative rate of 25% of the treated tumors, whereas the GcMAF regimen boosted the cures to 100%. The PDT-induced immunosuppression, assessed by the evaluation of the delayed-type contact hypersensitivity response in treated mice, was greatly reduced with the combined GcMAF treatment.

Antitumor activity of GcMAF. Koga *et al.* (28) reported that Ehrlich ascites tumor-bearing mice treated with GcMAF (100 pg/mouse) showed increased survival time compared with the control. Although this *in vitro* antitumor effect of GcMAF is very interesting, it must be viewed with caution because the data were obtained from experiments using a small number of mice.

Antiangiogenic activity of GcMAF. Recently two groups independently reported the antiangiogenic activity of GcMAF. Kanda *et al.* (29) observed that GcMAF inhibited endothelial cell proliferation, chemotaxis and tube formation, all stimulated by fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor-A, or angiopoietin 2. The proposal was made that this antiangiogenic activity of GcMAF was mediated through the CD36 receptor. Similarly, Kisker *et al.* (30) reported the same antiangiogenic activity for GcMAF and, more interestingly, they also reported a potent inhibition of the growth of human pancreatic cancer in immune compromised mice (SCID). At higher doses (4 ng/kg/day for 28-30 days), GcMAF caused tumor regression in SCID mice-implanted human pancreatic tumors (BxPC-3 and SU88.86). Histological examination revealed that the treated tumors had a higher number of infiltrating macrophages, as well as a reduced microvessel density and an increased level of apoptosis relative to untreated tumors. Recently, Onizuka *et al.* (31) reported that GcMAF and antithrombin III (aaAT-III), as anti-angiogenic molecules, were able to cause regression of tumors in SCID mice, demonstrating a potent inhibition of endothelial cell proliferation. Moreover, these angiogenesis inhibitors induced tumor dormancy in the animal model. They thus suggested that therapy using angiogenesis inhibitors may become a new strategy for the treatment of pancreatic cancer in the near future. These interesting results prompted us to explore the development of new antiangiogenic drugs based on GcMAF as a lead compound.

Serum alpha-*N*-acetylgalactosaminidase (alpha-NaGalase), Gc protein and GcMAF

The extracellular matrix-degrading enzyme, alpha-*N*-acetylgalactosaminidase (alpha-NaGalase), is generally produced in liver cells. Yamamoto *et al.* (23) reported that alpha-NaGalase deglycosylation of serum Gc protein results in loss of its precursor activity (shown in Figure 2), which, in turn, contributes to immunosuppression in cancer patients. Thus, the levels of serum alpha-NaGalase of individual patients have an inverse correlation with the precursor activities of their serum Gc protein. Surgical removal of the tumor resulted in a subtle decrease in serum alpha-NaGalase activity, with a concomitant increase in the precursor activity. This indicated that serum alpha-NaGalase activity is directly proportional to the tumor burden. From these results, they suggested that alpha-NaGalase activity in the blood can serve as a diagnostic/prognostic index (24, 32). For example, the decrease in serum alpha-NaGalase activity was more rapid after the treatment of SCCVII and EMT6 tumors by photodynamic therapy (PDT) and was dependent on the PDT dose. The treatments (based on the photosensitizers Photofrin or mTHPC) that were fully curative resulted in a reduction of alpha-NaGalase activity to background levels within 2 or 3 days after PDT (33). Matsuura *et al.* (34) delineated the effects of alpha-NaGalase produced by human salivary gland adenocarcinoma (SGA) cells on the activity of GcMAF. High exo-alpha-NaGalase activity was detected in the SGA cell line HSG. HSG alpha-NaGalase had both exo- and endo-enzyme activities, cleaving the Gal-GalNAc and GalNAc residues linked to Thr/Ser, but

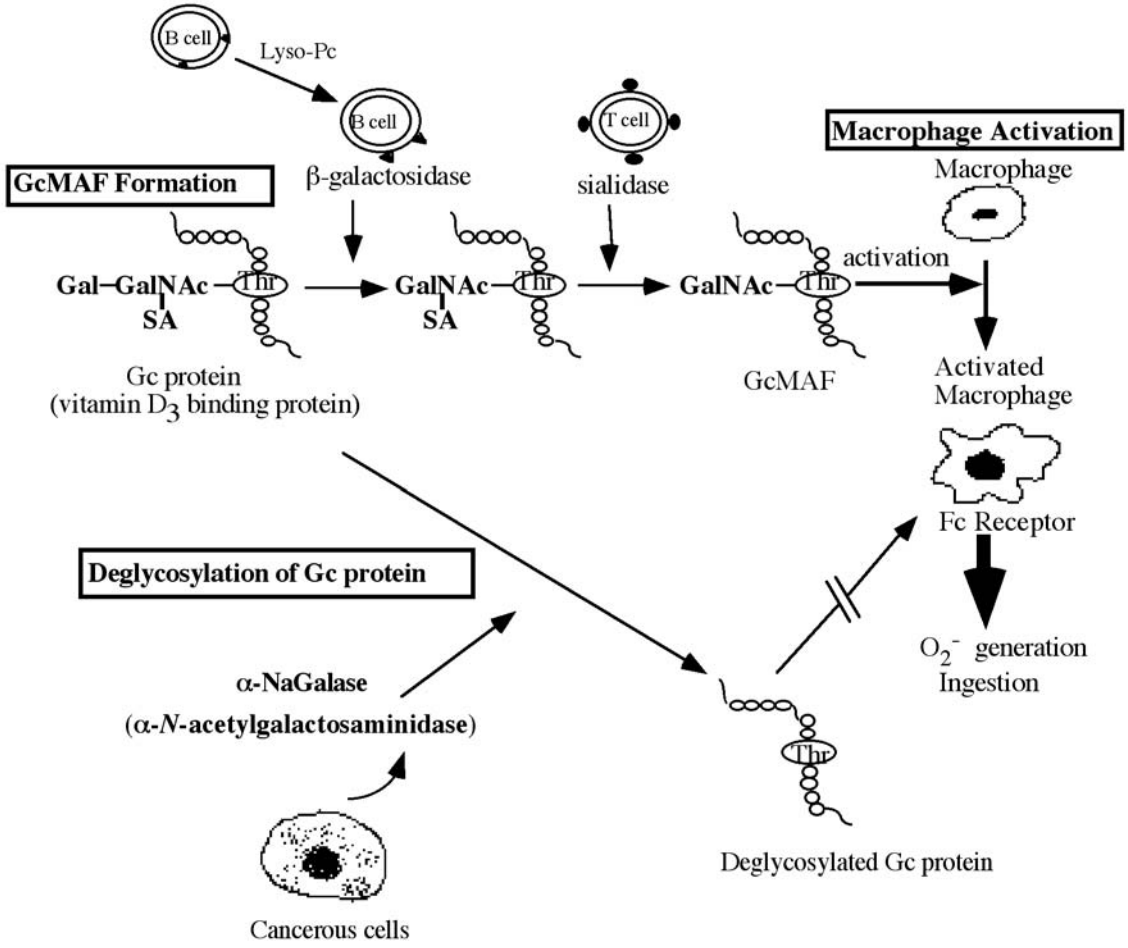

Figure 2. *Cascade from Gc protein to GcMAF and deglycosylation of Gc protein.*

not releasing the [NeuAc2-6]GalNac residue. Furthermore, GcMAF, enzymatically prepared from the Gc protein, enhanced the superoxide-generation capacity and phagocytic activity of monocytes/macrophages. However, GcMAF treated with purified alpha-NaGalase did not exhibit these effects. They concluded that HSG possessed the capacity to produce larger quantities of alpha-NaGalase, which inactivates GcMAF produced from the Gc protein, resulting in a reduced phagocytic activity and superoxide-generation capacity of monocytes/macrophages. They strongly suggested, from their data, that HSG alpha-NaGalase acts as an immunodeficiency factor in cancer patients. Their finding of HSG alpha-NaGalase having both exo- and endo-enzyme activities is a very interesting result, since normal human alpha-NaGalase possesses only exo-type processing activity. We hope that further investigation will confirm these results, thus indicating a greater contribution of GcMAF and its precursor, the Gc protein, to the quality of life of cancer patients.

Design of small molecules mimicking GcMAF

GcMAF is a macromolecule with diverse and critical physiological functions. As such, the development of small molecules mimicking GcMAF, more appropriate for clinical use, represents an inviting strategy for medicinal chemists. In an initial approach, Yamamoto *et al.* (24), who discovered GcMAF, designed and prepared a cloned GcMAF construct. It consisted of Gc protein domain III (macrophage activating site) that had 85 amino acids from the C-terminal (458) to the 85th amino acid (374) having the GalNAc moiety. The protein was cloned *via* a baculovirus vector and treated with immobilized beta-galctosidase and sialidase to yield the cloned, completely glycosylated (GalNAc moiety-containing) GcMAF. They reported that four administrations of cloned GcMAF (100 pg/mouse) to mice transplanted with $5x10^5$ Ehrlich ascites tumor cells with 4-day intervals produced an extended survival of at least 90 days and lowered serum alpha-

NaGalase to a non-detectable level between days 30 and 90. However, no data were provided comparing the macrophage activation of cloned GcMAF to that of full-size GcMAF, an issue that would be important in drug development. Recently, Schneider *et al.* (35) developed a small molecule 14mer-peptide GcMAF mimic and its GalNAc-containing glycopeptide, that was equivalent to the amino acid sequence between 418 and 431 of Gc protein domain III. The administration of this 14mer peptide, with or without GalNAc, at 0.4 ng/g body weight, to a rat for 2 weeks result in increased bone density comparable to that elicited by GcMAF. This result stands in contrast to previous data, which indicated that the GalNAc sugar group is essential to the macrophage-activating function of GcMAF (see ref. 34). However, these experiments did not include an *in vitro* study of macrophage activation, so direct comparison of the results is not possible.

Our GcMAF research and design of "dramatype" glycodendripeptides

Based on the results of our recent research (3-8), we are now focusing on GcMAF and its precursor Gc protein as leads to develop small-molecule immunopotentiators for potential use in cancer adjuvant therapy. In this section, our drug-discovery strategy for chemical modification, that targets the sugar processing of Gc protein, is described.

First, we prepared GcMAF (Gc1F-1F) by treating human serum Gc protein, purified using our homemade 25-hydroxyvitamin D_3 affinity chromatography, with immobilized beta-glycosidase and sialidase (3). We then compared the effect of GcMAF on activation of mouse peritoneal macrophage compared with LPS and IFN-alpha as general macrophage activators. After 3-hour incubation, GcMAF displayed potent macrophage activation, as shown by enhancement of the mouse peritoneal macrophage superoxide generation. As expected, 10 pg/ml GcMAF treatment demonstrated higher activity than did 10 pg/ml Gc protein. In mice, peritoneal macrophage GcMAF (10 pg/ml) showed a macrophage-activating function at the lowest concentration compared to the other general macrophage activators LPS (10 μg/ml) and IFN-gamma (500 U/ml) (4).

With mouse peritoneal macrophage, GcMAF (10 pg/ml) also enhanced the phagocytosis activity more than that seen in either control (non-treatment), 10 μg/ml LPS or 10 pg/ml Gc protein (4). Very interestingly, GcMAF activated the mouse peritoneal macrophage with no release of nitric oxide (with 1-100 pg/ml GcMAF) and TNF (with 100-1000 pg/ml GcMAF), in contrast to LPS (5). Consistent with the results of Matsuura, Uematsu and others (34), we also found that alpha-NaGalase activity at pH 7.0 was retained in the lysate of the human hepatoma cell line HepG2 (lysosomal glycosylases generally lose their activity at pH 7.0) (6).

We also postulated that the degree of macrophage activation might correlate with the changes in CD4 counts and inhibition of progress of AIDS, whereas alpha-NaGalase activity levels should be inversely proportional to CD4 counts. Therefore, we examined the relationship between alpha-NaGalase activity and the quality of life status of Tai HIV-infected patients (7). As expected, we found that the serum alpha-NaGalase activities in the patient groups with increased CD4 counts were lower than those in the patient groups with steady or reduced CD4 counts. Thus, there was a negative correlation between alpha-NaGalase activity and CD4 counts.

In order to elucidate the relationship between Gc polymorphism and Gc MAF precursor activity, we estimated the phagocytic ability of three homotypes of the Gc protein, Gc1F-1F, Gc1S-1S and Gc2-2, through processing of their carbohydrate moiety. The Gc1F-1F phenotype was shown to possess a Gal-beta 1-4 GalNAc linkage by the analysis of GcMAF precursor activity using beta 1-4 linkage-specific galactosidase from the jack bean. The GcMAF precursor activity of the Gc1F-1F phenotype was the highest of the three Gc homotypes. We suggest that the Gc polymorphism and carbohydrate diversity of the Gc protein are significant factors involved in its pleiotropic effects.

We have previously used a core structure with dendrimeric lysine residue to successfully design an endostatin-surface-mimic antiangiogenic/heparin-binding arginine dendrimer (36). Based on this same strategy, we plan to synthesize similar GcMAF-mimic dendrimers or GalNAcSer dendrimers (these glycopeptidic dendrimers are termed "glycodendripeptides"). The glycodendripeptides will be designed so as to possess an amino acid sequence that includes Thr/Ser substituted with a GalNAc-sugar moiety. A prototypical glycodendripeptide, $GalNAc_8S_8K_4K_2KG$-OH, is shown in Figure 3.

Conclusion

The Gc protein alleles, Gc*1F, Gc*1S and Gc*2, are important genetic risk factors for COPD. The Gc protein is also a very attractive and interesting molecule that acts as a vitamin D-binding protein and actin-scavenger protein. In addition, there are pleiotropic effects (corresponding to the dramatype), probably controlled by the sugar moiety, that include macrophage activation, antiangiogenic activity and antitumor activity. We intend to study the possible use of the Gc protein and GcMAF for the development of new antitumor agents based on the sugar-processing of these proteins. Collaborative, multidisciplinary approaches would be most effective, and it is hoped that this review paper will stimulate such research.

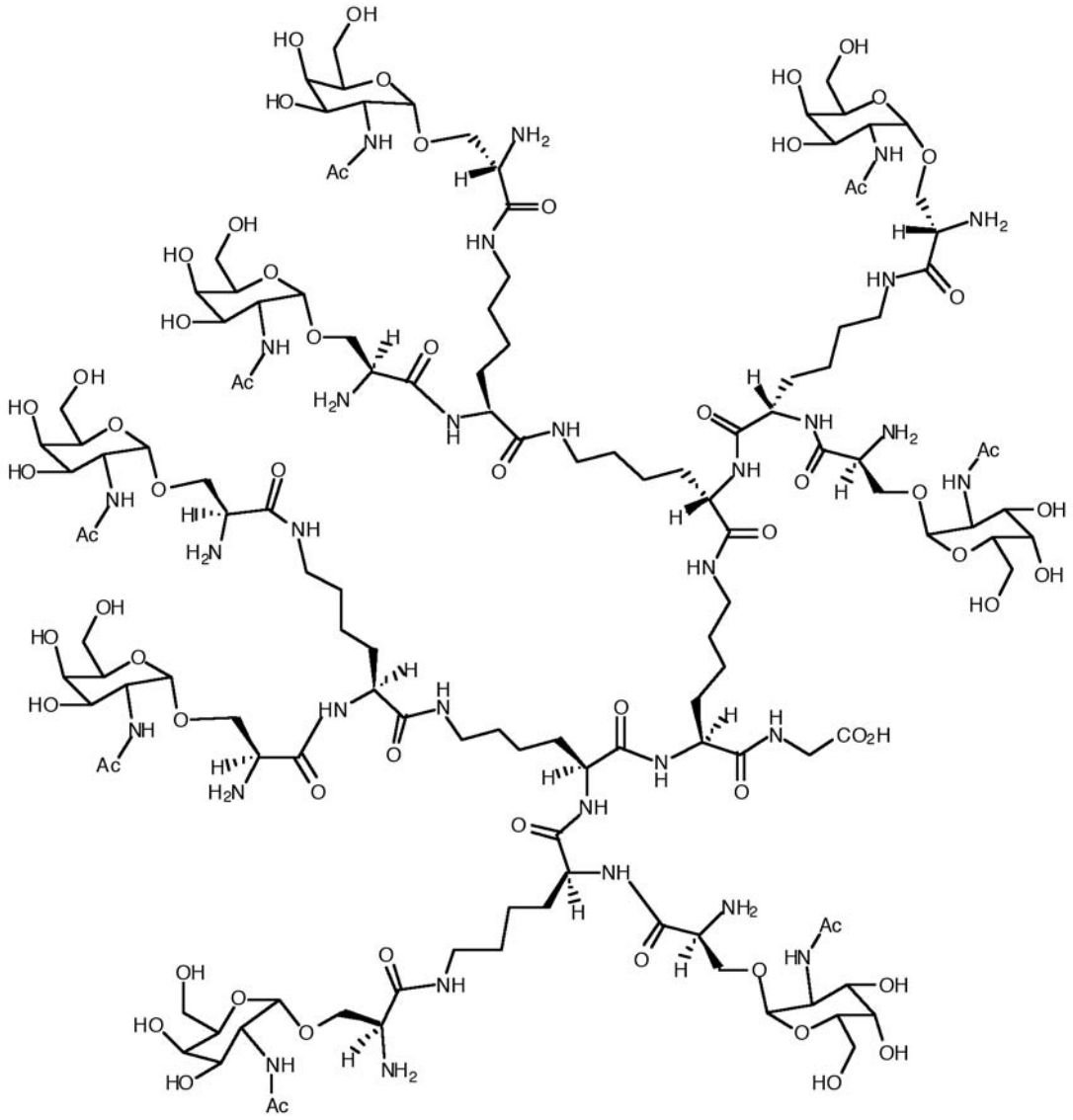

Figure 3. *Structure of GcMAF mimic dendrimer $GalNAc_8S_8K_4K_2KG$-OH.*

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Received June 3, 2005
Accepted July 12, 2005