Title: Molecular Imaging of N-linked Glycosylation Suggests Glycan Biosynthesis is a Novel Target for Cancer Therapy

Running Title: Imaging N-Linked Glycosylation In Vivo

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**Statement of Translational Relevance:**

Identifying novel targets for cancer therapy requires that preclinical models can demonstrate the following: 1- That the new target can be blocked in vivo. 2- That target blockade has mechanistic consequences for tumor cells. 3- That target blockade has efficacy. We investigated a new target for enhancing tumor radiosensitivity (N-linked glycosylation) by using molecular imaging to generate a tumor model that permits analysis of the above criteria. We propose that inhibition of NLG is an alternative strategy to reduce RTK expression in tumors and enhance radiation therapy, and is advantageous by reducing redundant and compensatory RTK survival signals. By using this pre-clinical model we demonstrate for the first time that inhibition of NLG: 1- Is feasible in vivo. 2- Reduces EGFR and Met RTKs in tumors. 3- Significantly reduces tumor growth when delivered with radiation therapy. This work provides direct evidence that inhibition of NLG is a promising approach to enhance current radiation therapy treatment regimens.
Abstract:

Purpose: Redundant receptor tyrosine kinase (RTK) signaling is a mechanism for therapeutic resistance to EGFR inhibition. A strategy to reduce parallel signaling by co-expressed RTKs is inhibition of N-linked glycosylation (NLG), an endoplasmic reticulum (ER) co-translational protein modification required for receptor maturation and cell surface expression. We therefore investigated the feasibility of blocking NLG in vivo to reduce over-expression of RTKs.

Experimental design: We developed a model system to dynamically monitor NLG in vitro and in vivo using bioluminescent imaging techniques. Functional imaging of NLG is accomplished with a luciferase reporter (ER-LucT) modified for ER-translation and glycosylation. After in vitro validation, this reporter was integrated with D54 glioma xenografts to perform non-invasive imaging of tumors, and inhibition of NLG was correlated with RTK protein levels and tumor growth.

Results: The ER-LucT reporter demonstrates the ability to sensitively and specifically detect NLG inhibition. Using this molecular imaging approach we performed serial imaging studies to determine safe and efficacious in vivo dosing of the GlcNAc-1-phosphotransferase inhibitor, tunicamycin, which blocks N-glycan precursor biosynthesis. Molecular analyses of tunicamycin treated tumors showed reduced levels of EGFR and Met, two RTKs over-expressed in gliomas. Furthermore, D54 and U87MG glioma xenograft tumor experiments demonstrated significant reductions in tumor growth following NLG inhibition and radiation therapy, consistent with an enhancement in tumor radiosensitivity.
Conclusions: This study suggests NLG inhibition is a novel therapeutic strategy for targeting EGFR and RTK signaling in both gliomas and other malignant tumors.
Introduction:

Previous work has shown that inhibition of N-linked glycosylation (NLG), a sequence specific, co-translational modification that occurs in the endoplasmic reticulum (ER), reduces protein levels of over-expressed RTKs (ie EGFR, ErbB2, Erbb3, and IGF-1R) in vitro (1). As a result, signaling through both dominant and redundant RTK signaling pathways are reduced, suggesting that inhibition of NLG is an alternative mechanistic approach for targeting RTK signaling in tumors. Like other strategies for targeting multiple RTKs in glioblastoma (2-5), NLG inhibition produced marked radiosensitization in cancer cell lines but did not radiosensitize non-transformed cells. Although in vitro experiments suggest the potential for a therapeutic ratio, further evaluation of whether this biosynthetic process can be blocked at tolerable levels requires an in vivo experimental model.

A major barrier for evaluating novel molecular targets in cancer therapy has been the inability to measure target activity and to assess inhibition by pharmacologic agents in vivo. Xenograft tumor models, which have been used to measure tumor growth, display heterogeneity and are not optimal for analyses performed at the molecular level. To address the deficiencies of the xenograft tumor model, techniques for molecular imaging using engineered reporter genes in small animals have been developed (see (6) for review). The underlying principle of these techniques is based upon unique modifications to genes such as firefly luciferase (Luc), that transform the reporter into a specific molecular sensor that transmits a quantifiable signal to a detection system. In tumor xenografts, Luc was initially employed as a dynamic marker for tumor growth and metastases, but quickly modified and adapted in approaches to report specific cellular
events such as apoptosis (7) or endoplasmic reticulum induced stress (8-9). Luc has also been used in vivo to measure changes at the protein level, such as the turnover rate of CDK2 dependent p27 levels (9). Luker et. al. pioneered a split Luc design (or luciferase complementation) to measure in vivo protein-protein interactions for CDC25c and 14-3-3 among others (10), a design recently used to demonstrate radiation-induced EGFR signaling (11). Post-translational modifications, such as site specific Akt phosphorylation (12), have also been successfully measured using Luc-based reporter vectors, demonstrating the flexibility of Luc as a tool for noninvasive imaging strategies.

We hypothesized that in vivo imaging of protein N-linked glycosylation (NLG) would be an invaluable model system to investigate the feasibility of blocking this process as a potential cancer therapy. Because our aim is to radiosensitize tumor cells with this maneuver, unlike most cancer therapies which are intended to eliminate all viable tumor cells, we sought to develop a sensitive model system to determine a threshold for NLG inhibition in tumors, to perform serial non-invasive measurements of this biologic process, and to guide dose and schedule optimization for in vivo experiments.

We now report on a bioluminescent reporter engineered to exploit the sequence specificity of NLG and the biophysical properties of Luc to measure inhibition of NLG. We have validated this reporter in vitro and used it in a glioma xenograft tumor model to demonstrate the feasibility of NLG inhibition in animals. This non-invasive imaging platform became instrumental as a method for testing the hypothesis that inhibition of NLG in vivo reduces RTK protein levels and radiosensitizes tumors. Our results demonstrate the power of molecular imaging techniques to efficiently evaluate novel
targets for cancer therapy (in this case NLG) as well as the crucial role this knowledge plays in developing new strategies for multimodality therapy.

**Materials and Methods:**

**Reagents:** Unless otherwise stated, all reagents were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased from Chemicon (luciferase), c-Met (Santa Cruz Biotechnology), and Invitrogen (EGFR). Tunicamycin and the Concanavalin A-agarose conjugate were purchased from Calbiochem. Luciferin was supplied by Promega (Madison, WI).

**Vectors:** The 24 amino acid EGFR endoplasmic reticulum translation sequence was added in frame to the NH2-terminus of the luc gene by sequential PCR reactions. The amplification product from primer 1: (5’CTGGCGCTGCTGGCTGCGCTCTGCCGCCTCGAGAGCTATGGAAGACGCCAAAAAC 3’) and primer 2 (5’ACGCGTCGACTTACACGGCGATCTTTCCGCCCTTCTTGGC 3’), was further amplified using primer 3: (CGGATCCACCATGCGACCCTCCGGGACGGCCGGGGCAGCGCTCCTGGCGCTGCTGGCTGC) and primer 2. The resultant PCR product was digested with BamHI and SalI and cloned into BamHI and XhoI sites of pcDNA3. The cleavage site of the ER translation sequence was engineered to precede the first Luc methionine so that following cleavage of the leader sequence in the ER, the Luc amino acid sequence would be unchanged. The ER-LucT sequence contains three (T) potential glycosylation sites and was amplified from the original pyralis luciferase sequence as found in PUHD10-3. The ER-LucS sequence contains a single (S) potential glycosylation site and...
was amplified from pGL3 (Promega), which is a modified luciferase with elimination of two of the three potential glycosylation sites inherent in wild type Luc. Luc is not normally glycosylated because it does not enter the ER lumen. The Luc-T was constructed by removal of the ER translation sequence through digestion of ER-LucT with Apa I and Xhol (a restriction site we nested in the ER translation sequence). Vectors were sequenced to insure that no mutations were introduced during the PCR reaction.

**Cell Culture:** Cell lines were maintained in RPMI 1640 media supplemented with 10% FCS and 100ug/ml each of penicillin and streptomycin at 37 degrees with 5% CO2. 293T cells were used for transient transfection experiments. CHO and D54 cell lines with stable expression of the luciferase plasmids were generated through transfection with Lipofectamine and selection with G418 (800ug/ml and 200ug/ml) respectively.

**Immunoblot:** Lysates were prepared using Western lysis buffer (25 mmol/L Tris, 10 mmol/L EDTA, 15% glycerol, 0.1% Triton X-100, 1X protease inhibitor cocktail (Roche), and 1X phosphatase inhibitor cocktails 1 and 2 (Sigma)). For tumor lysates, 2% SDS was used as a detergent and inhibitors were used at a 2.5X final concentration. Glycosylated proteins were removed from protein lysates by precipitation with Concanavalin A-agarose for 60 minutes with gentle agitation at 4 degrees C followed by centrifugation at 5000 RPM. Supernatants from these samples were used for western blot analysis.
In Vitro Bioluminescence: Cell lines with stable expression of luciferase plasmids were plated at a density of 40,000 cells/well in a twenty-four well dish. Inhibitors were added the following day, and imaging was performed 24 hours after drug treatment. Prior to bioluminescent imaging, the media was aspirated and replaced with fresh media containing 100ug/ml luciferin. Photon counts were then collected 10 minutes after the addition of luciferin using the Xenogen IVIS charge-coupled device camera system. A signal averaging time of 1 minute was used for all experiments and signal intensity was quantified as the sum of all detected photons per well. A pseudocolor image representing the detected photons was produced as an overlay on a gray-scale image of the plate. Triplicate wells for each treatment condition were analyzed for each experiment and untreated wells were used as baseline controls to calculate induction of bioluminescence.

In vivo Bioluminescence: All mouse experiments were approved by the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan. The protocol for bioluminescent imaging of mice bearing D54-ERlucT flank tumors has been previously reported (7). Briefly, tumors were grown in athymic nude mice (Charles River; Wilmington, MA) by bilateral subcutaneous implantation of 1X 10^7 cells. Ten days following injection, mice bearing palpable tumors were anesthetized with a 1% isoflurane/air mixture and given a single i.p. dose of 150 mg/kg luciferin in normal saline. Bioluminescent imaging was performed from 5 to 20 minutes after luciferin administration, and mice were anesthetized and kept warm with a temperature controlled bed during image acquisition. Signal intensity was quantified for a region of interest (ROI) for each tumor over the imaging time period to determine the peak of
bioluminescent activity. Tumor bioluminescence prior to drug treatment was used to establish a baseline of activity and to calculate induction of Luc activity. After obtaining base line images, mice were treated with IP tunicamycin (0.25-1mg/kg) or 150 mM dextrose as a control and underwent repeated daily imaging. Tunicamycin was prepared by dissolving the compound in DMSO to give a final concentration of 5mg/ml and then diluted 1:50 in 150mM Dextrose.

**Immunohistochemistry and Immunofluorescence:** To evaluate EGFR protein expression, immunohistochemistry was performed using a standard protocol (DAKO EnVision +System, Peroxidase (DAB), K4011). 5-um thick paraffin embedded tissue was dewaxed and hydrated in xylene and ethanol respectively. Antigen retrieval was performed in citrate buffer in microwave at pH 6.0. The primary antibody was a rabbit polyclonal antibody to EGFR (Santa Cruz) which was added to the tumor sections at a 1:70 dilution, and incubated for 45 minutes at room temperature. The section was then treated with a horseradish peroxidase-labeled secondary antibody for 30 minutes, followed by peroxide/diaminobenzidine substrate/chromagen. The slides were counterstained with hematoxylin. For quantitation of EGFR distribution in control and treated samples, random fields were imaged in sections from three animals in each group and the number of cells with membrane, cytosolic or no staining were counted. For Met immunofluorescence staining, deparaffinization, and antigen retrieval were similar as described above. Met antibody was incubated at dilution of 1:100 followed by cy3-coupled secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA secondary). The slides were then counter-stained with 1 μg/mL 4',6-diamidino-2-
phenylindole, mounted, and visualized under a fluorescence Nikon Eclipse TE2000-U microscope (Nikon, Melville, NY). All fluorescence images were acquired and quantitated using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA).

**Tumor growth:** D54 and U87MG xenograft tumors were generated by inoculation of 1X10^7 cells into the flanks of athymic nude mice. When tumors became palpable, 4-6 mice were randomized to each of four groups (i. untreated control, ii. 0.75mg/kg Tunicamycin, iii. Radiation, iv. Radiation + 0.75 mg/kg Tunicamycin). Median tumor volumes were 477 mm^3 for D54 tumors and 359 mm^3 for U87MG tumors, with no significant differences in tumor size between groups. Tunicamycin treatment consisted of a single IP injection on the day of randomization (Day 0). For those animals randomized to receive radiation, therapy began on Day 1 and consisted of five daily fractions of 2Gy, delivered at a dose rate of 2 Gy/min using a Pantak DXT 300 Orthovoltage Unit (East Haven, CT) producing 300 kV X-rays. Lead shielding was used to reduce animal exposure. The treatment schedule was designed to both optimize NLG inhibition during RT and to be similar to clinical delivery of RT with a weekly systemic agent. Tumor dimensions were measured three times weekly and tumor volume was calculated according to the equation: TV = (π/6) X (L X W^2), where L and W are the longer and shorter dimensions of the tumor, respectively. Data are expressed as the ratio of tumor volume at various times after treatment compared with the day of drug administration.
Statistics: Data points are reported as experimental averages and error bars represent the standard error of the mean (SEM). Statistical significance was determined using a two sided Student’s t test. A p value < 0.05 was considered to be statistically significant.

Results:

NLG Reporter Design:

N-Linked glycosylation is a co-translational protein modification that occurs in the endoplasmic reticulum (ER). Because of our interest in disrupting this biologic process as a means of sensitizing cancer cells to cytotoxic therapies, we sought to develop a simple method for measuring NLG. We have used firefly Luc as a bioluminescent reporter previously for both in vitro and in vivo cancer models (7), and therefore chose this gene to develop a way to monitor inhibition of NLG in living cells. We hypothesized that the co-translational transfer of the 14 sugar glycan precursor from its lipid precursor to the glycosylation sites of Luc in the ER would disrupt the ability of this enzyme to use luciferin and ATP as substrates to generate bioluminescence. The design predicts that a Luc enzyme translocated into the ER and engineered to contain consensus NLG sites (NXS/T) would have low base line levels of bioluminescent activity, but that inhibition of NLG (and loss of the added glycan moiety) would therefore enhance bioluminescent activity. Furthermore, the design accounts for the possibility of reduced signal secondary to cellular effects of NLG inhibition. Although aberrant glycosylation has the potential to reduce protein translation, the ‘gain of function’ premise of this reporter enhances the sensitivity for detecting inhibition of this biosynthetic process.
To test this design, luciferase cDNAs were constructed with an in-frame ER translation leader sequence from the EGFR and expressed in 293T cells. The reporters are designated ER-LucT and ER-LucS, and contain three consensus NLG sites or a single consensus NLG site, respectively (Fig. 1A). A control, cytoplasmic expressed luciferase (LucT) was also prepared. The ER signal peptide is cleaved just prior to the initiation methionine of the wild type Luc, yielding a protein identical to that of a cytoplasmic Luc (Fig. 1B). As predicted, expression of each ER-Luc vector increased the molecular weight of the Luc protein, consistent with its glycosylation (Fig. 1C). The increase in molecular weight for each ER-expressed construct was proportional to the number of glycosylation sites in each protein sequence, demonstrating that ER-LucT is more heavily glycosylated than the ER-LucS. Pretreatment of CHO cells expressing either the ER-LucT or ER-LucS constructs with the GlcNAc-1-phosphotransferase inhibitor, tunicamycin, reversed this increase in molecular weight consistent with blockade of the protein’s glycosylation. In comparison, the cytoplasmic expressed LucT construct maintained identical gel mobility after tunicamycin treatment.

**NLG Reporter Activity:**

Functional studies to quantify the effect of glycosylation on Luc activity were also undertaken. To quantitate changes in bioluminescence, we generated pooled clones of CHO cells with stable expression of each vector. Both ER-LucS and ER-LucT expressing CHO cells demonstrated increased bioluminescence when treated with tunicamycin (Fig. 2A). Because each N-linked glycan has the potential to disrupt Luc enzymatic activity, we hypothesized that the ER-LucT construct would display greater
sensitivity to NLG inhibition with tunicamycin. This was confirmed in the CHO cell clones, where ER-LucT demonstrated a 4.2 ± 0.1 fold induction compared to the 2.6 ± 0.1 fold induction measured for the ER-LucS. In contrast, control experiments with CHO cells expressing the cytoplasmic Luc (LucT) did not show an increase in bioluminescence following tunicamycin treatment (Fig. 2B). These results demonstrate that ER-translated Luc, but not cytoplasmic Luc, can be used to measure inhibition of NLG by tunicamycin. They also suggest that due to the presence of multiple NLG sites, the ER-LucT is a superior design for measuring NLG inhibition.

To further investigate the capability of the ER-LucT to serve as a sensitive reporter for NLG, we isolated an ER-Luc-T CHO clone by serial dilution. This clone displayed dose-response activity to tunicamycin treatment over several orders of magnitude (10nM to 10μM) and an 11.3 ± 0.4 fold maximal induction of Luc activity (Fig. 2C). Western blot analysis of protein lysates from tunicamycin treated cultures demonstrated that luminescence measurements directly corresponded to the glycosylation status of the protein (inset). These experiments confirm that glycosylation of Luc in the ER is a sensitive mechanism to measure the state of this co-translational event in living cells.

The ER-LucT reporter detects inhibition of enzymatic steps that precede transfer of the glycan chain to the consensus NLG site or those that contribute to glycan synthesis. The goal of imaging this biosynthetic process is based upon previous findings that disrupting core NLG, but not later NLG processing steps, sensitizes tumor cells to other cytotoxic therapies including ionizing radiation (1). To test the specificity of the ER-LucT reporter for discriminating between protein NLG and N-linked glycan processing,
we compared the effects of tunicamycin to castanospermine and swainsonine, compounds that block processing of N-linked glycans by inhibiting the α-glucosidases and α-mannosidase-II, respectively (Fig. 2D). Neither of these inhibitors enhanced Luc activity confirming that only inhibition of NLG can induce bioluminescent activity of the reporter.

**Imaging NLG In Vivo:**

Tunicamycin is the only known inhibitor of the lipid linked oligosaccharide precursors of N-linked glycans, and little is known regarding its in vivo pharmacology. Toxicity studies using intraperitoneal administration of tunicamycin in mice have estimated LD1 and LD50 doses to be approximately 1mg/kg and 1.5 mg/kg respectively (13). However these studies have not answered the fundamental question of whether a low dose of tunicamycin can disrupt NLG, but remain tolerable for the animal. Furthermore it is unknown whether systemic delivery of tunicamycin would reach the tumor, as it could be metabolized prior to entering the arterial blood supply or it could be prevented from perfusing the tumor and reaching the target tumor cells. To answer these questions we integrated the ER-LucT NLG reporter into a glioma xenograft tumor model with the goal of determining whether systemic delivery of tunicamycin could impair NLG in xenograft tumors. D54 glioma cells with stable expression of the ER-LucT reporter were generated for these in vivo experiments. Like the CHO cell line, Luc activity in the D54-ER-LucT cells could be induced ~10 fold with tunicamycin in vitro (data not shown). After subcutaneous inoculation, this cell line produced flank tumors with 100% take rate and a low background level of bioluminescence (Fig. 3A).
To assess the ability of tunicamycin to activate the reporter in vivo, we performed dose response experiments by delivering single injections of 0, 0.25, 0.5, 0.75, and 1mg/kg. We found a significant enhancement of bioluminescence for doses of tunicamycin ≥0.5 mg/kg (Fig. 3B), and a 7 fold induction at 0.75 mg/kg. To minimize potential toxicity, we selected this as a standard dose for subsequent experiments. To estimate the duration of NLG inhibition, we also performed daily imaging on the mice after tunicamycin injection to establish the time course of NLG inhibition in vivo (Fig. 3C). We found that a single dose of 0.75 mg/kg tunicamycin had sustained effects on NLG for several days, with peak luminescence occurring 48-72 hours following treatment. In comparison, control treated animals did not demonstrate enhanced luminescence over this time period.

To confirm that in vivo bioluminescence correlated with the glycosylation status of Luc an analysis of tumor lysates was also performed (Fig. 4A). We were unable to resolve the glycosylated and non-glycosylated forms of Luc from tumor samples by western blot (data not shown). Therefore Concanavalin A-agarose, a lectin that binds to high-mannose and hybrid-type N-linked glycans, was used to separate glycosylated Luc from the sample prior to western blotting. We found that tunicamycin treated animals had tumors with non-glycosylated Luc but control treated tumors did not. In summary low, tolerable doses of tunicamycin can reduce NLG in tumor cells and effect the function of target proteins (i.e. luciferase) for up to 96 hours after IP administration.

Inhibition of NLG Reduces RTK Levels In Vivo:
Based upon our prior in vitro experiments demonstrating that NLG inhibition reduces RTK protein levels (1), we next investigated whether inhibition of NLG would reduce RTK protein levels in xenograft tumor cells. We performed immunohistochemistry on tumor specimens treated with tunicamycin to evaluate the effects on EGFR and Met, two receptors over-expressed in wild type D54 tumors. We found that inhibition of NLG reduced protein levels of both receptors in tumors from tunicamycin treated animals as compared to controls. For the EGFR (Fig. 4B), characteristic membrane staining was apparent in 80 ± 9% of cells from control tumors. In contrast tumors from tunicamycin treated animals had a marked decrease of EGFR protein levels with only 18 ± 9% of cells demonstrating membrane EGFR staining. A similar reduction in receptor protein levels was also observed following immunofluorescence for Met (Fig. 4C). Tunicamycin treated tumors displayed a 50 ± 2% reduction in fluorescence intensity and membrane staining as compared to control animals. These results confirm that tunicamycin inhibits NLG in vivo, which reduces RTK protein levels in tumor cells.

**Xenograft Tumor Radiosensitization through NLG inhibition:**

To test the hypothesis that inhibition of NLG in vivo can sensitize tumors to radiation, we designed tumor growth delay experiments based upon data obtained from characterizing the dose response and time course for IP administration of tunicamycin. Based on this data, we selected an experimental treatment regimen for D54 or U87MG glioma xenografts of 0.75mg/kg tunicamycin followed by a 5 day fractionated course of radiation (Fig. 5A,B). We found that tunicamycin treatment alone had only a small effect
on tumor volume tripling (TVT) in D54 tumors (8± 1 vs. 11± 1 days) and no measureable
effect in U87MG tumors (14± 2 vs 15± 2 days). Radiation alone, delivered in 2Gy daily
fractions for a total of 10 Gy, reduced tumor growth significantly in both cell lines with
TVT of 15± 2 days for D54 and 28 ± 2 days for U87MG (p<.05 for both). However the
greatest delay in tumor growth was achieved with combined radiation and tunicamycin
treatment, which significantly extended TVT time to 24± 2 and 37± 2 days, respectively
(p<.05 when compared to RT only group). We conclude that low doses of tunicamycin
rationally selected by using in vivo imaging can radiosensitize glioma tumor xenografts.

Discussion:

NLG is a complex biosynthetic process that regulates maturation of proteins
through the secretory pathway. This co-translational modification is regulated by a series
of enzymatic reactions, culminates in the transfer of a core glycan from the lipid carrier to
a protein substrate, and has been identified as a target to enhance cytotoxic cancer
therapies (1). To determine NLG activity in vivo, we designed a non-invasive imaging
technique with the goal of examining this molecular event in a sensitive and specific
manner. To this end we developed and validated the ER-LucT, a bioluminescent NLG
reporter, and used it as a surrogate for measuring inhibition of NLG activity. In vitro,
induction of reporter activity was achieved with nanomolar concentrations of
tunicamycin, was dependent upon ER localization and the number of glycosylation sites
in the protein, was specific for inhibiting the addition (not processing) of N-linked
glycans, and corresponded directly to the glycosylation state of the protein. Using the
ER-LucT reporter, we developed a glioma xenograft tumor model to perform non-
invasive, serial imaging of NLG in tumor cells. We found that tolerable doses of tunicamycin (0.5-1mg/kg) significantly activate the NLG reporter when given by a single IP administration and have prolonged inhibitory effects, with quantifiable changes in bioluminescence for up to 4 days. Analysis of RTK protein levels of EGFR and Met confirmed that inhibition of NLG in vivo is an alternate strategy for reducing over-expressed RTKs in gliomas. Using this in vivo data, we then tested the hypothesis that inhibition of NLG sensitizes tumors to ionizing radiation and found that this treatment significantly delayed tumor growth. In summary, we present evidence that in principle NLG can be targeted in vivo to enhance the radiation sensitivity of tumor cells, and these results have the strength of correlating real time in vivo monitoring of target inhibition with improvements in tumor control.

N-linked glycosylation, like receptor phosphorylation, plays a major role in modulating protein function. While it is now understood that RTK phosphorylation is the underlying molecular mechanism for pro-proliferative and anti-apoptotic signal transduction in cancer cells, the contributions of NLG to protein function are just beginning to be understood. NLG alters protein conformation and folding (14), is involved in membrane receptor trafficking (15), modulates receptor function (16), and has been implicated in tumor cell migration and invasion (17). For the EGFR, which has been a successful molecular target in cancer therapy (18-19), elucidating the contribution of this co-translational modification to the biochemical and biophysical properties of the receptor may provide valuable information for potential therapeutic interventions. Although the EGFR has been successfully targeted by small molecule tyrosine kinase inhibitors (TKIs) and receptor specific antibodies, therapeutic resistance to EGFR
targeted therapies ultimately develops (20), and similar redundant RTK signaling cascades have also been implicated in radioresistance (21-22). In this study we found that disruption of NLG in vivo is an alternative method for reducing protective RTK signaling and increasing the radiosensitivity of gliomas. In fact, NLG inhibition has the advantage of reducing protein levels of both EGFR and other over-expressed RTKs such as Met, a kinase implicated in resistance to EGFR targeted therapies (23-24). This common effect on over-expressed RTKs also suggests that targeting NLG may combine favorably with other classes of EGFR inhibitors to reduce both oncogenic signaling as well as the mechanisms of therapeutic resistance. Although the concept for NLG inhibition was conceived to reduce redundant RTK signaling, we do not exclude the possibility that NLG inhibition may have other anti-tumor effects through functional disruption of other membrane proteins or activation of endoplasmic reticulum signaling.

NLG has been studied in the context of congenital disorders of glycosylation (CDGs), a group of disorders characterized by neuro-muscular abnormalities and developmental delay and defined by reduced activity of enzymes required for glycosylation. Additionally, three mouse models of genes involved in N-linked glycan synthesis; GlcNAc-1-phosphotransferase (GPT/ALG7), phosphomannose isomerase (PMI) and phosphomannomutase (PMM2), show embryonic lethality following knockout of each respective gene (25-27). This clinical and experimental data suggest that NLG is an essential cellular process, an important concept for cancer therapeutics as the viability of targeting essential cellular functions has been questioned (28). However, clinical and animal data for CDGs also demonstrate that low levels of PMI or PMM2 enzymatic activity (5-25% of normal) are not lethal. In fact patients or animals with a recessive
mutation (and ~50% activity) have virtually no discernable phenotype. Thus it appears that mammalian cells have a threshold for loss of enzymatic activity that is required before deficits in NLG biosynthesis become evident. This threshold is also likely to be different between tissue types and vary with proliferation status, as the requirements for cell surface protein production during cell cycle progression and cell division are increased.

A second advantage for managing potential side effects of NLG inhibition is the combination of NLG targeting with radiation therapy. In this combined modality approach, the goal of drug therapy is not direct cytotoxicity, but rather to enhance the effects of focal radiation therapy. This strategy, which depends upon reaching the threshold for NLG inhibition, would employ lower concentrations of an inhibitor and be less likely to produce systemic toxicity. But how could these NLG thresholds be determined in human tumors? One possibility is based upon the work of several groups attempting to develop radioactive tracers for EGFR imaging. Using the specificity of EGFR TKIs or EGFR antibodies, these PET and SPECT based techniques have shown promise in human clinical trials (29-30). Because EGFR levels are reduced by NLG inhibition, an EGFR imaging technique could be used as a surrogate for determining dosing and the efficacy of NLG inhibitors and to facilitate phase I clinical investigations.

Tunicamycin itself is not a potential therapeutic agent due to its narrow window of efficacy (13). However, this compound proved to be an excellent experimental tool to inhibit NLG in vitro and in vivo and to test our hypothesis. It allowed us to show three principles crucial for evaluating this potential therapeutic approach: 1. That we could hit the target in vivo and reduce NLG in tumor cells 2. That hitting the target had
consequences for RTK expression levels and 3. That disrupting NLG enhanced tumor cell radiosensitivity. Although tunicamycin is the only known inhibitor of lipid linked oligosaccharide precursor synthesis, our data suggest that a compound with similar biological effects, but a broader therapeutic window and potentially a superior toxicity profile, could be useful for radiosensitization. In this context we are currently evaluating the NLG reporter as a tool to identify novel NLG inhibitors. Secondary to its gain of function design, the NLG reporter provides a robust signal over several orders of magnitude and has been optimized for a high throughput screening format. Paired with the in vivo capabilities demonstrated herein, the NLG reporter has the potential to streamline in vitro/in vivo drug discovery for NLG inhibitors.

In summary this work describes a novel bioluminescent imaging technique to measure changes in NLG in living cells. This technique provides a real time method to quantify changes in this co-translational modification in vitro as well as in xenograft tumors. We have used this model system to investigate the feasibility of targeting NLG in vivo, and we anticipate the ability to image NLG will have applications for identifying novel NLG inhibitors as well as for developing a better understanding of NLG deficiency syndromes (ie CDGs) and other factors that affect glycan precursor biosynthesis.

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Immunoperoxidase Lab, and University of Michigan Microscopy and Imaging Analysis Laboratory for assistance with experimental procedures.
**Figure Legends:**

**Figure 1:** Schematic diagram illustrating the NLG reporter design (A,B). The ER translation sequence of the epidermal growth factor receptor is fused to the N-terminus of the Luc enzyme. Constructs contain either three (ER-LucT) sites for N-linked glycosylation or a single (ER-LucS) site. C. Constructs were transiently expressed in 293T cells and lysates of samples treated with or without 500nM tunicamycin (Tn) were analyzed by western blotting.

**Figure 2:** Validation of the NLG reporter. A. Dose response induction of Luc activity by tunicamycin in pooled CHO clones that have stable expression of either the ER-LucS (upper panel) or ER-LucT (lower panel) constructs. B. Effect of tunicamycin on CHO cell clones expressing cytoplasmic Luc (LucT). Activity is reported as fold increase of relative light units (RLU) of experimental to control samples. C. Induction of Luc activity in an ER-LucT cell line clone with correlation to Luc glycosylation (inset). D. In vitro IVIS imaging of luminescence for 20 µM castanospermine (Cs), 20µM swainsonine (Sw), and 500nM tunicamycin (Tn). Results represent the average and standard error of three independent experiments performed in triplicate.

**Figure 3:** In Vivo imaging of NLG in D54 xenograft tumors. A. Induction of bioluminescence by IP administration of 1mg/kg tunicamycin. Images are shown for peak induction of bioluminescence at 48 hrs for control and experimentally treated animals. B. Dose response induction of bioluminescence by tunicamycin for IP treatment with 0, 0.25, 0.5, 0.75, and 1mg/kg measured at 48hrs. Data represent the average and standard error 3 animals (6 tumors) per group. C. Time course for tunicamycin induced luminescence for mice treated with 0.75mg/kg followed by daily imaging. Data represent the average and standard error for 4 animals (8 tumors) for each group.

**Figure 4:** Ex Vivo analysis of NLG inhibition in D54 tumors. A. Inhibition of Luc glycosylation in tumors. Glycosylated forms of Luc were separated by ConA-agarose precipitation and lysates were analyzed by western blot for in vitro samples (left panel) and tumor samples (right panel). B. EGFR staining by immunohistochemistry in representative control and Tn treated tumors. Percent membrane staining with the standard error is also reported. C. Met fluorescence by immunofluorescence in representative control and Tn treated tumors. Fluorescence intensity values and standard error for each treatment is also reported.

**Figure 5:** Tumor growth experiments. Mice bearing wild type D54 (A.) and U87-MG (B.) xenograft tumors were randomized to four treatment groups (1) control (2) 0.75mg/kg Tn (3) Radiation (RT, 5 daily fractions of 2Gy) (4) Radiation + Tn, and followed with tumor measurements. Data points represent relative tumor growth compared to the tumor volume on Day 0, and error bars report the standard error.
References:


Figure 1:

A. 

- LucT
  - Luciferase
  - VNITY, MNISQ, MNSSG
- ER-LucT
  - ER
  - Luciferase
  - VNITY, MNISQ, MNSSG
- ER-LucS
  - ER
  - Luciferase
  - VDITY, MGISQ, MNSSG

B. 

- EGFR endoplasmic reticulum translation sequence
- cleavage site
- luciferase start

C. 

- Empty Vector
- LucT
- ER-LucT

Tn - + - + - + - +
Figure 2:

A. ER-LucS

B. ER-LucT

B. LucT

Tunicamycin (uM)

Fold Increase

Luminescence (RLU x 10^5)

Tunicamycin (uM)
Figure 2:

C.  

ER-LucT

![Graph showing Fold Increase versus Tunicamycin (μM)]

D.  

![Image of a cell culture dish with different treatments labeled C, Cs, Sw, Tn]
Figure 3:

A. [Images of mice showing luciferase expression on Day 0 and Day 2 for Control and Tn groups]

B. [Bar chart showing relative luminescence at different Tn doses (mg/kg)]

C. [Graph showing relative luminescence over time (days) with Tn and Control groups]
Figure 4:

A. In Vitro In Vivo

Tn: ++ + + + + +
Con A: − − − − + + +

B. Control Tn

α EGFR

C. Control Tn

α Met Nuclei Overlay

% Cells Stained

Overlay overlay overlay
Figure 5:

A.

B.
Molecular Imaging of N-linked Glycosylation Suggests Glycan Biosynthesis is a Novel Target for Cancer Therapy

Joseph N Contessa, Mahaveer Bhojani, Hudson H Freeze, et al.

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