

Type of the Paper (Research Article)

# *In vivo* Glycan Engineering via the Mannosidase I Inhibitor (Kifunensine) Improves Efficacy of Rituximab Manufactured in *Nicotiana benthamiana* Plants

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**Abstract:** N-glycosylation has been shown to affect the pharmacokinetic properties of several classes of biologics including monoclonal antibodies, blood factors, and lysosomal enzymes. In the last two decades, N-glycan engineering has been employed to achieve a N-glycosylation profile that is either more consistent or aligned with a specific improved activity (i.e. effector function or serum half-life). In particular, attention has focused on engineering processes *in vivo* or *in vitro* to alter the structure of the N-glycosylation of the Fc region of anti-cancer monoclonal antibodies in order to increase antibody-dependent cell-mediated cytotoxicity (ADCC). Here we applied the mannosidase I inhibitor kifunensine to the *Nicotiana benthamiana* transient expression platform to produce an afucosylated anti-CD20 antibody (rituximab). We determined the optimal concentration of kifunensine used in the infiltration solution, 0.375  $\mu$ M, which was sufficient to produce exclusively oligomannose glycoforms, at a concentration 14 times lower than previously published levels. The resulting afucosylated rituximab revealed a 14-fold increase in ADCC activity targeting the lymphoma cell line Wil2-S when compared with rituximab produced in the absence of kifunensine. When applied to the cost-effective and scalable *N. benthamiana* transient expression platform, the use of kifunensine allows simple in-process glycan engineering without the need for transgenic hosts.

**Keywords:** ADCC, Glycosylation, Kifunensine, Plant made pharmaceuticals, Monoclonal Antibody

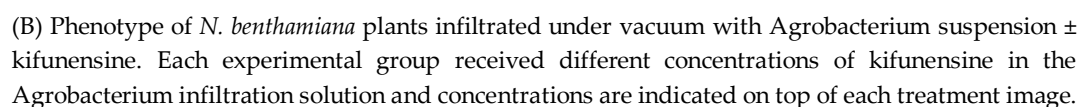
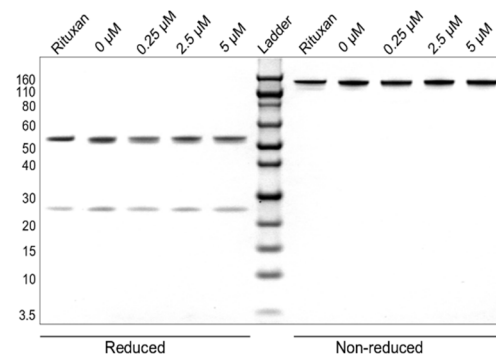
## 1. Introduction

Monoclonal antibodies (mAbs) represent the fastest growing class of therapeutics [1,2] and have been especially beneficial in the treatment of cancer [3]. Since the approval of the first anti-cancer monoclonal antibody in 1986 [4], several innovations have improved the potency of monoclonal antibodies used in immunotherapies that offer increased drug efficiency and/or lower drug dosage for a specific treatment. Among them, glycan engineering of the oligosaccharides attached to Asn297 of the Fc region of the heavy chain has been shown to affect antibody-dependent cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and binding to the neonatal Fc

receptor, FcRn [5–7]. Specific oligosaccharides influence the affinity of the antibody Fc domain to Fc receptor present on effector cells resulting in altered biological functions. For example, the removal of terminal galactose residues on mammalian cell-derived antibodies lowered C1q binding [8,9], while ADCC activity is almost completely dependent on the presence or absence of fucose residues bound to the glycosylation core [10–12]. Several approaches have been employed to manufacture monoclonal antibody with a decreased or absent core fucosylation. One strategy is to use cell lines or organisms with modified glycosylation pathways [13–16]. The alteration of the expression of key enzymes in the host glycosylation pathway such as the mammalian  $\alpha$ 1,6-fucosyltransferase [15], the plant  $\alpha$ 1,3-fucosyltransferase [17–21], the GDP-mannose 4,6-dehydratase [11,22], or the  $\beta$ 1,4-N-Acetylglucosaminyltransferase III [14,23] led to afucosylated antibodies with improved anti-tumor activity. This led to the approval of mogamulizumab (POTELIGIO®, Kyowa Hakko Kirin) [24] and obinutuzumab (Gazyva®, Roche) [25] in 2012 and 2013, respectively, both produced in glycoengineered mammalian cell lines. Another approach to alter the antibody glycosylation profile is to modify the culture conditions of the host cells by adjusting the growth environment [26] or supplementing the media with inhibitors of enzymes in the glycosylation pathway such as N-butyldeoxynojirimycin (NB-DNJ), mannosatin A, swainsonine or kifunensine [27–31]. Kifunensine from the actinomycete *Kitasatosporia kifunense* 9482 inhibits class I  $\alpha$ -mannosidases and blocks N-glycan synthesis at the Man<sub>8</sub>GlcNAc<sub>2</sub> (Man<sub>8</sub>) or Man<sub>9</sub>GlcNAc<sub>2</sub> (Man<sub>9</sub>) stage before the core fucose is added [28,32]. In mammalian cell culture, kifunensine was successfully employed to produce protein with >90% high-mannose content [28,29,33,34]. This effect was similar across many different proteins including antibodies, suggesting that this simple treatment could be applied broadly. Compared to other  $\alpha$ -mannosidase I inhibitors, kifunensine is highly effective on mammalian cell culture without significantly affecting cell growth or protein yield, even at concentrations as low as 100 ng/mL culture [29,33,34]. Similar to mammalian cell studies, kifunensine was used in conjunction with the *Nicotiana benthamiana* transient protein expression systems to produce proteins with >98% afucosylated high-mannose glycans [35–37]. In plants, the non-human  $\alpha$ 1,3-fucose and  $\beta$ 1,2-xylose residues are commonly added in the Golgi apparatus after mannose trimming by mannosidases in the endoplasmic reticulum. Upon kifunensine treatment, addition of  $\alpha$ 1,3-fucose and  $\beta$ 1,2-xylose residues were not observed on the Man<sub>3</sub> to Man<sub>9</sub> structures [35,37]. However, the amount of kifunensine used in these studies was at or above 1.16  $\mu$ g/mL (5  $\mu$ M) [35,37] which significantly increases production costs at manufacturing scale.

Here, we determined the optimal concentration of kifunensine and demonstrate that kifunensine addition at a concentration of 0.375  $\mu$ M (87 ng/mL) in the *Agrobacterium* infiltration solution of *N. benthamiana* plants during the vacuum infiltration process allows the production of exclusively high-mannose rituximab, an anti-CD20 monoclonal antibody approved for the treatment of non-Hodgkin's lymphoma (NHL) [38]. More importantly, we demonstrate that afucosylated high-mannose decorated antibody derived from the treated plants exhibits increased ADCC effector function as compared with rituximab derived from non-treated plants. The increased ADCC activity was verified using effector cells carrying both Fc $\gamma$ RIIIa-V158 and Fc $\gamma$ RIIIa-F158 allotypes. Although afucosylated high-mannose antibody could also be generated by incorporating an ER retention signal, our approach eliminates the need for modification of the primary sequence and also allows for recovery of the secreted product from the apoplast wash fluid without homogenizing the plant tissue which can simplify downstream processing [39–41]. Combined with the scalability [41] and

Figure1



(C) Quantification of rituximab in crude protein extracts using Biolayer interferometry (BLItz®, ForteBio). Expression levels of rituximab in 7 dpi plant extracts are reported in mg rituximab/kg fresh weight (FW). Error bars represent standard deviations of duplicated expression measurements, where n=3.

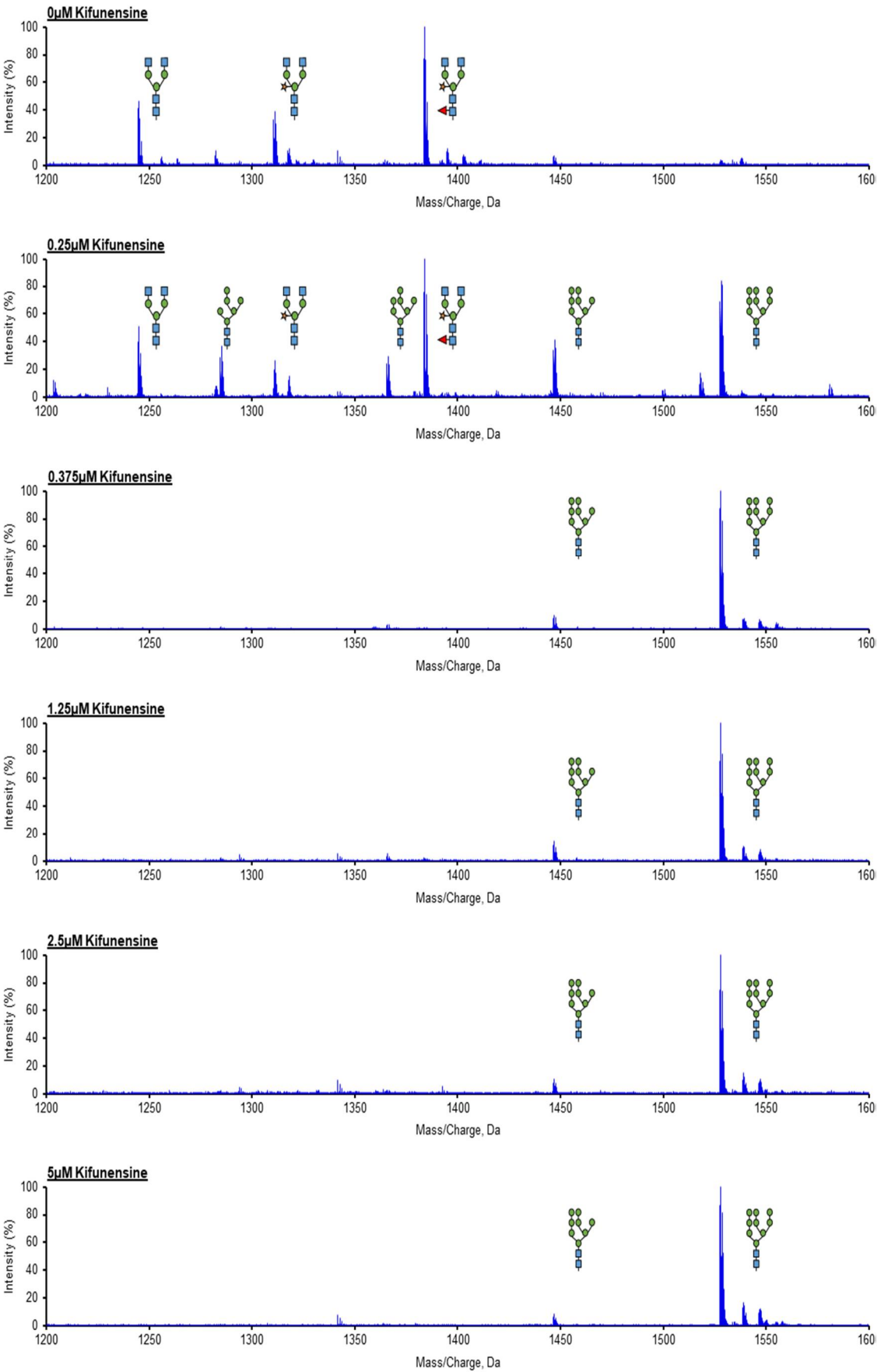
(D) SDS-PAGE analysis of purified rituximab samples under reduced and non-reduced conditions. Rituxan, plant-made rituximab with no kifunensine, 0.25  $\mu$ M kifunensine, 2.5  $\mu$ M kifunensine, and 5  $\mu$ M kifunensine were separated on a 4-12% Bis-Tris gel along with Novex sharp pre-stained protein standard.

We have hypothesized that a treatment with kifunensine would inhibit trimming of mannose residues in the endoplasmic reticulum (ER), subsequently preventing the addition of  $\alpha$ 1,3-fucose and  $\beta$ 1,2-xylose residues on the polysaccharide core (Figure 1A). To do so, plants were vacuum infiltrated in a solution of *Agrobacteria* with or without kifunensine, ranging from 0 to 5  $\mu$ M (0  $\mu$ g/mL to 1.16  $\mu$ g/mL). Visual observation of infiltrated plants from 3 to 7 days post infiltration (dpi) revealed no noticeable phenotypic or morphological differences between treated and untreated control plants (Figure 1B). Rituximab expression levels were quantified at 7 dpi and revealed a low to moderate increase (up to 34%) in antibody expression between untreated and treated plants, with the average rituximab expression level ranging from 288 mg/kg to 385 mg/kg whole plant fresh weight (FW) in treated plants, compared to 287 mg/kg whole plant FW in untreated plants (Figure 1C). These observations demonstrated that the kifunensine treatments were not detrimental to plant growth or protein expression. To evaluate the effect of kifunensine treatments on the integrity and assembly of rituximab, SDS-PAGE analysis was carried out under reduced and non-reduced conditions. As illustrated in Figure 1D, rituximab derived from kifunensine treated and untreated conditions appeared intact and fully assembled. Non-reduced rituximab migrated at the expected molecular weight (MW) of ~145 kDa, while the reduced heavy and light chains migrated at the expected MW of ~50kDa and ~25kDa, respectively (Figure 1D).

## 2.2. A concentration of 0.375 $\mu$ M of kifunensine is sufficient to produce rituximab with afucosylated, oligomannose-type glycoforms exclusively

The N-glycosylation profiles of purified rituximab expressed in *N. benthamiana* were evaluated by LC-MS/MS analysis (Figure 2).

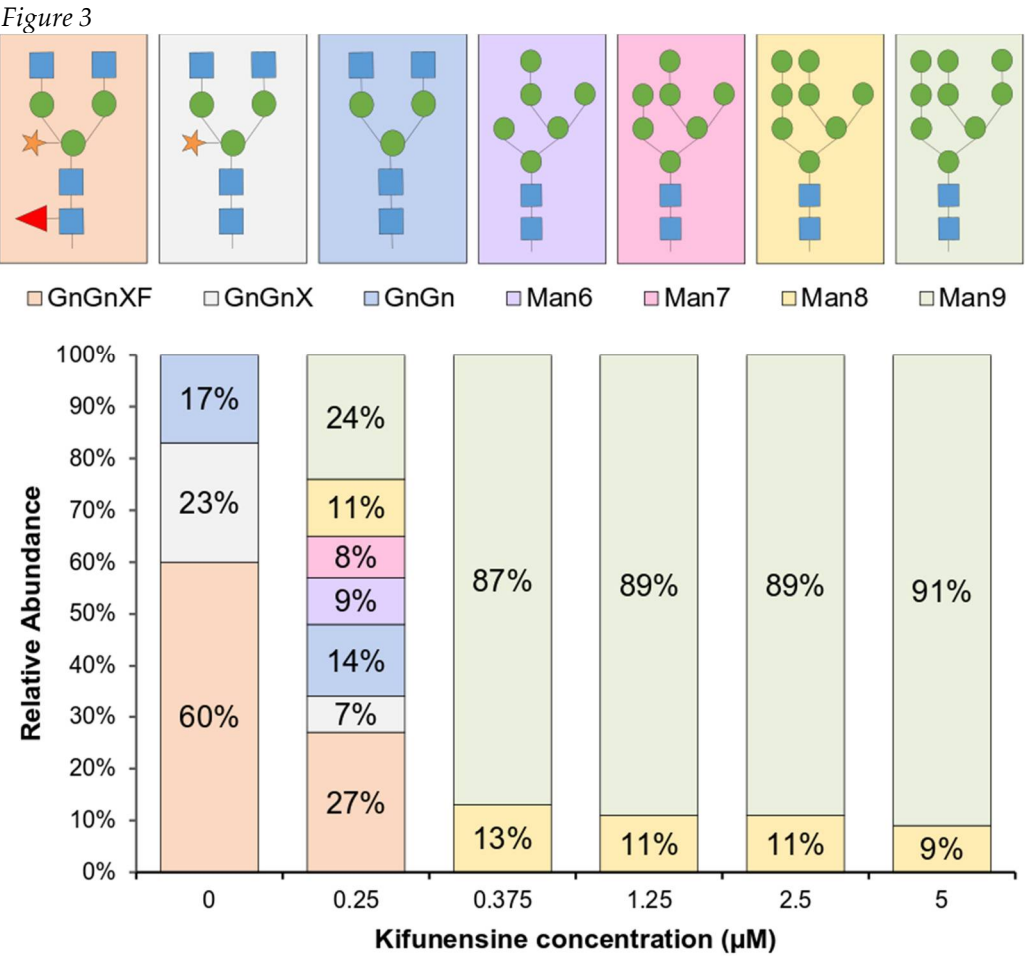
Figure 2.





**Figure 2.** N-Glycan analysis of rituximab expressed in *N. benthamiana* plants with/without kifunensine. LC-MS glycopeptide profiling of rituximab expressed in control and kifunensine treated plants. The distribution of glycoforms in each sample is illustrated and kifunensine concentration are indicated on each image.

The major glycoforms were compared based on the relative intensity of the Asn297 glycopeptide masses identified by LC-MS/MS. We first established the glycoform distribution of rituximab derived from untreated plants (Figure 2; 0  $\mu$ M kifunensine). As previously described in the literature, this plant-derived rituximab control exhibited primarily complex-type N-glycans [42], with the most abundant N-glycan structure being GlcNAc<sub>2</sub>(Xyl)Man<sub>3</sub>(Fuc)GlcNAc<sub>2</sub> (Figure 3).



**Figure 3.** LC-MS glycopeptide profiling of rituximab samples. The ratio between oligomannose glycoforms (Mans, Man<sub>9</sub>) and hybrid glycoforms (GnGn, GnGnXF and GnGnX) are represented in histograms. Kifunensine concentrations are indicated on the X axis and glycoform percentages are indicated on each sample.

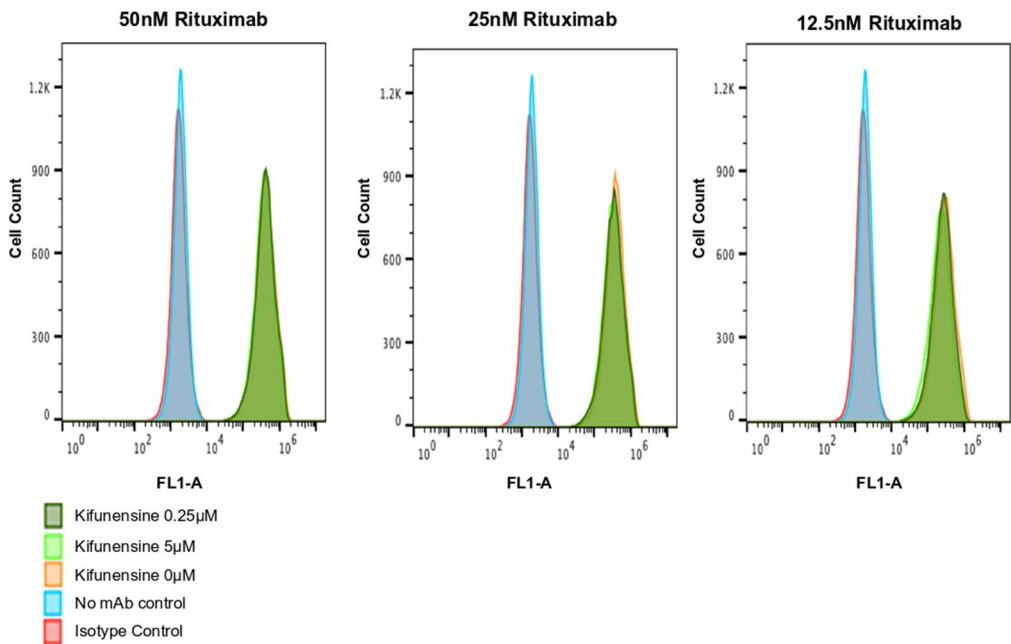
On the other hand, there were significant differences in rituximab N-glycan profiles between untreated and treated samples. Complete conversion of plant complex glycans to oligomannose-type glycans was observed when *N. benthamiana* plants were infiltrated with 'higher range' and 'medium range' concentrations of kifunensine (from 5  $\mu$ M to 0.375  $\mu$ M). The GlcNAc<sub>2</sub>Man<sub>9</sub> (Man<sub>9</sub>) and GlcNAc<sub>2</sub>Man<sub>8</sub> (Man<sub>8</sub>) were the major glycoforms observed (Figure 2; 0.25 to 5  $\mu$ M kifunensine) with Man<sub>9</sub> being the most abundant. In fact, the same oligomannose-type glycoform distribution was observed whether 0.375  $\mu$ M or higher kifunensine concentration was used, indicating that 0.375  $\mu$ M is sufficient to provide homogeneous rituximab with oligomannose-type glycans (Figure 3). When

lower concentrations of kifunensine were used, a mixture of oligomannose, hybrid, and complex glycans was detected (Figure 2; 0.25  $\mu$ M kifunensine). For instance, the glycosylation profile of rituximab from plants treated with 0.25  $\mu$ M contained more than ~48% hybrid/complex glycan modifications (GnGn, GnGnX and GnGnXF) and ~52% oligomannose (Man<sub>6</sub>, Man<sub>7</sub>, Man<sub>8</sub> and Man<sub>9</sub>) glycosylation (Figure 3). Importantly, no  $\alpha$ 1,3-fucose or  $\beta$ 1,2-xylose residues were detected in rituximab derived from plants treated with  $\geq$ 0.375  $\mu$ M kifunensine.

2.3 Rituximab decorated with oligomannose residues binds target CD20 as efficiently as Rituxan®

The mode of action of rituximab is characterized first by binding to the tumor cell surface antigen CD20 present of lymphoma cells before cell cytotoxicity is induced. Thus, to confirm that rituximab derived from kifunensine-treated plants binds CD20, a flow cytometry analysis using the human B cell line Wil2-S was performed. Rituximab samples purified from plants treated with 0, 0.25, 0.375, 1.25, 2.5, and 5  $\mu$ M of kifunensine was compared to Rituxan® for the proteins' ability to bind CD20 on Wil2-S cells. This analysis revealed no difference in the binding affinity of rituximab derived from plants treated with 5  $\mu$ M or 0.25  $\mu$ M kifunensine as compared with Rituxan® (Figure 4). In all antibody independent assays and isotype controls no CD20 binding signal was observed.

Figure 4



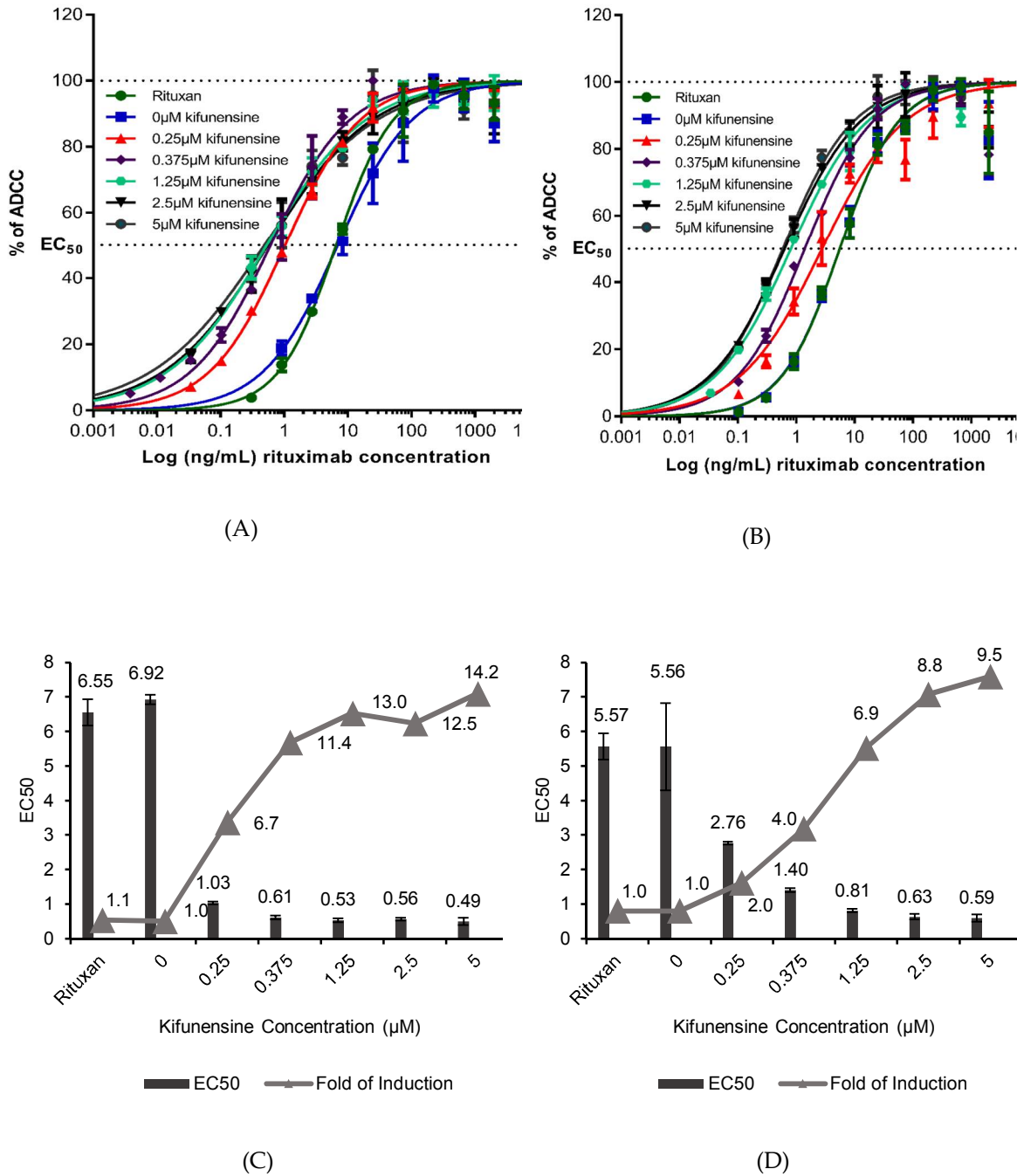
**Figure 4.** CD20 binding assay of rituximab treated with kifunensine 5  $\mu$ M, 0.25  $\mu$ M and untreated controls with Flow Cytometry analysis. Plant-made rituximab was used at concentrations of 50 nM, 25 nM and 12.5 nM. Antibodies bound to CD20 on Wil2-S were detected with goat anti-human IgG polyclonal antibodies conjugated with FITC. Median Fluorescence intensity (MFI) was derived from the median value of the fluorescence histogram.

2.4 Effect of kifunensine on biological activity of rituximab produced in *N. benthamiana*

Rituximab samples purified from plants treated with 0, 0.25, 0.375, 1.25, 2.5, and 5  $\mu$ M of kifunensine as well as a commercially available rituximab (Rituxan®) were assessed for their ability to generate *in vitro* ADCC activity against the Wil2-S human B cell line using modified Jurkat effector

cells expressing either the F158 or V158 variant of the Fc receptor RIIIa (FcγRIIIa or CD16a). In the human population, FcγRIIIa polymorphism is observed at amino acid 158 [Valine (V)/Phenylalanine (F)] of the FcγRIIIa with the V158 allotype exhibiting a higher affinity for IgG1. The relative ADCC activity between the samples was represented by non-linear regression activity curves comparing normalized ADCC activities (as measured by induced florescence) to rituximab concentrations (Figure 5A-B).

Figure 5



**Figure 5.** Antibody-dependent cell-mediated cytotoxicity of rituximab samples expressed in the presence or absence of kifunensine. Assay was performed using Wil2-S target cells along with either high affinity V/V 158 FcγRIIIa variant (A, C) or low affinity F/F 158 FcγRIIIa variant (B, D) engineered Jurkat cells. The effector cell: target cell ratio was 10:1. Values are expressed as normalized RLU's (A,



B) and represent the mean  $\pm$  S.D. for triplicate analyses. Summary of ADCC activity represented as EC<sub>50</sub> values (C, D), normalized to the control 0 $\mu$ M kifunensine control value, indicating relative activity. The error bars of each EC<sub>50</sub> value correspond to the standard error of the mean.

A robust, dose-dependent reporter signal was produced when Wil2-S cells were co-incubated with either Fc $\gamma$ RIIIa-V158 (Figure 5A) or Fc $\gamma$ RIIIa-F158 (Figure 5B) effector cells and rituximab. The 50% effective concentrations (EC<sub>50</sub>) calculated from these curves were directly compared between samples to evaluate the efficacy of each antibody variant. Compared to rituximab from untreated plants and mammalian cell derived Rituxan<sup>®</sup>, rituximab derived from plants treated with  $\geq 0.25$   $\mu$ M kifunensine induced significantly greater ADCC activity with both Fc $\gamma$ RIIIa-V158 (Figure 5C) and Fc $\gamma$ RIIIa-F158 (Figure 5D) effector cell variants. The ADCC activity of rituximab with oligomannose-type glycans was increased by 14-fold and 9.5-fold when using V158 and F158 variant effector cells, respectively (Figure 5C and 5D) as compared with Rituxan<sup>®</sup>. Interestingly, the increase in ADCC activity from rituximab derived from plants treated with 0.25  $\mu$ M kifunensine (Figure 5) was found to be intermediate between the non-treated rituximab and rituximab derived from plants treated with  $\geq 0.25$   $\mu$ M kifunensine (Figure 5C-D). This may be attributed to a similar intermediate content in  $\alpha 1,3$ -fucose on this rituximab sample compared to the other rituximab samples. In all antibody independent assays (AICC), no signal was observed in the absence of antibody.

### 3. Discussion

#### 3.1 Kifunensine treatment is well tolerated during expression of rituximab in *N. benthamiana*

To date, several inhibitors of the N-glycosylation pathway have been used to modulate the glycosylation profiles of recombinant proteins. Among them, kifunensine, an inhibitor of class I mannosidases, acts early in the N-glycosylation processing by blocking the trimming of Man<sub>9</sub> oligosaccharides to Man<sub>5</sub>. In comparison, application of swainsonine, an inhibitor of class II mannosidases, leads to the formation of a more heterogeneous population of N-glycans with fucose-containing hybrid structures as well as oligomannose structures [43]. Also, kifunensine has been shown to be very efficient at low concentrations as compared with swainsonine, thus becoming a good candidate as a medium supplement for alternation of host N-glycosylation pathways. Although it has been used in mammalian cells since 1990 [28], it was only recently applied in the plant expression systems [35–37].

In this study, we have investigated the use of kifunensine in a plant expression platform that is established for large-scale manufacturing for recombinant protein [41]. More specifically, we evaluated the concentration of kifunensine sufficient for the production of an afucosylated rituximab with enhanced biological activity. The vacuum infiltration of plants in a solution containing *Agrobacterium* culture supplemented with kifunensine at a concentration varying from 0.0625  $\mu$ M to 5  $\mu$ M did not affect the 7-day post-infiltration growth of the plants or the expression of rituximab. In fact, the expression of rituximab was slightly higher from kifunensine-treated plants. Similar tolerance to kifunensine treatment has also been described for the expression of antibodies in Chinese Hamster Ovary (CHO) cells [29,33]. However, it has been reported in one instance that kifunensine treatment of *N. benthamiana* plants via the growth medium led to a decrease in expression of a recombinant protein [35]. When applied during plant vacuum infiltration in the Agroinfiltration solution, kifunensine enters the interstitial spaces of the leaf tissue in contact with the host cells where

recombinant protein expression occurs, rather than through uptake via the root system. The positive impact of infiltrated kifunensine on host cell tolerance and protein production may be due to its suppressing effects on the ER-associated degradation pathway [44] where proteins with trimmed oligomannose glycans may be degraded if the polysaccharide chain is not further processed or proteins transported to the Golgi apparatus [45,46].

### 3.2 *Kifunensine has a strong effect on the plant N-glycosylation machinery, even at low concentration*

Kifunensine treatment during the agroinfiltration ultimately results in protein afucosylation as it stops mannose trimming in the endoplasmic reticulum, yielding Man<sub>5</sub>-Man<sub>9</sub> N-glycan structures. When delivered in this fashion, a minimum concentration of 0.375  $\mu$ M kifunensine was sufficient to generate rituximab harboring only oligomannose glycan structures lacking fucose residues. In agreement with reports using mammalian cell cultures [29,33], the minimum required kifunensine concentration to generate antibody devoid of fucose residues in *N. benthamiana* falls somewhere between 0.25 and 0.375  $\mu$ M (58 and 87 ng/mL). Kifunensine has the practical advantage of being active at 2- to 4-fold-lower concentrations than other inhibitors of the glycosylation pathway (e.g. swainsonine or N-butyldeoxynojirimycin) making it more cost-effective. Moreover, as described here and in mammalian cell cultures, treatments with kifunensine leads to a highly homogeneous product, with ultimately no formation of core-fucosylated hybrid structures [29,33–35,37].

### 3.3 *Kifunensine represents a cost-effective method to produced biobetter anti-cancer antibodies*

Proteins produced with glycan-engineering technologies not only lack potentially immunogenic plant-specific glycoforms but also provide enhanced effector function. The *in vitro* bioassay described in this study demonstrated enhanced ADCC activity from rituximab containing high-mannose glycoforms. It is expected that the reason for this increased ADCC activity lies in the absence of fucose residues on the glycosylation core rather than the high content of mannose residues, as many studies have reported the effect of afucosylation on the ADCC activity of anti-cancer antibodies [47] including rituximab [10,20,48,49]. In fact, similar ADCC results were obtained with antibodies derived from CHO cell cultures treated with kifunensine [33,34]. It is important to note that ADCC activity was linearly proportional to the relative abundance of oligomannose glycoforms. This was particularly evident with ‘low-range’ concentrations of kifunensine applied which generated a mixture of complex, hybrid, and oligomannose structures. With rituximab derived from a treatment of 0.25  $\mu$ M kifunensine, generating a relatively small increase (24%) of oligomannose glycoforms, there was a significant but lower increase in ADCC activity (6.7-fold and 2-fold increase in ADCC activity using the Fc $\gamma$ RIIIa-V158 and Fc $\gamma$ RIIIa-F158 effector cell variants, respectively) (Figure 5). Importantly, plant-derived high-mannose rituximab glycoforms exhibited the same affinity for CD20 as Rituxan<sup>®</sup>, the commercial standard. Thus, the kifunensine treatment does not affect the paratope conformation of the plant-derived antibody. Because the glycosylation profile of an anti-cancer antibody is correlated to its biological activity it is therefore considered a critical quality attribute that needs to be maintained during the manufacturing process [50]. To that end, the glycosylation profile of kifunensine-treated antibodies is homogeneous, consistent, and easy to control at scale, which represent a significant advantage for this technology. There are reports that antibodies carrying high

mannose glycans have a shorter serum half-life as compared with other glycoforms [33,51,52]. However, other pharmacokinetic studies with afucosylated high mannose antibodies indicated no impact on clearance [29,34,53]. Thus, the pharmacokinetics property of any antibody will have to be evaluated based on its biological activity, the target indication and dosage regimen.

In conclusion, the application of kifunensine during transient agroinfiltration of the *N. benthamiana* host leverages the scalability and cost-effectiveness of the plant expression platform for the production of biobetter anti-cancer antibodies. Using the process model described by Holtz *et al.*, 2015 [41], and the finding from this study (a kifunensine concentration of 0.375  $\mu$ M), the cost of kifunensine to produce high-mannose, afucosylated antibodies at manufacturing scale (~100 kg/year of rituximab) was estimated to be less than \$0.80/g of antibody produced. Thus, kifunensine can be incorporated into already established manufacturing protocols without affecting production cost significantly. Further studies will focus on determining how long the inhibition effect of kifunensine lasts during and after the plant infiltration process. This attempt to increase anti-cancer efficacy of recombinant antibodies through in-process glycan engineering represents a promising alternative to meet unmet medical needs.

#### 4. Materials and Methods

##### 4.1. Construction of Heavy chain and Light chain Expression Vectors

The genetic sequence of rituximab heavy and light chains was obtained from DrugBank (Accession DB00073). The rituximab heavy chain (HC) and light chain (LC) genes were fused to the Pathogenesis-related protein (PR1a) signal peptide sequence (UniProtKB/Swiss-Prot: P08299.1). Rituximab HC and LC genes were codon optimized for plant expression using the *Nicotiana tabacum* codon usage table (DNA2.0, Menlo Park, CA, USA) and independently cloned into proprietary plant viral-based expression vectors (iBio Inc., New York, NY, USA). Vectors were then mobilized in *Agrobacterium tumefaciens* strain GV3101 for transient expression in *Nicotiana benthamiana*.

##### 4.2. Expression and Extraction

*Agrobacterium* clones harboring HC and LC expression vectors were grown individually in culture flasks containing Luria-Bertani (LB) medium supplemented with 50 mg/L kanamycin, 50 mg/L gentamycin and 25 mg/L rifampicin at 28°C with agitation of 225 rpm. Cultures reaching an OD<sub>600nm</sub> of ~3.0 were collected and diluted in 2 mM MES to a final OD<sub>600nm</sub> of 0.05 for each culture. *N. benthamiana* 4-week old plants, hydroponically grown under red/blue LED light were vacuum infiltrated with *Agrobacterium* culture as described previously [41], along with varying concentrations of kifunensine mixed in the infiltration solution. Kifunensine concentrations used for infiltrations included a 'higher range' at 5  $\mu$ M, 2.5  $\mu$ M, 1.5  $\mu$ M; 'medium range' 1.25  $\mu$ M, 0.75  $\mu$ M, 0.375  $\mu$ M; and 'lower range' 0.25  $\mu$ M, 0.125  $\mu$ M and 0.0625  $\mu$ M. Control infiltration was carried out without kifunensine. Each infiltration was carried out with 60-plant batches and kifunensine was freshly prepared for each infiltration. Agro-infiltrated plants were then incubated under constant LED light at ~22°C with relative humidity of ~50% for rituximab production. After 7 days post-infiltration (dpi), plants were harvested, and proteins extracted. *Agrobacterium* infiltrated *N. benthamiana* biomass was mechanically homogenized in three volumes (v:w) of extraction buffer (50mM sodium phosphate, 150 mM sodium chloride, 60 mM ascorbic acid, 5 mM EDTA, 1 mM PMSF, pH 5.5) using a Waring blender at maximum speed for one minute. Primary clarification was achieved by centrifugation at 14,000  $\times$  g for 20 minutes at 4°C. To determine the concentration of the rituximab in the samples, the supernatant was harvested and a 4  $\mu$ L sample was analyzed with the

BLItz system using protein A sensors (Pall ForteBio, Menlo Park, CA, USA). After data acquisition, the concentration of rituximab in the extracts was calculated based on a rituximab reference standard.

#### 4.3. Purification and SDS-PAGE Analysis

Rituximab purification was carried out as described earlier [54]. Antibody samples were analyzed on a 4-12% Bis-Tris gradient NuPAGE gel under reducing conditions according to manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

#### 4.4. Intact protein analysis

Purified rituximab samples were exchanged into 50 mM ammonium bicarbonate, pH 8.0 buffer and adjusted to 1 mg/mL. A 5  $\mu$ L aliquot was injected into a BEH C4 column 300  $\text{\AA}$ , 1.7  $\mu$ m 2.1 mm x 100 mm (Waters Corporation, Milford, MA, USA) equilibrated with 0.1% (v/v) formic acid in water at a flow rate of 200  $\mu$ L/min. Proteins were eluted using a gradient of 2–85% acetonitrile (v/v) over the course of 30 min. A blank solution using acetonitrile was utilized between each sample to avoid peak carry-over between runs. The mass spectrometer was calibrated using PPG positive ion calibration solution from SCIEX (AB SCIEX LLC, Framingham, MA, USA). The eluted proteins were introduced into the mass spectrometer and analyzed using the mass spectrometer in positive ion mode with the following settings: intact protein mode, scan range 1500–4000 m/z, Accumulation Time 0.5 s, GS1 = 45, GS2 = 50, CUR = 30, source temperature = 400  $^{\circ}$ C, DP = 225V, CAD = 6, CE = 15V and ISVF = 4800. The raw data of intact protein analysis were processed using the software Unidec (University of Oxford, Oxford, UK) with subtract curved of 1, Gaussian smoothing of 5, bin every 1, peak FWHM of 2 and peak detection threshold of 0.1.

#### 4.5. Glycopeptide profiling

The purified rituximab samples were analyzed with LC/MS/MS based peptide mapping method as previously described [55]. The glycopeptides were identified manually by screening the N-Acetylglucosamine residue reporter ion (m/z=204.0794) in MS/MS ion fragmentation spectrum of the second run. Glycopeptide candidates were further filtered manually by matching fragment ions with predicted glycopeptide sequences. The retention time of a positive candidate was used to determine the retention time for all other glycopeptides in the results of the first run. All other glycopeptides were determined by matching the observed molecular weights with predicted ones in the results of first run. The relative abundance of each glycopeptide was calculated by its corresponding peak areas of extracted ion chromatograph.

#### 4.6. Cell lines and cell culture conditions

The hereditary spherocytosis cell line Wil2-S was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Wil2-S cells were cultured in RPMI (Corning, NY, USA) media supplemented with 10% fetal bovine serum (VWR, Radnor, PA, USA). Cells were seeded in culture flasks at a density of  $1 \times 10^5$  cells/mL, incubated at 37 $^{\circ}$ C with 7% CO<sub>2</sub> and sub-cultured when the cell density reached approximately  $1.5\text{--}2 \times 10^6$  cells/mL.

#### 4.7. CD20 binding assay

The binding of plant-made rituximab to target cells (Wil2-S) was determined with flow cytometry analysis using a Becton Dickinson Accuri C6 flow cytometer. Wil2-S lymphoma cells at  $1 \times 10^6$  cells/mL were incubated with different concentrations of Rituxan<sup>®</sup> (Genentech, South San Francisco, CA, USA), or plant-made rituximab treated either with 5  $\mu$ M, 0.25  $\mu$ M or no kifunensine for 45 min at 4 $^{\circ}$ C. Cells



were washed and incubated with FITC conjugated anti-human IgG Fc (BioLegend, San Diego, CA, USA) in PBS with 2% FBS. FITC-labeled Mouse IgG2a, kappa (FC) (BioLegend, San Diego, CA, USA) was used as an isotype control. Cells were analyzed by flow cytometry after briefly washing them with PBS containing 2% FBS. 7-AAD exclusion dye was used for the quantification and segregation of dead cells in each sample. Cell Quest data acquisition software (BD Biosciences, San Jose, CA, USA) and Flowjo FACS analysis software (Tree Star Inc., Ashland, OR, USA) were used to derive data plots. Cell labelling and Flowjo FACS analysis was as described earlier [54].

#### 4.8. Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) assay

The ADCC reporter assay was performed using Wil2-S cells as targets along with Jurkat-CD16 reporter cell lines. Two reporter cell lines stably expressing the Fc $\gamma$ RIIIa receptor, V158 (high affinity) or F158 (low affinity) variants (Promega, Madison, WI, USA) were used. Wil2-S cells were plated in a 96-well white bottom assay plate at 5,000 cells per well. Serial dilutions of test antibodies were added to the plates containing the target cells and incubated at 4°C for 15 min to allow opsonization. Jurkat-CD16 reporter cells were then added to assay plates already containing Wil2-S cells and antibodies. The final concentration of antibodies ranged from 2 to 0.0003  $\mu$ g/mL following several 3-fold dilutions. The effector:target cell ratio was 10:1. After a 6 hr. incubation at 37°C, One-Glo<sup>TM</sup> Luciferase Assay Reagent (Promega, Madison, WI, USA) was added and luminescence was determined using a Gen5 microplate reader. Samples and controls were tested in triplicate, and the mean reporter signals of sample dilutions in Relative Luminescence Units (RLU) were plotted against the antibody concentration. Antibody independent cellular cytotoxicity (AICC) was measured in wells containing target and effector cells without antibodies. GraphPad prism software (GraphPad, La Jolla, CA, USA) was used to plot normalized RLU versus Log<sub>10</sub>. The half maximal effective concentration (EC<sub>50</sub>) values of plant-made rituximab and Fc variants were derived as dose response obtained from non-linear regression curves. Fold of induction was calculated by taking the ratio of background subtracted induced RLU and background subtracted untreated control.

## 5. Conclusions

Glyco-engineering methods represent promising means to enhance biological activities of therapeutic glycoproteins. In this study, we evaluated the effectiveness of  $\alpha$ -mannosidase-I inhibitor kifunensine, to modulate the metabolic pathway of the *Nicotiana benthamiana* transient expression platform. We demonstrated that 0.375  $\mu$ M of kifunensine applied to the infiltration solution was sufficient to block the trimming of Man<sub>9</sub> and Man<sub>8</sub> oligosaccharides and resulted in the production of completely afucosylated rituximab, an anti-CD20 antibody. Oligomannose rituximab glycoforms did not possess any plant-specific  $\alpha$ (1,3)-fucose and  $\beta$ (1,2)-xylose and showed an increase antibody-dependent cell-mediated cytotoxicity (ADCC) when compared to Rituxan® or rituximab with plant complex-glycans. In addition, kifunensine modulated the plant N-glycosylation pathway without affecting plant growth or protein expression. Incorporating kifunensine into the *N. benthamiana* transient expression system, a cost-effective and scalable expression system, provides a simple alternative to the generation of transgenic plants, both expensive and time-consuming. This technology is applicable to many therapeutic glycoproteins for which oligomannose glycosylation offers a therapeutic advance, like the anti-cancer antibody rituximab.

**Author Contributions:** Vally Kommineni conceptualized, designed and performed some of the experiments. Sreenath Palle infiltrated plants; Berenice Carrillo, Jasmine Deng, Armando Tejada purified the proteins; Matthew Markert performed ADCC assays and edited figures; Zhongjie Ren performed intact protein analysis and glycoform analysis; Vally Kommineni and Sylvain Marcel wrote the manuscript; Karen A. McDonald and Somen Nandi conceptualized and edited the draft. All authors read, revised and approved the manuscript.



399     **Acknowledgments:**

400     The authors would like to acknowledge Dr Gus Wright, Flow Cytometry Core Facility, VTPB Department, Texas  
401     A&M University for CD20 binding assay analysis.

402     **Conflict of interest:**

403     The authors declare no financial or commercial conflict of interest.

404     **Abbreviations**

mAb	monoclonal antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Man <sub>8</sub>	mannose-8 glycan
Man <sub>9</sub>	mannose-9 glycan
EC <sub>50</sub>	half maximal effective concentration
PGG	polypropylene glycol

405     **References**

406     1.     Ecker, D.M.; Jones, S.D.; Levine, H.L. The therapeutic monoclonal antibody market. *MAbs* **2015**, *7*, 9–14,  
407     doi:10.4161/19420862.2015.989042.

408     2.     Morgan, C.; Fernandes, D. Designing Biobetter Monoclonal Antibody Therapeutics By  
409     Glycoengineering. *Int. Pharmaceutical Ind.* **2008**, 1–5.

410     3.     Cai, H.H. Therapeutic Monoclonal Antibodies Approved by FDA in 2015. *MOJ Immunol.* **2016**, *3*, 2015–  
411     2016, doi:10.15406/moji.2016.03.00087.

412     4.     Liu, J.K.H. The history of monoclonal antibody development - Progress, remaining challenges and future  
413     innovations. *Ann. Med. Surg.* **2014**, *3*, 113–6, doi:10.1016/j.amsu.2014.09.001.

414     5.     Vidarsson, G.; Dekkers, G.; Rispens, T. IgG subclasses and allotypes: From structure to effector functions.  
415     *Front. Immunol.* **2014**, *5*, doi:10.3389/fimmu.2014.00520.

416     6.     Pincetic, A.; Bournazos, S.; Dilillo, D.J.; Maamary, J.; Wang, T.T.; Dahan, R.; Fiebiger, B.M.; Ravetch, J. V.  
417     Type i and type II Fc receptors regulate innate and adaptive immunity. *Nat. Immunol.* **2014**, *15*, 707–716.

418     7.     Jennewein, M.F.; Alter, G. The Immunoregulatory Roles of Antibody Glycosylation. *Trends Immunol.*  
419     **2017**, *38*, 358–372.

420     8.     Boyd, P.N.; Lines, A.C.; Patel, A.K. The effect of the removal of sialic acid, galactose and total  
421     carbohydrate on the functional activity of Campath-1H. *Mol. Immunol.* **1995**, *32*, 1311–1318,  
422     doi:10.1016/0161-5890(95)00118-2.

423     9.     Malhotra, R.; Wormald, M.R.; Rudd, P.M.; Fischer, P.B.; Dwek, R.A.; Sim, R.B. Glycosylation changes of

- 424 IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein.  
425 *Nat. Med.* **1995**, *1*, 237–243, doi:10.1038/nm0395-237.
- 426 10. Shinkawa, T.; Nakamura, K.; Yamane, N.; Shoji-Hosaka, E.; Kanda, Y.; Sakurada, M.; Uchida, K.;  
427 Anazawa, H.; Satoh, M.; Yamasaki, M.; Hanai, N.; Shitara, K. The absence of fucose but not the presence  
428 of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the  
429 critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem.* **2003**, *278*, 3466–3473,  
430 doi:10.1074/jbc.M210665200.
- 431 11. Shields, R.L.; Lai, J.; Keck, R.; O'Connell, L.Y.; Hong, K.; Gloria Meng, Y.; Weikert, S.H.A.; Presta, L.G.  
432 Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and  
433 antibody-dependent cellular toxicity. *J. Biol. Chem.* **2002**, *277*, 26733–26740, doi:10.1074/jbc.M202069200.
- 434 12. Rothman, R.J.; Perussia, B.; Herlyn, D.; Warren, L. Antibody-dependent cytotoxicity mediated by natural  
435 killer cells is enhanced by castanospermine-induced alterations of IgG glycosylation. *Mol. Immunol.* **1989**,  
436 *26*, 1113–1123, doi:10.1016/0161-5890(89)90055-2.
- 437 13. Davies, J.; Jiang, L.; Pan, L.Z.; Labarre, M.J.; Anderson, D.; Reff, M. Expression of GnTIII in a recombinant  
438 anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an  
439 increase in ADCC through higher affinity for FcγRIII. *Biotechnol. Bioeng.* **2001**, *74*, 288–294,  
440 doi:10.1002/bit.1119.
- 441 14. Umaña, P.; Jean-Mairet, J.; Moudry, R.; Amstutz, H.; Bailey, J.E. Engineered glycoforms of an  
442 antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.*  
443 **1999**, *17*, 176–180, doi:10.1038/6179.
- 444 15. Yamane-Ohnuki, N.; Kinoshita, S.; Inoue-Urakubo, M.; Kusunoki, M.; Iida, S.; Nakano, R.; Wakitani, M.;  
445 Niwa, R.; Sakurada, M.; Uchida, K.; Shitara, K.; Satoh, M. Establishment of FUT8 knockout Chinese  
446 hamster ovary cells: An ideal host cell line for producing completely defucosylated antibodies with  
447 enhanced antibody-dependent cellular cytotoxicity. *Biotechnol. Bioeng.* **2004**, *87*, 614–622,  
448 doi:10.1002/bit.20151.
- 449 16. Mori, K.; Kuni-Kamochi, R.; Yamane-Ohnuki, N.; Wakitani, M.; Yamano, K.; Imai, H.; Kanda, Y.; Niwa,  
450 R.; Iida, S.; Uchida, K.; Shitara, K.; Satoh, M. Engineering Chinese hamster ovary cells to maximize  
451 effector function of produced antibodies using FUT8 siRNA. *Biotechnol. Bioeng.* **2004**, *88*, 901–908,  
452 doi:10.1002/bit.20326.
- 453 17. Strasser, R.; Altmann, F.; Mach, L.; Glössl, J.; Steinkellner, H. Generation of *Arabidopsis thaliana* plants  
454 with complex N-glycans lacking β1,2-linked xylose and core α1,3-linked fucose. *FEBS Lett.* **2004**, *561*,  
455 132–136, doi:10.1016/S0014-5793(04)00150-4.
- 456 18. Strasser, R.; Stadlmann, J.; Schähs, M.; Stiegler, G.; Quendler, H.; Mach, L.; Glössl, J.; Weterings, K.; Pabst,  
457 M.; Steinkellner, H. Generation of glyco engineered *Nicotiana benthamiana* for the production of  
458 monoclonal antibodies with a homogeneous human like N glycan structure. *Plant Biotechnol. J.* **2008**, *6*,  
459 392–402.

- 460 19. Koprivova, A.; Stemmer, C.; Altmann, F.; Hoffmann, A.; Kopriva, S.; Gorr, G.; Reski, R.; Decker, E.L.  
461 Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. *Plant Biotechnol.*  
462 *J.* **2004**, *2*, 517–523, doi:10.1111/j.1467-7652.2004.00100.x.
- 463 20. Cox, K.M.; Sterling, J.D.; Regan, J.T.; Gasdaska, J.R.; Frantz, K.K.; Peele, C.G.; Black, A.; Passmore, D.;  
464 Moldovan-Loomis, C.; Srinivasan, M.; Cuison, S.; Cardarelli, P.M.; Dickey, L.F. Glycan optimization of  
465 a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat. Biotechnol.* **2006**, *24*, 1591–1597,  
466 doi:10.1038/nbt1260.
- 467 21. Sourrouille, C.; Marquet-Blouin, E.; D'Aoust, M.A.; Kiefer-Meyer, M.C.; Seveno, M.; Pagny-Salehabadi,  
468 S.; Bardor, M.; Durambur, G.; Lerouge, P.; Vezina, L.; Gomord, V. Down-regulated expression of plant-  
469 specific glycoepitopes in alfalfa. *Plant Biotechnol. J.* **2008**, *6*, 702–721, doi:10.1111/j.1467-7652.2008.00353.x.
- 470 22. Imai-Nishiya, H.; Mori, K.; Inoue, M.; Wakitani, M.; Iida, S.; Shitara, K.; Satoh, M. Double knockdown of  
471  $\alpha$ 1,6-fucosyltransferase (FUT8) and GDP-mannose 4,6-dehydratase (GMD) in antibody-producing cells:  
472 A new strategy for generating fully non-fucosylated therapeutic antibodies with enhanced ADCC. *BMC*  
473 *Biotechnol.* **2007**, *7*, 84, doi:10.1186/1472-6750-7-84.
- 474 23. Ferrara, C.; Brünker, P.; Suter, T.; Moser, S.; Püntener, U.; Umaña, P. Modulation of therapeutic antibody  
475 effector functions by glycosylation engineering: Influence of golgi enzyme localization domain and co-  
476 expression of heterologous 1, 4-N-acetylglucosaminyltransferase III and Golgi-mannosidase II.  
477 *Biotechnol. Bioeng.* **2006**, *93*, 851–861, doi:10.1002/bit.20777.
- 478 24. Remer, M.; Al-Shamkhani, A.; Glennie, M.; Johnson, P. Mogamulizumab and the treatment of CCR4-  
479 positive T-cell lymphomas. *Immunotherapy* **2014**, *6*, 1187–1206, doi:10.2217/imt.14.94.
- 480 25. Klein, C.; Bacac, M.; Umaña, P.; Wenger, M. Obinutuzumab (Gazyva®), a Novel Glycoengineered Type  
481 II CD20 Antibody for the Treatment of Chronic Lymphocytic Leukemia and Non-Hodgkin's  
482 Lymphoma. In *Handbook of Therapeutic Antibodies: Second Edition*; Wiley-VCH Verlag GmbH & Co. KGaA:  
483 Weinheim, Germany, 2014; Vol. 3–4, pp. 1695–1732 ISBN 9783527682423.
- 484 26. Zupke, C.; Brady, L.J.; Slade, P.G.; Clark, P.; Caspary, R.G.; Livingston, B.; Taylor, L.; Bigham, K.; Morris,  
485 A.E.; Bailey, R.W. Real-time product attribute control to manufacture antibodies with defined N-linked  
486 glycan levels. *Biotechnol. Prog.* **2015**, *31*, 1433–1441, doi:10.1002/btpr.2136.
- 487 27. Platt, F.M.; Neises, G.R.; Dwek, R.A.; Butters, T.D. N-butyldeoxynojirimycin is a novel inhibitor of  
488 glycolipid biosynthesis. *J. Biol. Chem.* **1994**, *269*, 8362–8365.
- 489 28. Elbein, A.D.; Tropea, J.E.; Mitchell, M.; Kaushal, G.P. Kifunensine, a potent inhibitor of the glycoprotein  
490 processing mannosidase I. *J. Biol. Chem.* **1990**, *265*, 15599–15605.
- 491 29. Zhou, Q.; Shankara, S.; Roy, A.; Qiu, H.; Estes, S.; McVie-Wylie, A.; Culm-Merdek, K.; Park, A.; Pan, C.;  
492 Edmunds, T. Development of a simple and rapid method for producing non-fucosylated oligomannose  
493 containing antibodies with increased effector function. *Biotechnol. Bioeng.* **2008**, *99*, 652–665.
- 494 30. Gross, V.; Tran Thi, T.A.; Vosbeck, K.; Heinrich, P.C. Effect of swainsonine on the processing of the

- 495 asparagine-linked carbohydrate chains of alpha 1-antitrypsin in rat hepatocytes. Evidence for the  
496 formation of hybrid oligosaccharides. *J. Biol. Chem.* **1983**, *258*, 4032–4036.
- 497 31. Davis, S.J.; Davies, E.A.; Barclay, A.N.; Daenke, S.; Bodian, D.L.; Jones, E.Y.; Stuart, D.I.; Butters, T.D.;  
498 Dwek, R.A.; van der Merwe, P.A. Ligand binding by the immunoglobulin superfamily recognition  
499 molecule CD2 is glycosylation-independent. *J. Biol. Chem.* **1995**, *270*, 369–75, doi:10.1074/JBC.270.1.369.
- 500 32. Strasser, R. Plant protein glycosylation. *Glycobiology* **2016**, *26*, 926–939, doi:10.1093/glycob/cww023.
- 501 33. Yu, M.; Brown, D.; Reed, C.; Chung, S.; Lutman, J.; Stefanich, E.; Wong, A.; Stephan, J.P.; Bayer, R.  
502 Production, characterization and pharmacokinetic properties of antibodies with N-linked Mannose-5  
503 glycans. *MAbs* **2012**, *4*, 475–487, doi:10.4161/mabs.20737.
- 504 34. Kanda, Y.; Yamada, T.; Mori, K.; Okazaki, A.; Inoue, M.; Kitajima-Miyama, K.; Kuni-Kamochi, R.;  
505 Nakano, R.; Yano, K.; Kakita, S.; Shitara, K.; Satoh, M. Comparison of biological activity among  
506 nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: The high-  
507 mannose, hybrid, and complex types. *Glycobiology* **2007**, *17*, 104–118, doi:10.1093/glycob/cwl057.
- 508 35. Roychowdhury, S.; Oh, Y.J.; Kajiura, H.; Hamorsky, K.T.; Fujiyama, K.; Matoba, N. Hydroponic  
509 Treatment of *Nicotiana benthamiana* with Kifunensine Modifies the N-glycans of Recombinant  
510 Glycoprotein Antigens to Predominantly Man9 High-Mannose Type upon Transient Overexpression.  
511 *Front. Plant Sci.* **2018**, *9*, 1–10, doi:10.3389/fpls.2018.00062.
- 512 36. Hamorsky, K.T.; Kouokam, J.C.; Jurkiewicz, J.M.; Nelson, B.; Moore, L.J.; Husk, A.S.; Kajiura, H.;  
513 Fujiyama, K.; Matoba, N. N-Glycosylation of cholera toxin B subunit in *Nicotiana benthamiana*: Impacts  
514 on host stress response, production yield and vaccine potential. *Sci. Rep.* **2015**, *5*, 1–9,  
515 doi:10.1038/srep08003.
- 516 37. Xiong, Y.; Li, Q.; Kailemia, M.J.; Lebrilla, C.B.; Nandi, S.; McDonald, K.A. Glycoform modification of  
517 secreted recombinant glycoproteins through kifunensine addition during transient vacuum  
518 agroinfiltration. *Int. J. Mol. Sci.* **2018**, *19*, 890, doi:10.3390/ijms19030890.
- 519 38. Maloney, D.G.; Grillo-López, A.J.; White, C.A.; Bodkin, D.; Schilder, R.J.; Neidhart, J.A.; Janakiraman,  
520 N.; Foon, K.A.; Liles, T.-M.; Dallaire, B.K.; Wey, K.; Royston, I.; Davis, T.; Levy, R. IDEC-C2B8  
521 (Rituximab) Anti-CD20 Monoclonal Antibody Therapy in Patients With Relapsed Low-Grade Non-  
522 Hodgkin's Lymphoma. *Blood* **1997**, *90*.
- 523 39. Kingsbury, N.J.; McDonald, K.A. Quantitative evaluation of E1 endoglucanase recovery from tobacco  
524 leaves using the vacuum infiltration-centrifugation method. *Biomed Res. Int.* **2014**, *2014*,  
525 doi:10.1155/2014/483596.
- 526 40. Nandi, S.; Kwong, A.T.; Holtz, B.R.; Erwin, R.L.; Marcel, S.; McDonald, K.A. Techno-economic analysis  
527 of a transient plant-based platform for monoclonal antibody production. *MAbs* **2016**, *8*,  
528 doi:10.1080/19420862.2016.1227901.
- 529 41. Holtz, B.R.; Berquist, B.R.; Bennett, L.D.; Kommineni, V.J.M.; Munigunti, R.K.; White, E.L.; Wilkerson,

- 530 D.C.; Wong, K.Y.I.; Ly, L.H.; Marcel, S. Commercial-scale biotherapeutics manufacturing facility for  
531 plant-made pharmaceuticals. *Plant Biotechnol. J.* **2015**, *13*, 1180–1190, doi:10.1111/pbi.12469.
- 532 42. Montacir, O.; Montacir, H.; Eravci, M.; Springer, A.; Hinderlich, S.; Saadati, A.; Parr, M.K. Comparability  
533 study of Rituximab originator and follow-on biopharmaceutical. *J. Pharm. Biomed. Anal.* **2017**, *140*, 239–  
534 251, doi:10.1016/j.jpba.2017.03.029.
- 535 43. Chang, V.T.; Crispin, M.; Aricescu, A.R.; Harvey, D.J.; Nettleship, J.E.; Fennelly, J.A.; Yu, C.; Boles, K.S.;  
536 Evans, E.J.; Stuart, D.I.; Dwek, R.A.; Jones, E.Y.; Owens, R.J.; Davis, S.J. Means Glycoprotein Structural  
537 Genomics: Solving the Glycosylation Problem. *Structure* **2007**, *15*, 267–73, doi:10.1016/j.str.2007.01.011.
- 538 44. Wang, F.; Song, W.; Brancati, G.; Segatori, L. Inhibition of endoplasmic reticulum-associated degradation  
539 rescues native folding in loss of function protein misfolding diseases. *J. Biol. Chem.* **2011**, *286*, 43454–  
540 43464, doi:10.1074/jbc.M111.274332.
- 541 45. Ellgaard, L.; Molinari, M.; Helenius, A. Setting the standards: Quality control in the secretory pathway.  
542 *Science (80-. )*. **1999**, *286*, 1882–1888, doi:10.1126/science.286.5446.1882.
- 543 46. Fagioli, C.; Sitia, R. Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by  
544 endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled  
545 immunoglobulin subunits. *J. Biol. Chem.* **2001**, *276*, 12885–12892, doi:10.1074/jbc.M009603200.
- 546 47. Yu, X.; Marshall, M.J.E.; Cragg, M.S.; Crispin, M. Improving Antibody-Based Cancer Therapeutics  
547 Through Glycan Engineering. *BioDrugs* **2017**, *31*, 151–166, doi:10.1007/s40259-017-0223-8.
- 548 48. Huang, W.; Giddens, J.; Fan, S.; Toonstra, C.; Wang, L.X. Chemoenzymatic glycoengineering of intact  
549 IgG antibodies for gain of functions. *J. Am. Chem. Soc.* **2012**, *134*, 12308–12318.
- 550 49. Li, T.; DiLillo, D.J.; Bournazos, S.; Giddens, J.P.; Ravetch, J. V.; Wang, L.-X. Modulating IgG effector  
551 function by Fc glycan engineering. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 3485–3490,  
552 doi:10.1073/pnas.1702173114.
- 553 50. Reusch, D.; Tejada, M.L. Fc glycans of therapeutic antibodies as critical quality attributes. *Glycobiology*  
554 **2015**, *25*, 1325–1334.
- 555 51. Alessandri, L.; Ouellette, D.; Acquah, A.; Rieser, M.; Leblond, D.; Saltarelli, M.; Radziejewski, C.;  
556 Fujimori, T.; Correia, I. Increased serum clearance of oligomannose species present on a human IgG1  
557 molecule. *MAbs* **2012**, *4*, 509–20, doi:10.4161/mabs.20450.
- 558 52. Goetze, A.M.; Liu, Y.D.; Zhang, Z.; Shah, B.; Lee, E.; Bondarenko, P. V.; Flynn, G.C. High-mannose  
559 glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology*  
560 **2011**, *21*, 949–959, doi:10.1093/glycob/cwr027.
- 561 53. Millward, T.A.; Heitzmann, M.; Bill, K.; Längle, U.; Schumacher, P.; Forrer, K. Effect of constant and  
562 variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice. *Biologicals* **2008**,  
563 *36*, 41–47, doi:10.1016/j.biologicals.2007.05.003.



- 564 54. Bennett, L.D.; Yang, Q.Qi. of G.R. to P.-M.T.A.; Berquist, B.R.; Giddens, J.P.; Ren, Z.; Kommineni, V.;  
565 Murray, R.P.; White, E.L.; Holtz, B.R.; Wang, L.-X.X.; Marcel, S. Implementation of glycan remodeling  
566 to plant-made therapeutic antibodies. *Int. J. Mol. Sci.* **2018**, *19*, 421, doi:10.3390/ijms19020421.
- 567 55. Bennett, L.L.D.; Yang, Q.; Berquist, B.B.R.; Giddens, J.J.P.; Ren, Z.; Kommineni, V.; Murray, R.R.P.;  
568 White, E.L.E.; Holtz, B.; Wang, L.-X.L.-X.; Marcel, S. Implementation of Glycan Remodeling to Plant-  
569 Made Therapeutic Antibodies. *Int. J. Mol. Sci.* **2018**, *19*, 421, doi:10.3390/ijms19020421.
- 570