Research Article

Inhibition of N-Linked Glycosylation Disrupts Receptor Tyrosine Kinase Signaling in Tumor Cells

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Abstract

Receptor tyrosine kinases (RTK) are therapeutic targets for the treatment of malignancy. However, tumor cells develop resistance to targeted therapies through the activation of parallel signaling cascades. Recent evidence has shown that redundant or compensatory survival signals responsible for resistance are initiated by nontargeted glycoprotein RTKs coexpressed by the cell. We hypothesized that disrupting specific functions of the posttranslational machinery of the secretory pathway would be an effective strategy to target both primary and redundant RTK signaling. Using the N-linked glycosylation inhibitor, tunicamycin, we show that expression levels of several RTKS (EGFR, ErbB2, ErbB3, and IGF-IR) are exquisitely sensitive to inhibition of N-linked glycosylation. Disrupting this synthetic process reduces both cellular protein levels and receptor activity in tumor cells through retention of the receptors in the endoplasmic reticulum/Golgi compartments. Using U251 glioma and BXPC3 pancreatic adenocarcinoma cell lines, two cell lines resistant to epidermal growth factor receptor–targeted therapies, we show that inhibiting N-linked glycosylation markedly reduces RTK signaling through Akt and radiosensitizes tumor cells. In comparison, experiments in nontransformed cells showed neither a reduction in RTK-dependent signaling nor an enhancement in radiosensitivity, suggesting the potential for a therapeutic ratio between tumors and normal tissues. This study provides evidence that enzymatic steps regulating N-linked glycosylation are novel targets for developing approaches to sensitize tumor cells to cytotoxic therapies. [Cancer Res 2008;68(10):3803–9]

Introduction

Receptor tyrosine kinases (RTK) are transmembrane glycoproteins that initiate downstream signaling cascades, resulting in both proproliferative and antiapoptotic cellular programs. The epidermal growth factor receptor (EGFR) and ErbB2, two members of the ErbB family of RTKs, stimulate these downstream signaling pathways in multiple cancers, including squamous cell carcinomas, adenocarcinomas of the breast, prostate and pancreas, and malignant gliomas. Tumor-specific activation of these receptors can occur through receptor overexpression, activating mutations, autocrine/juxtacrine receptor stimulation, and receptor activation by ionizing radiation. Disrupting the function of the EGFR and/or ErbB2 has therefore become a therapeutic strategy for blocking cell growth as well as for sensitizing tumors to radiation therapy.

Specific inhibitors of either EGFR or ErbB2 block carcinoma cell growth in both *in vitro* and *in vivo* experimental systems (1, 2). These findings have led to the development of two mechanistically distinct therapeutic agents to block EGFR or ErbB2 signaling; monoclonal antibodies that specifically neutralize receptors, and small molecule inhibitors that inhibit RTK activity. Both classes of inhibitors have had recent clinical successes by demonstrating improvements in patient survival. Trastuzumab, an antibody that targets ErbB2, and lapatinib, an EGFR/ErbB2 small molecule inhibitor, increases overall survival in patients with breast cancer (3–5). Erlotonib, a small molecule EGFR inhibitor provides a small but statistically significant benefit for the treatment of advanced NSCLC (6), and cetuximab, an antibody that targets the EGFR, improves survival in combination with radiotherapy in patients with head and neck cancer (7), and alone in metastatic colorectal cancer (8). However, the results of targeting these receptors in other cancer cell types that overexpress ErbB receptors, such as gliomas (9, 10) and pancreatic cancers (11) have been less impressive. These mixed clinical results show that neither the tumor cell type nor the presence of EGFR or ErbB2 expression absolutely predicts the effectiveness of these agents, and suggests that alternate cellular signal transduction cascades regulate and maintain carcinoma cell proliferation.

An evolving literature has shown carcinoma cell resistance to strategies that target single or even multiple ErbB RTKs. These resistance mechanisms are mediated through other RTK glycoproteins that initiate parallel signal transduction cassettes and promote cell growth and proliferation despite EGFR or ErbB2 blockade. For example, the insulin-like growth factor-I receptor (IGF-IR) has been implicated as a parallel signaling pathway that produces resistance to EGFR inhibition through increased phosphoinositide-3-kinase signaling (12), and coexpression of ErbB2 and ErbB3 has been shown to rescue carcinoma cells from EGFR inhibition (13). Resistance to ErbB2 inhibition has also been shown through either IGF-IR or ErbB receptor–dependent mechanisms (14, 15). We have previously shown that the kinase-inactive ErbB3 combines with the non-receptor tyrosine kinase, c-Src, to produce antiapoptotic signaling that compensates for inhibition of EGFR and ErbB2 (16, 17). These findings provide evidence that both the ErbB family and other RTKs act in a flexible signaling network that maintains mitogenic signaling when a single receptor is targeted. Therefore, a therapeutic strategy that disrupts signaling from multiple RTKs may have the potential advantage of blocking both the primary and the compensatory signaling mechanisms that have been shown to contribute to tumor cell resistance.

N-linked glycosylation is a highly regulated and critical step in the maturation of transmembrane RTK glycoproteins. This

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posttranslational modification is initiated upon entry of the polypeptide into the lumen of the endoplasmic reticulum (ER) and involves transfer of a carbohydrate moiety to an asparagine (N) residue within a specific amino acid consensus sequence NXS/T (reviewed in ref. 18). The carbohydrate side chain is then processed in the ER and Golgi network to produce a mature glycoprotein that is exported through the secretory machinery to the plasma membrane. N-linked glycosylation is a common synthetic step for many transmembrane receptor families that are targets for inhibition in cancer therapy including the IGF-IR, vascular endothelial growth factor RTK family, and the ErbB RTK family. Inhibition of N-linked glycosylation has been shown to reduce IGF-IR protein levels (19), and prevent both ligand-induced activation of the EGFR as well as constitutive EGFR-VIII signaling (20). More recently, EGFR activity has been shown to be regulated by specific glycosylation side chain modifications (21, 22), and to require glycosylation as part of the transitional state conformation required for signaling (23).

Inhibiting the synthetic pathway for N-linked glycosylation thus represents a novel, targeted approach to disrupt signaling through multiple RTKs in an effort to sensitize tumors to concomitant therapy. Therefore, we investigated the role of N-linked glycosylation in RTK signaling and tested whether inhibition of this posttranslational event sensitizes tumor cells known to be resistant to current anti-EGFR therapies to radiation therapy. Our results show the viability of inhibiting the N-linked glycosylation machinery as a tool to down-regulate RTK signaling activity and thereby sensitize tumor cells to cytotoxic therapies such as ionizing radiation.

Materials and Methods

Reagents. Unless otherwise stated, all reagents were purchased from Invitrogen. Antibodies were purchased from Cell Signaling (pY1173 EGFR, pY1248 ErbB2, pS473 Akt, total Akt, Hsp70), Santa Cruz Biotechnology (EGFR, IGF-IR), and LabVision (ErbB2, ErbB3). Tunicamycin, swainsonine, and castansospermine were purchased from Calbiochem.

Vectors. The EGFR-EGFP was constructed by subcloning a *Ban*II/*Xho*I COOH-terminal EGFR PCR fragment engineered to read through the stop codon with the *Xba*I/*Ban*II NH_2-terminal fragment. This full-length EGFR cDNA was then cloned into the *Nde*I/*Xho*I of the EGFP-N3 vector (Clontech) and sequenced.

Cell lines. The BXPC3 and U251 cell lines were purchased from American Type Culture Collection. Normal primary fibroblasts were derived from human foreskin, and all experiments were performed using cells from passages 6 to 8. Cells were cultured in RPMI medium supplemented with 5% (U251, BXPC3) or 10% (fibroblasts) FCS (Hyclone) with penicillin and streptomycin.

The BXPC3-EGFR-GFP cell line was isolated following Lipofectamine transfection of the BXPC3 parental cell line with the EGFR-EGFP vector. Colonies were selected with 500 μg/mL of G418 and evaluated for EGFP expression. A single clone was further selected by flow cytometry to recover GFP-positive cells.

Microscopy. For time-lapse microscopy, BXPC3-EGFR-GFP cells were grown in 35 or 50 mm glass-bottomed dishes (MatTek Corporation) for 24 to 48 h. Cells were then transferred to the WeatherStation environmental chamber of the Delta Vision RT Image Restoration Microscopy System (Applied Precision, Inc.) which maintained cells at 37°C in a humidified environment with 5% CO_2. Time-lapse images were acquired every 30 min using the 60× objective lens (NA1.42) and SoftWoRx software (Applied Precision). For visualization of Golgi bodies and the ER, cells were treated with 1 μm of ER-Tracker Blue-White DPX dye for 10 min prior to imaging with a long-pass 4′,6-diamidino-2-phenylindole filter on a Nikon fluorescence microscope.

^{35}S-Met labeling of EGFR. Cell cultures were pulsed with 50 μCi/mL of ^{35}S-Met (MP Biomedicals) for 6 h in RPMI plus 10% dialyzed FCS. Tunicamycin treatment (500 nmol/L) was performed as specified. Cells plates were snap-frozen after washing twice with PBS and immunoprecipitates were prepared in Western lysis buffer. Lysates were incubated with 10 μL of anti-EGFR antibody (SC-03, Santa Cruz Biotechnology) for 90 min and precipitated after a 60-min incubation with protein A-Sepharose beads. After washing twice in lysis buffer and once in PBS, samples were boiled in 1× SDS loading buffer and subjected to PAGE.

Western blot analysis. Cells were treated with inhibitors for specified times, washed with PBS, and harvested by snap-freezing on dry ice. Lysates were prepared using Western lysis buffer [25 mmol/L Tris, 10 mmol/L EDTA, 15% glycerol, 0.1% Triton X-100, 1× protease inhibitor cocktail (Roche), and 1× phosphatase inhibitor cocktails 1 and 2 (Sigma)]. Epidermal growth factor (Invitrogen) treatments were performed at a final concentration of 10 ng/mL for 10 min. Erlotonib (OSI Pharmaceuticals) pretreatment was for 1 h at a concentration of 5 μmol/L.

Quantitative real-time PCR. Cell conditions were identical to those of the time course for Western blots. RNA was harvested with Trizol, reverse-transcribed for 30 min at 54°C, and quantitative PCR was then performed using SYBR green dye on an Applied Biosystems 7300 real-time PCR system as described previously (24). Primers for EGFR (5′cgcaagtgtaagaagtgcgaa, 5′cgtagcatttatggagagtgagtct) and glyceraldehyde-3-phosphate dehydrogenase (5′tgagcgatgtggctcggct, 5′ctctctgctcctcctgttcga) were purchased from Integrated DNA Technologies.

Clonogenic survival. Dose-response colony formation assays were performed as previously reported (25) with modifications. Cell cultures were pretreated with 500 nmol/L of tunicamycin 18 h prior to irradiation. Cells were then trypsinized prior to irradiation. Cell numbers required to establish >50 colonies per 6 cm dish were plated in triplicate plates and allowed to grow for 10 days. Colonies were fixed with ethanol–acetic acid and stained with crystal violet. Irradiation was performed at a dose rate of ∼2 Gy/min using a Pantak orthovoltage unit producing 250 kV X-rays. Clonogenic survival curves were calculated according to the linear quadratic formula [$S = e^{-(y)}$, where $y = \alpha D + \beta D^2$].

Statistics. Data points are reported as experimental averages and error bars represent the SE. Statistical significance was determined using a two-sided Student's t test. $P < 0.05$ was considered to be statistically significant.

Results

Disruption of RTK protein through inhibition of N-linked glycosylation. We hypothesized that disruption of N-linked glycosylation would lead to a reduction in mature RTKs and thus result in a reduction of RTK signaling in cancer cells. We used BXPC3 pancreatic adenocarcinoma and U251 malignant glioma cell lines in our experiments due to their overexpression of multiple RTKs (EGFR, ErbB2, ErbB3, and IGF-IR), resistance to EGFR-targeted therapies, and differing primary tumor sites. Western blot analysis of cell extracts showed that nanomolar concentrations of tunicamycin, a nucleoside antibiotic that blocks GlcNAc-1-phosphotransferase and thus core glycan synthesis, reduced protein levels of each RTK (Fig. 1*A*). These results also revealed a tunicamycin-dependent decrease in tyrosine-phosphorylated EGFR (Y1173) and ErbB2 (Y1248), that paralleled the reduction of receptor protein levels. Furthermore, disruption of N-linked glycosylation using tunicamycin also decreased ErbB3 and IGF-IR, two RTKs implicated in the resistance of tumors to ErbB inhibitors. Tunicamycin treatment produced RTK proteins with faster gel mobility, consistent with smaller molecular weight isoforms of hypoglycosylated receptors. These receptor isoforms are visible in Fig. 1*A* and were more prominent with longer exposures (data not shown). In comparison, cancer cells treated with inhibitors of enzymes that modify the carbohydrates of existing glycoproteins such as castanospermine, a specific inhibitor

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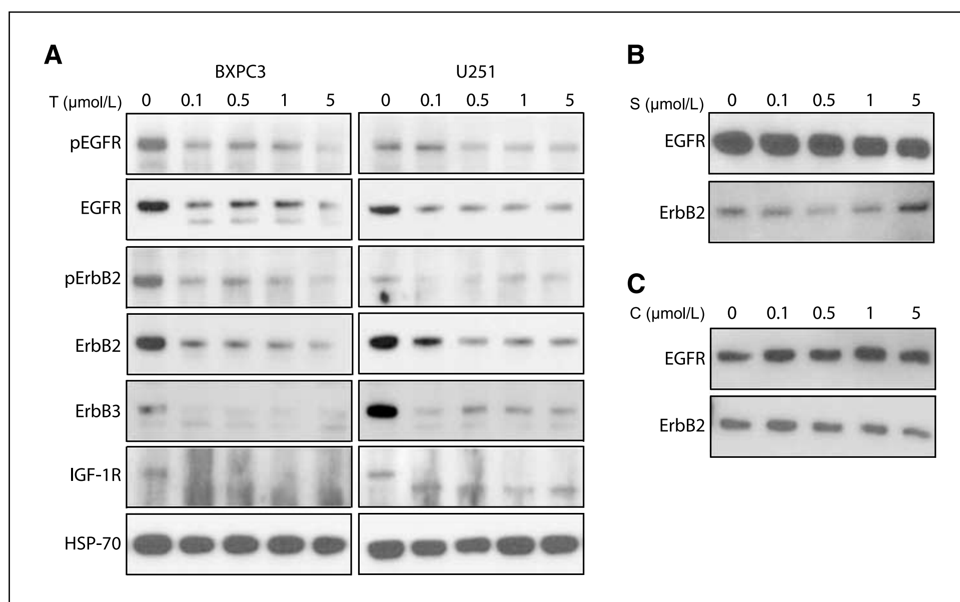

Figure 1. Tunicamycin reduces RTK protein and phosphorylation levels. *A,* BXPC3 and U251 cell lines were exposed to increasing concentrations of tunicamycin (*T*) for 18 h. Western blot analysis was performed for EGFR, ErbB2, ErbB3, and IGF-IR, as well as phosphorylated forms of EGFR (Y1173) and ErbB2 (Y1248). Blots were also probed for Hsp70 to ensure equal protein loading. *B* and *C,* parallel experiments performed with swainsonine (*S*) and castanospermine (*C*) in U251 cells.

of glucosidase, or swainsonine, a specific inhibitor of mannosidase, did not reduce RTK protein levels (Fig. 1*B* and *C*).

These results show that steps in core glycan synthesis, which occur prior to carbohydrate transfer to the protein, are required to produce mature and functional RTKs. The absence of an effect by inhibitors of later enzymatic steps involved in glycoprotein carbohydrate processing provides evidence that once transfer of the core glycan to the asparagine has occurred, further modifications of N-linked glycosylation do not significantly influence RTK protein levels. Together, these findings support the hypothesis that disruption of specific steps in N-linked glycosylation impairs RTK signaling through a reduction in RTK protein levels. Furthermore, they show that tunicamycin-induced inhibition of N-linked glycosylation abrogates the level of overexpressed RTKs in tumor cells, and is therefore a method for blocking multiple prosurvival signaling pathways with a single biochemical manipulation.

Mechanism of RTK inhibition by tunicamycin. Time course experiments were performed in BXPC3 cells to further characterize the inhibition of RTK protein overexpression. Western blot analysis of receptor protein levels following treatment with 500 nmol/L of tunicamycin showed a progressive reduction in total EGFR and ErbB2 protein over 18 h (Fig. 2*A*). Reduction in ErbB3 and IGF-IR receptors was seen somewhat sooner, with measurable reductions in total cellular receptor protein levels by 6 h. The time courses for reduced receptor levels were consistent with the individual half-lives of the respective RTKs, suggesting that tunicamycin reduced the levels of newly synthesized proteins.

To confirm that tunicamycin did not have unexpected effects on RTK protein levels through enhanced receptor degradation or reduced transcription, we performed experiments using EGFR as a prototypical RTK. ^{35}S-Met metabolic labeling of BXPC3 cells followed by immunoprecipitation of EGFR showed that 500 nmol/L of tunicamycin administered concurrently with ^{35}S-Met prevented the appearance of ^{35}S-labeled EGFR (Fig. 2*B*). In comparison, BXPC3 cells treated with tunicamycin after ^{35}S-Met labeling did not show a reduction in EGFR protein. This data shows that tunicamycin does not increase EGFR degradation and that it reduces total RTK levels by preventing the accumulation

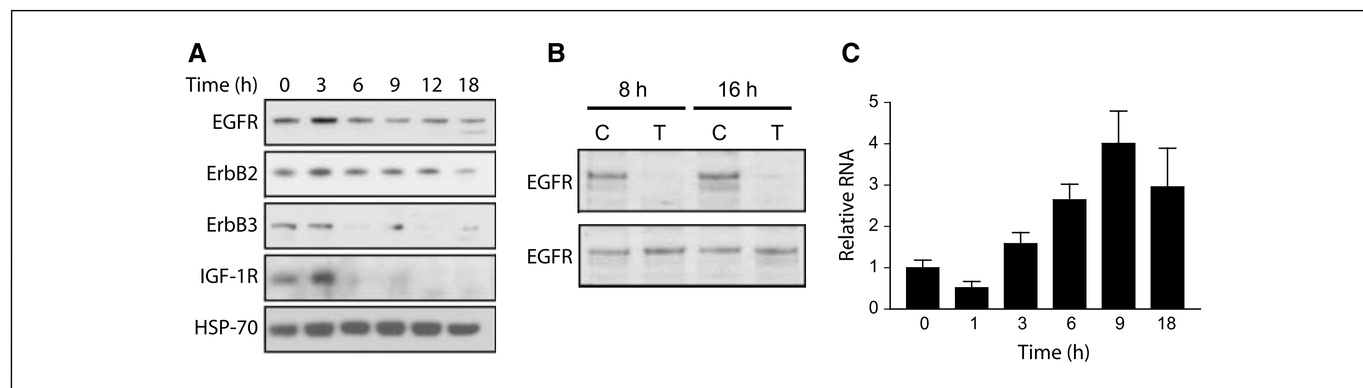

Figure 2. Mechanism of reduced RTK protein production. *A,* Western blot analysis showing the time course of reduced RTK protein levels in BXPC3 cells following treatment with 500 nmol/L of tunicamycin. *B,* cells were labeled with ^{35}S-Met for 6 h during 500 nmol/L tunicamycin treatment (*top*) or tunicamycin (*T*) was added after labeling was complete (*bottom*), and cultures were harvested at 8-h and 16-h time points after removal of the ^{35}S-Met label. EGFR was immunoprecipitated, separated by SDS-PAGE, and gels were exposed to film for 18 h. *C,* quantitative real-time PCR of EGFR mRNA after 500 nmol/L of tunicamycin treatment. Relative RNA represents the ratio of EGFR RNA/GAPDH RNA. *Columns,* averages of two experiments performed in triplicate; *bars,* SE.

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of newly synthesized receptors. Furthermore, quantitative real-time PCR experiments with BXPC3 cells treated under identical conditions showed no reduction in EGFR mRNA during this 18-h time period (Fig. 2*C*). EGFR mRNA increased over this time period, consistent with prior findings following EGFR protein depletion (24).

Cellular localization of RTKs in response to tunicamycin treatment was also characterized. We employed a strategy of real-time, live-cell imaging of an EGFR-GFP fusion protein to dynamically monitor the fate of this receptor after tunicamycin treatment. This method of localizing the EGFR has the advantage of reducing both cell selection bias and nonspecific detection. The EGFR fusion protein could be detected immunologically by both anti-EGFR and anti–phosphorylated Y1173 EGFR antibodies (Fig. 3*C*). Like the wild-type receptor, the EGFR-GFP was activated in response to 10 ng/mL of epidermal growth factor and inhibited by 5 μmol/L of erlotonib. The EGFR-GFP was readily seen to be localized in live cells to the ER, Golgi, and plasma membrane (Fig. 3*A*). Single-cell imaging of untreated cells revealed no significant change in the cellular distribution of EGFR during the observation period. However, treatment with tunicamycin resulted in an increased accumulation of EGFR in the ER and Golgi and decreased levels in post-Golgi compartments, consistent with retention of the hypoglycosylated receptor. To confirm the cellular compartment in which the EGFR resided, an ER/Golgi tracker Blue-White DPX dye was added to live cells following tunicamycin treatment (Fig. 3*B*).

Inhibition of N-linked glycosylation radiosensitizes tumor cells. These findings confirm the hypothesis that the N-linked glycosylation pathway is a potential target for both disrupting RTK function and sensitizing tumor cells to cytotoxic therapies. We therefore performed radiation clonogenic survival experiments to determine the effect of tunicamycin treatment on tumor cell survival. To be consistent with our *in vitro* studies, colony formation assays were performed following an 18-h treatment with 500 nmol/L of tunicamycin. Tunicamycin treatment significantly reduced plating efficiency in BXPC3 cells by 45% ($P < 0.05$), but did not effect plating efficiency in U251 cells. In BXPC3 cells, tunicamycin also significantly enhanced radiosensitivity at doses from 1 to 8 Gy ($P < 0.05$; Fig. 4*A*). Similar results were found in the U251 glioma cell line, in which tunicamycin produced significant radiosensitization at each dose tested ($P < 0.05$). Cell survival curves were determined using the linear quadratic formula, and surviving fractions (SF) were also derived from this equation. In

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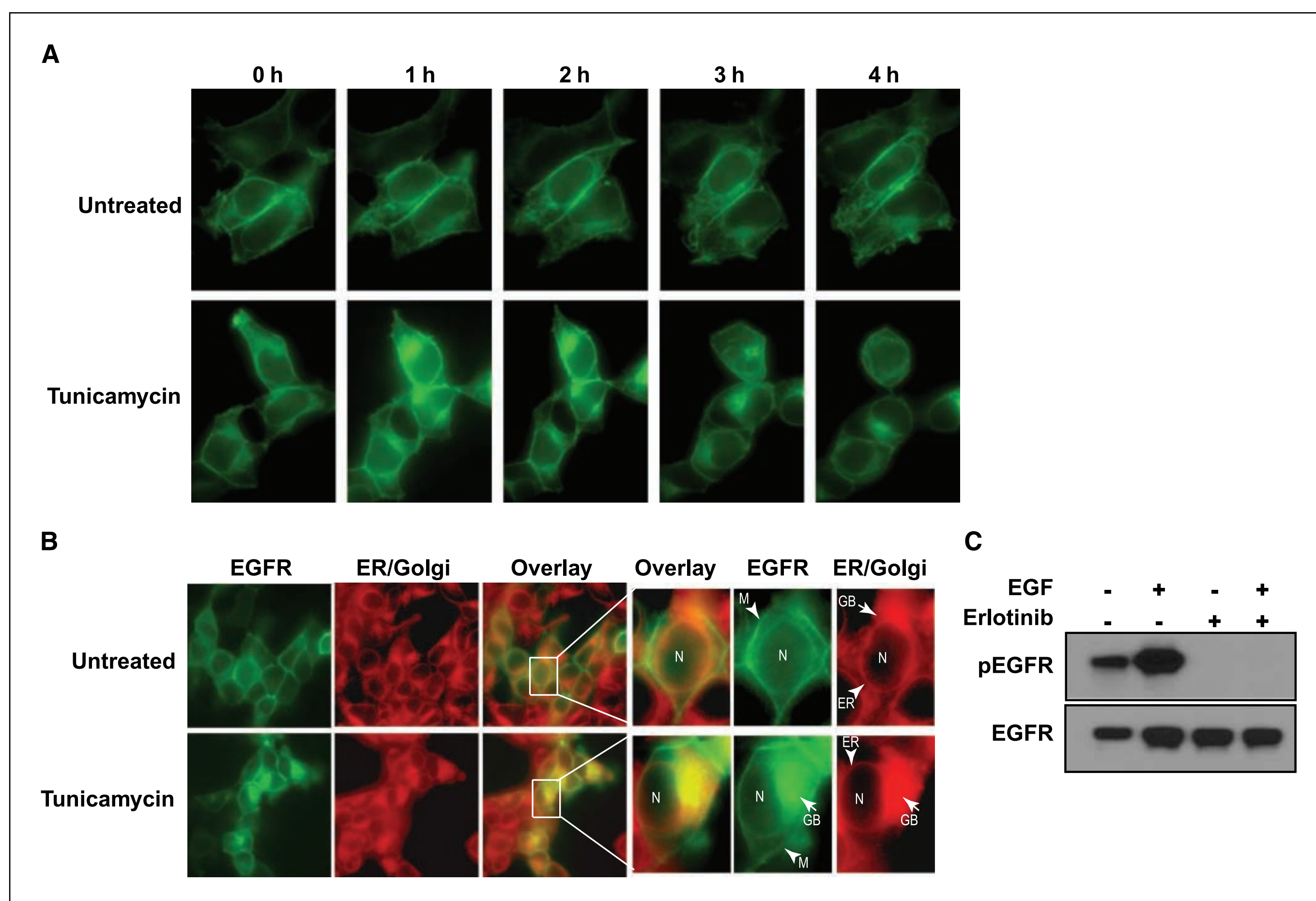

Figure 3. Live-cell EGFR imaging. BXPC3 cells with stable expression of an EGFR-GFP vector were generated as detailed in Materials and Methods. *A,* time-lapse images of EGFR localization in cells treated or untreated with tunicamycin and maintained at 37°C with 5% CO_2. Images were acquired every hour for 4 h. *B,* fluorescence micrograph of BXPC3-EGFR-GFP cells treated with or without tunicamycin for 6 h. Cells were counterstained with ER tracker to visualize ER and Golgi bodies. *Arrows,* Golgi bodies (*GB*); *arrowheads,* membrane staining (*M*) or the endoplasmic reticulum (*ER*); *N,* nucleus. *C,* BXPC3-EGFR-GFP cells overexpress the EGFR-EGFP fusion protein. This fusion protein is activated by 10 ng/mL of epidermal growth factor, as shown by Y1173 phosphorylation, and is inhibited by 1-h pretreatment with 5 μmol/L of erlotinib.

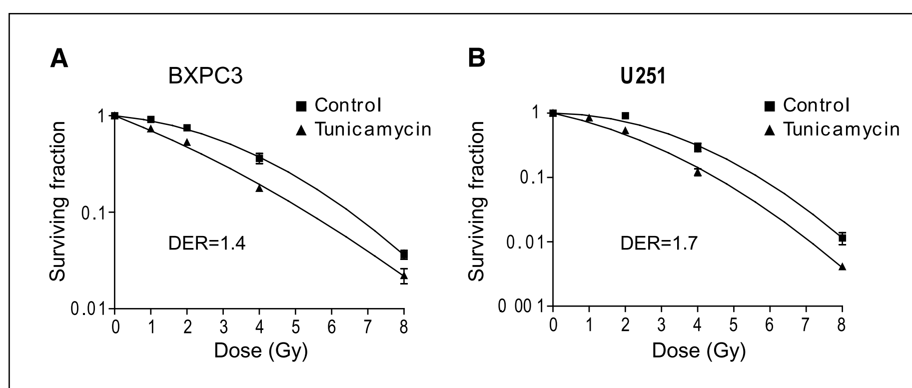

Figure 4. Radiosensitization of BXPC3 and U251 cells. *A,* clonogenic dose-response survival of BXPC3 cells pretreated for 18 h with 500 nmol/L of tunicamycin. *Points,* averages of three independent experiments performed in triplicate; *bars,* SE. *B,* clonogenic survival of U251 cells; conditions were identical to those in *A*. The dose enhancement ratio (*DER*) reports the comparative surviving fractions at 2 Gy.

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BXPC3 cells, the SF_{2Gy} was 75%, whereas tunicamycin treatment significantly reduced the SF_{2Gy} to 53% ($P < 0.05$). In U251 cells, SF_{2Gy} was 91% and tunicamycin significantly reduced survival to 53% ($P < 0.05$). The enhancement of radiation-induced cell death, or dose enhancement ratio, at 2 Gy is thus 1.4 (± 0.05) and 1.7 (± 0.04), respectively.

Inhibition of N-linked glycosylation in normal human fibroblasts. Although disruption of N-linked glycosylation in tumor cells with tunicamycin produced an enhancement in radiosensitivity, translational strategies for radiosensitization must consider the potential for a therapeutic ratio between tumor cells and normal tissues. We therefore characterized the effects of tunicamycin on normal primary human fibroblasts, an example of nontransformed cells which express the EGFR. Following tunicamycin treatment, normal fibroblasts also showed reduced EGFR protein levels, and time course experiments showed similar kinetics for the reduction of cellular EGFR protein levels (Fig. 5*A* and *B*). However, a striking difference between fibroblasts and tumor cells was that only tumor cells displayed constitutive phosphorylation of the EGFR (and ErbB2), whereas normal fibroblasts did not have detectable levels of EGFR phosphorylation. This inability to detect EGFR phosphorylation in fibroblasts was not an issue of sensitivity, as similar amounts of BXPC3 or U251 EGFR protein gave highly detectable signals. These results show that although tunicamycin reduced EGFR RTK protein levels in fibroblasts in a similar manner to tumor cells, the cellular consequences with regard to receptor activation and signaling were vastly different. In agreement with this finding, clonogenic survival experiments performed in normal fibroblasts did not show enhanced radiosensitivity by tunicamycin (Fig. 5*C*). In fact, in normal fibroblasts, tunicamycin treatment produced a trend towards protection from ionizing radiation without significantly reducing plating efficiency.

In summary, *in vitro* experiments show similar effect of tunicamycin on RTK protein levels for both tumor and non-transformed cells. In tumor cells, which are dependent on RTK-derived survival signaling, reduction of receptor number and signaling resulted in sensitization to radiation. However, in fibroblasts, which are not transformed and are not dependent on autocrine signaling for growth and survival, RTKs are not constitutively activated and these cells cannot be sensitized to radiation by reducing RTK protein levels.

N-linked glycosylation and Akt signaling. Although tunicamycin reduced RTK protein levels in both tumor cells and normal fibroblasts, only tumor cells had reduced RTK phosphorylation and enhanced sensitivity to the cytotoxic effects of ionizing radiation. We therefore hypothesized that prosurvival signals downstream of RTKs (i.e., EGFR, ErbB2, ErbB3, and IGF-IR) would also be abrogated following tunicamycin treatment. In lung, breast, and glioma model systems, phosphorylation of Akt has been used as a measure of

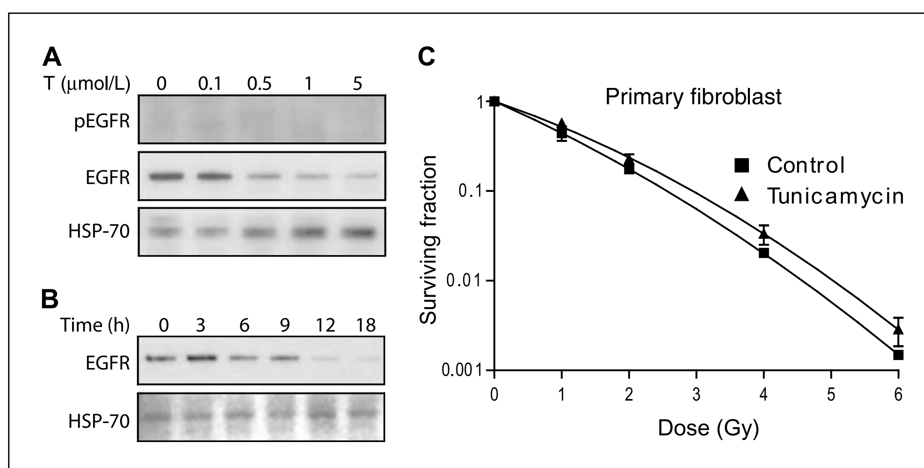

Figure 5. Differential effect of tunicamycin on normal fibroblasts. *A,* Western blot analysis of EGFR and phosphorylated EGFR (Y1173) in primary fibroblasts exposed to increasing doses of tunicamycin (*T*). *B,* time course of EGFR protein inhibition in primary fibroblasts following an 18-h treatment with 500 nmol/L of tunicamycin. Blots were also probed for Hsp70 to ensure equal loading. *C,* clonogenic survival of primary fibroblasts pretreated for 18 h with 500 nmol/L of tunicamycin. *Points,* averages of two independent experiments performed in triplicate.

effective upstream RTK inhibition. We therefore determined the effect of tunicamycin treatment on Akt phosphorylation in BXPC3, U251, and normal fibroblast cells (Fig. 6). In both tumor cell lines, Akt phosphorylation was not blocked by EGFR inhibition with cetuximab (data not shown). However, disrupting N-linked glycosylation did reduce Akt phosphorylation in BXPC3 and U251 cells. In comparison, tunicamycin treatment had no effect on Akt phosphorylation in normal fibroblasts.

Discussion

The contributions of coexpressed growth factor receptors to developing resistance to therapeutic RTK inhibitors have been mechanistically shown (12–15, 26, 27). This has confirmed the concept that inhibition of multiple RTKs may be of therapeutic benefit (28–30). In an effort to test this concept, we have undertaken a new strategy to disrupt the posttranslational machinery (i.e., N-linked glycosylation) to decrease the protein level and activity of a broad range of cell surface receptors. In this study, we found that nanomolar concentrations of the N-linked glycosylation inhibitor, tunicamycin, reduced the tumor cell protein levels of four RTKs (EGFR, ErbB2, ErbB3, and IGF-IR) that contribute to tumor cell proliferation and survival and have become targets for antineoplastic therapies. Under these conditions, tunicamycin reduced RTK overexpression without affecting the general translation of other proteins (i.e., Hsp70 and Akt). Biochemical analysis with ^{35}S-Met labeling showed that this drug inhibits the cellular accumulation of newly synthesized RTK proteins and these findings were confirmed by live-cell imaging experiments. Using the EGFR as a prototypical RTK, we show that inhibition of N-linked glycosylation results in the retention of receptors in the ER and Golgi prior to their down-regulation.

The consequence of reducing RTK protein levels in tumor cells is a commensurate reduction of both activated RTKs and downstream Akt phosphorylation. Consistent with other strategies that target multiple RTKs (25), disruption of RTK signaling correlated with enhanced radiosensitivity for both glioma and pancreatic cancer cell lines. These findings were in contrast with the effects of tunicamycin on normal fibroblasts, which express unactivated EGFR protein, and are therefore not dependent on this receptor for Akt phosphorylation. In these normal cells, disruption of N-linked glycosylation did not enhance radiosensitivity. Together, these experiments show that inhibition of N-linked glycosylation is a novel strategy for blocking RTK signaling both within and across RTK families and that this pathway is a target that may be exploited to enhance radiation therapy.

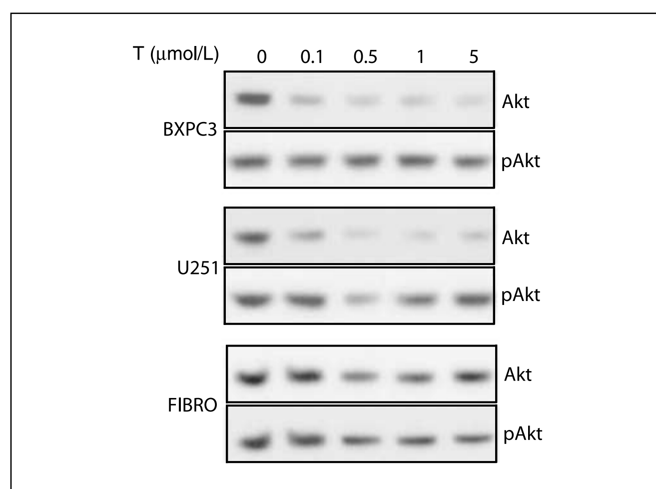

Figure 6. Differential effect of tunicamycin on Akt phosphorylation in tumor cells and fibroblasts. BXPC3, U251, and primary fibroblasts were treated for 18 h with increasing doses of tunicamycin. Western blot analysis was performed to determine the phosphorylation status of Akt (S473) compared with total Akt protein levels.

Many autocrine-regulated RTKs identified as therapeutic targets have increased expression or activity in tumor cells, yet successful targeting of an individual receptor does not predict tumor cell response to the therapy. In pancreatic tumor cells, this may be due to dysregulation of non-RTK signaling proteins such as k-Ras or SMAD transcription factors. In gliomas, there are known alternative pathways for tumor progression (i.e., p53 mutation) which occur independently of up-regulated EGFR signaling. However, the heterogeneity of overexpressed RTKs is another mechanism by which a given cancer cell produces both parallel and compensatory mitogenic signaling. The ErbB receptor family is a well-studied example of a coordinated signaling network that circumvents attempts at targeted RTK inhibition. For example, blockade of EGFR tyrosine kinase activity can be essentially replaced by activation of the ErbB2/ErbB3 coreceptor, and conversely, EGFR can substitute for the inhibition of ErbB2. Somewhat more surprising is that inhibition of both EGFR and ErbB2 can be compensated for by reconstitution of signaling through the kinase *inactive* ErbB3 with either the non-receptor tyrosine kinase (16) or the MET RTK (26). Although there have been great strides in empirically defining the appropriate RTK target for a given tumor type, the above examples of cellular flexibility to maintain oncogenic signaling within just a single RTK family make the prospect of choosing the appropriate RTK target a priori unlikely. Thus, the strategy of targeting N-linked glycosylation has a considerable advantage by both targeting multiple receptor types and having a more pronounced effect on those that are overexpressed.

A current concept with regard to RTKs in cell signaling is that cancer cells become “addicted” to the powerful growth-promoting cascades initiated by these receptors (31). A reliance on RTK signaling (or networks of RTKs) is thus a feature of the transformed phenotype and is not seen in normal cells. Our findings in tumor cells and normal fibroblasts are consistent with this concept. We show that disruption of N-linked glycosylation reduces up-regulated RTK signaling in tumor cells by reducing RTK protein levels, whereas in fibroblasts, this manipulation does not alter RTK activation levels. This effect on signaling was confirmed at both the level of the receptor and downstream at the level of Akt phosphorylation, where inhibition of N-linked glycosylation disrupted Akt activity only in tumor cells. This differential response between tumor cells and normal fibroblasts thus suggests the basis for an exploitable therapeutic ratio. Taken together, our data show that low doses of tunicamycin can be used to reduce up-regulated or addicted RTK signaling in tumor cells and make them more sensitive to the toxic effects of ionizing radiation. Importantly, this therapeutic ratio does not require that N-linked glycosylation is inhibited to the point where it causes toxicity, but only to the point where it normalizes RTK expression and signaling.

Is the N-linked glycosylation pathway a practical target in cancer therapy? Tunicamycin used at micromolar concentrations (5 μmol/L≈4.2 μg/mL) induces ER stress, which might suggest that toxicity for normal cells could be prohibitive. However, there are successful examples of agents that induce cellular stress, such as proteosome inhibitors and Hsp90 inhibitors, currently

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undergoing clinical or preclinical investigations. Furthermore, our results show that RTK protein levels in tumor cells are exquisitely sensitive to concentrations of tunicamycin that are at least 10- to 50-fold lower than those used to induce ER stress. Although inhibition of N-linked glycosylation is a process common to all cells, targeting this pathway with the goal of reducing RTK protein levels and sensitizing cells to a second, cytotoxic therapy may be feasible. Ultimately, the success of any agent that targets the N-linked glycosylation pathway will be dependent on a therapeutic ratio between the tumor and the normal tissues. In this work, we show no enhancement of radiosensitivity by tunicamycin in nontransformed fibroblasts; however, the final answer to this question of practicality will require evaluation in animal models. The development of precise molecular tools to measure the effects of disrupting N-linked glycosylation in tumors *in vivo* is currently under way.

Although we have shown the mechanism by which inhibition of N-linked glycosylation disrupts RTK signaling, we cannot exclude effects on other classes of glycoproteins which could also contribute to tumor cell radiosensitivity. For example, secreted protein ligands can also require glycosylation for activity, and the function of other transmembrane proteins involved in both intercellular and intracellular signaling could be compromised by disrupting N-linked glycosylation. Nevertheless, our findings provide strong evidence that disruption of N-linked glycosylation inhibits multiple prosurvival receptors both within and across RTK families as well as sensitizing tumor cells to radiation. A recent molecular study that confirmed evidence for parallel signaling through RTKs in both glioma and pancreas cell lines concluded that effective clinical therapies might require the administration of multiple small molecule RTK inhibitors (27). Our work provides the basis for an alternative approach to targeting multiple overexpressed RTKs in cancer cells and suggests that selective targeting of a biosynthetic pathway involved in the production of multiple RTKs (i.e., N-linked glycosylation) could be developed into a therapy that blocks the primary, dominant signaling RTK (e.g., EGFR), as well as the secondary, compensatory signaling, RTKs coexpressed by the cell.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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