Efficacy of Combining GMX1777 with Radiation Therapy for Human Head and Neck Carcinoma

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Abstract

Purpose: Rapidly metabolizing tumor cells have elevated levels of nicotinamide phosphoribosyltransferase, an enzyme involved in NAD+ biosynthesis, which serves as an important substrate for proteins involved in DNA repair. GMX1777, which inhibits nicotinamide phosphoribosyltransferase, was evaluated in two human head and neck cancer models in combination with radiotherapy.

Experimental Design: Effects of GMX1777-mediated radiosensitization were examined via metabolic and cytotoxicity assays in vitro; mechanism of action, in vivo antitumor efficacy, and radiosensitization were also investigated.

Results: IC50 values of GMX1777 for FaDu and C666-1 cells were 10 and 5 nmol/L, respectively, which interacted synergistically with radiotherapy. GMX1777 induced a rapid decline in intracellular NAD+ followed by ATP reduction associated with significant cytotoxicity. These metabolic changes were slightly increased with the addition of radiotherapy, although poly(ADP-ribose) polymerase activity was significantly reduced when GMX1777 was combined with radiotherapy, thereby accounting for the synergistic cytotoxicity of these two modalities. Systemic GMX1777 administration with local tumor radiotherapy caused complete disappearance of FaDu and C666-1 tumors for 50 and 20 days, respectively. There was also significant reduction in tumor vascularity, particularly for the more sensitive FaDu model. [18F]FDG positron emission tomography/computed tomography images showed reduction in [18F]FDG uptake after GMX1777 administration, showing decreased glucose metabolism in vivo.

Conclusions: Our data represent the first report showing that GMX1777 plus radiotherapy is an effective therapeutic strategy for head and neck cancer, mediated via pleiotropic effects of inhibition of DNA repair and tumor angiogenesis, while sparing normal tissues. Therefore, GMX1777 combined with radiotherapy definitely warrants clinical evaluation in human head and neck cancer patients. Clin Cancer Res; 16(3); 898–911. ©2010 AACR.

Head and neck cancer is the fifth most common neoplasm worldwide (1, 2), with an estimated 644,000 new cases diagnosed each year wherein 60% of such patients present with stage III or IV disease (1, 2). Such locally advanced head and neck squamous cell cancers are treated with either hyperfractionated radiotherapy (3) or chemoradiotherapy (1, 2) but still associated with >50% mortality rate (1, 2), underscoring an urgent need to develop novel molecular therapies.

Cancer cells have a dysregulated metabolism, which provides a survival advantage despite a noxious environment (4, 5). Consequently, there is increased demand for energy consumption and DNA repair. One of the key cofactors in cellular respiration is NAD+, which plays several essential roles, including serving as a coenzyme in redox reactions, providing substrates for the poly(ADP-ribose) polymerase (PARP), and sirtuins (6), a newly described group of proteins involved in multiple functions including DNA repair that requires NAD+ to deacetylate (7). All these processes are involved in stress response, genomic instability, and apoptosis. In the mammalian system, the three major precursors of NAD+ synthesis are tryptophan, nicotinic acid, and nicotinamide (8). In the salvage pathway of NAD+ resynthesis, one rate-limiting enzyme is nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the transfer of the phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate to nicotinamide, forming nicotinamide mononucleotide, and pyrophosphate. In turn, nicotinamide

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angiogenesis. Growth, associated with significant reduction in tumor NAD+ (10, 12, 13). FK866 has been combined with ionizing antitumor efficacy, associated with depletion of cellular FK866 (10) and CHS828 (11); both of which have shown groups have developed inhibitors of this target, such as expression of NAMPT in human cancers (9), several mononucleotide adenylyltransferase. mononucleotide is converted to NAD+ by nicotinamide mononucleotide adenyltransferase.

Given the metabolic demands of cancer cells and over-expression of NAMPT in human cancers (9), several groups have developed inhibitors of this target, such as FK866 (10) and CHS828 (11); both of which have shown antitumor efficacy, associated with depletion of cellular NAD+ (10, 12, 13). FK866 has been combined with ionizing radiation (14) in a mouse mammary tumor model, showing a delay in tumor growth, associated with reduction in bioenergetic status (14). This current study is the first evaluation of GMX1777 (15), a prodrug of GMX1778 (CHS828), in conjunction with radiotherapy, examined in two human head and neck cancer models. Synergistic cytotoxicity was observed for this combination, associated with rapid depletion of intracellular NAD+, followed by ATP reduction. This therapeutic combination was effective in vitro, causing significant delay in tumor growth, associated with significant reduction in tumor angiogenesis.

Materials and Methods

Cell lines. The two human head and neck cancer models were FaDu (human hypopharyngeal squamous cancer) and C666-1 (EBV-positive nasopharyngeal cancer), obtained from the American Type Culture Collection and the Chinese University of Hong Kong (16), respectively. GM05757, a primary normal human fibroblast strain, was obtained from the Coriell Institute for Medical Research. C666-1 and FaDu cells were cultured as described previously (17, 18). All cell lines were authenticated via microscopic morphology check and growth curve analysis.

Compound dilutions. GMX1777 (EB1627) and its active metabolite, GMX1778 (CHS828), were both obtained from Gemin X Pharmaceuticals. For in vitro experiments, GMX1778 was dissolved in DMSO at a concentration of 1000 μmol/L, with subsequent dilutions in medium. For negative control (vehicle alone), DMSO was diluted in medium to a concentration corresponding to the DMSO in the respective drug-treated groups. For in vivo experiments, GMX1777, the prodrug of GMX17778, which is metabolized by liver enzymes into the active GMX1778 compound, was dissolved in 5% dextrose and administered as described.

Cell viability and clonogenic assays. FaDu, GM05757, and C666-1 cells were seeded in 96-well plates at 5,000 or 8,000 per well in 200 μL growth medium and then incubated for 24 h. GMX1778 was subsequently added to a total volume of 10 μL. After 72 h, the viability MTS assay was conducted according to the manufacturer’s specifications, with 0.1% DMSO-treated cells serving as a negative control.

Colony formation assays for FaDu cells were conducted as described previously (18). GMX1778 or vehicle control was added at the specified concentrations. Surviving fractions were determined as the ratio of the number of colonies in the treated sample to that of untreated controls. Clonogenic assays were not conducted for C666-1 cells because they are not amenable to forming colonies in vitro.

Radiation treatments. For in vitro experiments, cells were irradiated at room temperature using a 137Cs unit (Gammar Cell 40 Extractor; Nordion International) at a dose rate of 0.86 Gy/min. For in vivo experiments, mice were immobilized in a Lucite box, and the tumor-bearing leg was exposed to 225 kVp (13 mA) at a dose rate of 3.37 Gy/min using an X-ray irradiator C (X-RAD 225; Precision X-ray).

Morphologic assessment of apoptosis. FaDu and C666-1 cells were seeded (0.5 × 10^6 per T-25 flask), allowed to incubate for 1 day, and treated with 10 nmol/L GMX1778. After 24 h for FaDu and 48 h for C666-1, cells were stained with Hoechst 33342 (Invitrogen) and visualized by fluorescence microscopy as described previously (18).

Quantitative real-time PCR. Forward (5′-GCCCAGGGAAATTTGTTA-3′) and reverse (5′-TGATGCTGCTTCAGTTTC-3′) primer pairs were designed for human NAMPT mRNA using the Primer Express software (Applied Biosystems). β-Actin primers were purchased from Applied Biosystems. FaDu, C666-1, and GM05757 cells were seeded (3 × 10^5 per well of six-well plates) and 2 days later lysed for total RNA extraction using the RNeasy Mini kit (Qiagen). Reverse transcription was conducted using SuperScript II reverse transcriptase (Invitrogen) as specified by the manufacturer. Quantitative real-time PCR was done using SYBR Green (Applied Biosystems) and a PerkinElmer/ABI Prism 7900 sequence detection system (PE Biosystems). The mean fold change in mRNA expression was calculated using the 2^(-ΔΔCt) method (19).

Flow cytometric analysis. After 24 h following seeding in six-well plates (3 × 10^5 per well), FaDu cells were treated with GMX1778 (10 nmol/L) ± radiation (4 Gy) 24 h later. Two days after radiation, cells were processed for cell cycle analysis as described previously (18). For determination of propidium iodide (PI) positivity, FaDu cells were harvested and washed with fluorescence-activated cell sorting buffer (0.5% bovine serum albumin-PBS) after treatment.
followed by incubation with 1.0 μg/mL PI (Sigma) on ice for 5 min, and then analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**ATP, NAD+, and PARP measurements.** Intracellular ATP content was determined with a luciferase-based ATP assay (Calbiocem) according to the manufacturer's instructions. Luciferase activity was measured in the cell lysates of FaDu, C666-1, and GM05757 cells at 12 to 96 h post-GMX1778 treatment. ATP content was also assessed in FaDu and GM05757 cells treated with GMX1778 ± radiotherapy at 8 to 72 h posttreatment. Intracellular NAD+ content was analyzed in the supernatant after cell lysis, homogenization, and centrifugation at 14,000 rpm for 5 min using the NAD+/NADH Quantification kit (Medical Biological Laboratories). PARP activity in treated FaDu cells was determined by measuring net PAR expression using the PARP In vivo Pharmacodynamic Assay ( Trevigen) as specified by the manufacturer.

**γ-H2AX nuclear foci.** FaDu cells seeded and treated on coverslips were fixed with 2% paraformaldehyde-0.2% Triton X-100 for 20 min, washed with PBS, and permeabilized with 0.3% NP-40 for 20 min. Cells were blocked with 2% bovine serum albumin-1% normal donkey serum for 1 h and then incubated with γ-H2AX monoclonal primary antibody (clone JBW301; Upstate Biotechnology) overnight at 4°C. Coverslips were washed with 0.175% Tween 20-0.5% bovine serum albumin-PBS and incubated with donkey anti-mouse Alexa 488 secondary antibody (1:100 dilution; BD Pharmingen) and mouse anti-human Ki-67 antibody (clone JBW301; Upstate Biotechnology) with microwave antigen retrieval. Tumor nuclear staining was considered positive for Ki-67. CD31+ microvessels were scored semiquantitatively in tumor areas. All slides were examined and scored independently by two observers.

**Normal gut epithelium toxicity assay.** Normal tissue toxicity was determined by measuring intestinal clonogenic survival in vivo as described previously (21). Briefly, tumor-bearing mice were treated with either 5% dextrose or 50 mg/kg GMX1777 daily for 5 days by intramuscular injection as per above. Where indicated, whole-body irradiation of 4 Gy (Gammacell 40 Extractor) was administered on days 2 and 5 following intramuscular injection. Three days after the final GMX1777 administration, the small intestines were removed, washed, and fixed in formalin. Gut cross-sections (six sections per mouse) were stained with Ki-67, and the number of surviving crypts in each treatment group was scored, defined as >5 Ki-67-positive intestinal epithelial cells.

**[18F]FDG-positron emission tomography/computed tomography imaging.** C666-1 and FaDu xenograft tumors were established in severe combined immunodeficient mice as described above and allowed to grow until the tumor plus leg diameter reached 9.75 mm. Scans were acquired at pretreatment and after 2 days of GMX1777 administration. On the day of imaging, food was removed 12 h before injection with [18F]FDG but with free water access. Blood glucose level was measured before injection using a glucometer (Ascensia Contour; Bayer) and determined to be within normal limits. [18F]FDG (9.66 ± 3.29 MBq in 0.2 mL) was injected via tail vein. During the [18F]FDG uptake period, mice were sedated with 1.5% to 2% isoflurane in 100% oxygen and placed in a temperature-controlled Lucite box. Positron emission tomography (PET) imaging was acquired 60 min after [18F]FDG injection using a micro-PET Focus 220 system (Siemens) immediately followed with a micro-CT scan using routine image acquisition parameters.
[18F]FDG-PET images were analyzed quantitatively (22) by manually drawing regions of interest for each image slice; for each tumor, the [18F]FDG uptake signal intensity was obtained from a total of ∼15 to 25 regions of interest. The maximum %ID/g was then determined. The change in [18F]FDG uptake for each tumor after treatment was thus calculated and compared with that of vehicle-treated mice. Maximum intensity projection images were used as they more accurately illustrate the overall [18F]FDG uptake changes in the entire tumor pretreatment and posttreatment versus coronal or axial single slices, which are ∼0.8 mm thick and only represent a small portion of the tumor.

Statistical analyses. All data were compiled using Microsoft Excel and graphed using GraphPad Prism (GraphPad Software). All experiments were done at least three independent times, with the data presented as mean ± SE. The statistical differences between treatment groups were determined using the Student’s t test and one-way ANOVA. Statistical significance was attained if *P < 0.05, **P < 0.01, or ***P < 0.001.

Results

Synergistic cytotoxicity of GMX1778 with irradiation. Head and neck cancer is a highly heterogeneous disease; hence, cell line models representing that spectrum, ranging from nasopharyngeal (C666-1; doubling time of 48 h) to hypopharyngeal (FaDu; doubling time of 21 h) subsites, were selected for examination. As shown in Fig. 1A, GMX1778 induced significant cytotoxicity in both FaDu and C666-1 cells, with IC50 values of 10 and 5 nmol/L, respectively. The combination of GMX1778 with radiotherapy was evaluated using both MTS viability (both FaDu and C666-1) and clonogenic assays (FaDu; Fig. 1B-D). Cells treated with GMX1778 (0.05-100 nmol/L) and radiotherapy (4 Gy), administered simultaneously or sequentially (radiotherapy-GMX or GMX-radiotherapy, 24 h apart), were subjected to the MTS assay 3 days after the last treatment. For C666-1 cells, the lowest viability of 3.2% was observed with GMX1778 followed 24 h later by radiotherapy (Fig. 1B). For FaDu cells, the lowest viability of 4.2% was observed with the simultaneous administration of GMX1778 and radiotherapy (Fig. 1C). However, clonogenic assays with FaDu cells, wherein GMX1778 (5 nmol/L) and radiotherapy (2-6 Gy) were administered with the aforementioned sequences, showed that GMX1778 first followed 24 h later by radiotherapy appeared to achieve the lowest survival levels (Fig. 1D). Hence, for the remainder of this study, the sequence examined for both cell lines was GMX1778 first followed 24 h later by radiotherapy.

Two additional clonogenic assays were conducted in FaDu cells, examining in further detail the interaction between GMX1778 (10 nmol/L) and radiotherapy (2-8 Gy; Fig. 1E and F). This interaction was determined to be synergistic based on the Chou-Talalay median-effect principle (23) wherein the combination index was consistently <1 for all surviving fractions (Supplementary Fig. S1).

GMX1778 induces apoptosis. Apoptosis appeared to be one mode of cell death in response to GMX1778 treatment wherein, based on morphologic assessment, both C666-1 and FaDu cells showed nuclear condensation with Hoechst 33342 staining (Supplementary Fig. S2A). Flow cytometric DNA content analyses also showed an increase in the sub-G1 population from 3.8% to 7.3% for FaDu and from 2.1% to 8.1% for C666-1 cells after GMX1778 treatment compared with controls (Supplementary Fig. S2B). The combination with radiotherapy, however, did not induce any further increase in the sub-G1 population for either cell line, but an increase in G2-M was observed.

GMX1778 induces cytotoxicity via reduction in NAD+ and ATP levels. Global cellular cytotoxicity of GMX1778 was assayed using PI staining in FaDu cells (Fig. 2A), showing a marked increase in the proportion of PI-positive cells starting with 12% at 48 h with GMX1778 alone (20 nmol/L) up to 25% by 72 h (P < 0.01). The addition of radiotherapy (4 Gy) at 4 h post-GMX1778 further increased the proportion of PI-positive cells, up to 30% by 72 h (P < 0.01). Given the inhibitory role of GMX1778 in NAD+ biosynthesis, total cellular NAD+ was measured over time in FaDu cells as a function of different treatments (Fig. 2B). Compared with vehicle-treated cells, GMX1778 (20 nmol/L) reduced NAD+ levels as early as 4 h, down to 0 by 24 h (P < 0.01). The addition of radiotherapy had no further effect, although, interestingly, radiotherapy alone increased NAD+ content at 24 h (P < 0.01). To better understand the effects of GMX1778 treatment, PI positivity, NAD+, and ATP content were assayed simultaneously, longitudinally, over a 5-day period (Fig. 2C). As already observed in Fig. 2B, NAD+ level was the first to decline in FaDu cells after GMX1778 exposure (20 nmol/L), down to 0 by 24 h. Subsequently, ATP started to fall to 58% of control cells at 24 h, progressively reduced to 19% by 48 h, and then approached 0 by 72 h. In turn, PI positivity became detectable as early as 24 h post-GMX1778 treatment, slowly increasing by 2.7-fold versus control at 72 h and then rapidly increasing to a 7.7-fold increase by 96 h, which remained at that level for another day.

Tumor selectivity of GMX1778 was addressed by measuring the relative basal transcript level of NAMPT, which was ∼6-fold higher in both head and neck cancer cell lines compared with GM05757 normal fibroblasts (Supplementary Fig. S3). This difference in NAMPT mRNA expression correlated with resistance to ATP perturbation in that cellular ATP levels in GM05757 fibroblasts did not budge until 96 h of GMX1778 exposure, in contrast to a much earlier reduction observed at 48 h for both head and neck cancer cell lines (Fig. 2D). This tumor selectivity remained consistent with the addition of radiotherapy wherein ATP levels remained unaltered when GM05757 fibroblasts were treated with GMX1778 ± radiotherapy up to 72 h (Fig. 2E). In contrast, when FaDu cells were thus treated, ATP declined for GMX1778 alone (20 nmol/L) at 24 h, which was significantly further reduced by the addition of radiotherapy (4 Gy; P < 0.05). By
48 and 72 h, ATP levels were drastically reduced by both treatments (P < 0.01), although radiotherapy alone had no effect on ATP level. In sum, these results showed that GMX1778 induced cell death via rapid depletion of NAD+ followed by reduction in intracellular ATP in head and neck cancer cells, which was slightly increased by the addition of radiotherapy.

**GMX1778 reduces PARP activity and inhibits DNA repair.** Given the clear demonstration of a synergistic interaction between GMX1778 with radiotherapy (Fig. 1E and F; Supplementary Fig. S1) and the requirement for NAD+ in poly(ADP)ribosylation of DNA repair enzymes, PARP activity in FaDu cells after GMX1778 ± radiotherapy was investigated (Fig. 2F); FaDu cells were treated with GMX1778 (20 nmol/L) followed 4 h later with radiotherapy. PARP activity was significantly reduced with GMX1778 alone, down to 52% versus control cells (P < 0.01). Interestingly, radiotherapy alone increased PARP activity, but the combination of GMX1778 with radiotherapy compromised PARP activity down to 68% versus vehicle-treated controls (P < 0.05), indicating that this combinatorial approach indeed interfered with DNA repair capacity. This effect was further corroborated by quantification of γ-H2AX foci, a commonly used marker for DNA double-strand breaks (24), showing a significantly higher number of foci when FaDu cells were treated with GMX1778 alone or when combined with radiotherapy (Supplementary Fig. S4).

**In vivo efficacy of GMX1777 with radiotherapy for head and neck cancer xenograft tumors.** GMX1777 (50 mg/kg/d, intramuscularly, days 1-5) with or without local tumor radiotherapy (4 Gy, days 2 and 3) was effective for both FaDu and C666-1 tumors in vivo (Fig. 3). This regimen was adapted from our previous in vivo therapeutic studies studying drug-radiotherapy combinations (18, 20), which follows the optimized in vitro treatment sequence described in Fig. 1: drug first followed 24 h later by irradiation, with the initial radiotherapy coinciding with the intracellular NAD+ depletion (Fig. 2C), and interference with DNA repair capacity. A second radiotherapy dose was administered following the final GMX1777 treatment to ensure irradiation of the remaining drug-exposed cells. Impressively, as early as 2 days after completion of treatment, tumor plus leg diameters between vehicle- and GMX1777-treated groups differed by ~3.5 mm (P < 0.001) for FaDu (Fig. 3A) and ~2.7 mm (P < 0.001) for C666-1 tumors (Fig. 3C). GMX1777 alone appeared to delay tumor growth more effectively than radiotherapy alone for FaDu (Fig. 3A) compared with C666-1 (Fig. 3C) tumors. However, the most efficacious treatment approach was the combination of GMX1777 with radiotherapy, particularly for the FaDu tumors, with complete tumor disappearance for 50 days (Fig. 3A). A similar effect was observed for the C666-1 model, although tumors started to regrow at day 20 (Fig. 3C).

This treatment regimen was well tolerated in that total body weight did not fluctuate significantly for either group of tumor-bearing mice (Fig. 3B and D). The low toxicity profile of the regimen was further validated in a murine normal tissue pelvic irradiation model based on intestinal crypt cell survival, a well-established method to assess normal tissue toxicity in vivo (21, 25). GMX1777 at 50 mg/kg/d for 5 days ± radiotherapy did not reduce the proportion of Ki-67-positive normal gut epithelial cells beyond the minimal effects observed with radiotherapy alone (Fig. 3E and F). These observations differed, however, when a higher dose of GMX1777 (100 mg/kg) was administered (Supplementary Fig. S5). With complete tumor regression observed for 50 and 70 days when GMX1777 was combined with radiotherapy for the C666-1 and FaDu tumors, respectively, this was associated with a reduction in mouse total body weight.

**GMX1777 reduces proliferation and vasculature in head and neck cancer models.** Given the impressive therapeutic efficacy of GMX1777 ± radiotherapy in vivo, tumor cell proliferation and microvessel density were assayed 24 h after treatment completion. Ki-67 expression was significantly reduced with GMX1777 treatment for both FaDu and C666-1 models (Fig. 4), down to 49% (P < 0.01) and 57% (P < 0.05), respectively (Fig. 4E and J), versus control or radiotherapy-alone tumors. Consistent with the greater sensitivity of FaDu tumors to GMX1777 plus radiotherapy (Fig. 3A), Ki-67 expression was further decreased with this combinatorial therapy down to 11% (Fig. 4E; P < 0.01). In contrast, Ki-67 was only slightly reduced to 51% when C666-1 tumors were treated with both drug and radiotherapy (Fig. 4; P < 0.05).

A similar pattern was observed with tumor microvessel density as evaluated by CD31 expression. For FaDu tumors, CD31 immunoeexpression was significantly reduced to 18% after GMX1777 alone (P < 0.01) and 4% when combined with radiotherapy (Fig. 5A-E; P < 0.01). In contrast, for C666-1 tumors, GMX1777 alone reduced CD31 expression down to 89% of control tumors (P < 0.05), but the combination with radiotherapy had minimal additional effect (Fig. 5F-I).

[18F]FDG-PET images reflect head and neck tumor response. Given the rapid decline in intracellular bioenergy levels in vitro, one would anticipate that [18F]FDG-PET images should reflect such changes in vivo (26). To that end, [18F]FDG-PET images were obtained at baseline and at post-GMX1777 treatment in tumor-bearing mice. [18F]FDG uptake relative to baseline for FaDu tumors indeed showed a reduction to 72% after 2 days of systemic administration of GMX1777 (Fig. 6A; Table 1). In contrast, minimal reduction (~6%) was observed for C666-1 tumors (Fig. 6B; Table 2), in part reflecting the reduced sensitivity of C666-1 tumors to GMX1777 treatment. Representative fused [18F]FDG-PET/computed tomography images of the FaDu tumor pre- and post-GMX1777 are presented in Fig. 6C, showing a reduction in signal intensity post-drug administration. It should be noted that one cannot accurately assess [18F]FDG biodistribution changes in other organs from Fig. 6C, without setting regions of interest on the organ of interest and obtaining maximum
Fig. 1. *In vitro* efficacy of GMX1778 with or without radiotherapy. A, MTS assay was conducted 72 h posttreatment for C666-1 and FaDu cells exposed to increasing concentrations of GMX1778 (GMX; 0.001–100 nmol/L). B, C666-1 cells were treated with three different sequences: GMX1778 (0.01–100 nmol/L) simultaneously with radiotherapy (RT; 4 Gy) or GMX1778 and radiotherapy delivered 24 h apart in either sequence. C, as in B but for FaDu cells. D, clonogenic assay for FaDu cells treated with GMX1778 (5 nmol/L) and radiotherapy (2–6 Gy) in the abovementioned sequences. E, clonogenic assay for FaDu cells treated with GMX1778 alone (0.1–50 nmol/L) or with radiotherapy (4 Gy) delivered 24 h after GMX1778. F, clonogenic survival of FaDu cells given radiotherapy (2–8 Gy) ± GMX1778 (10 nmol/L) with radiotherapy delivered 24 h post-GMX1778. All experiments were conducted at least three independent times in triplicate. Mean ± SE or the log of mean ± SE.
Fig. 2. Biochemical changes in cells treated with GMX1778 with or without radiotherapy. A, PI positivity of FaDu cells after GMX1778 (20 nmol/L) treatment ± radiotherapy (4 Gy) delivered at 4 h post-GMX1778 and monitored for 72 h. B, intracellular NAD+ level in FaDu cells treated with vehicle or GMX1778 (20 nmol/L) ± radiotherapy (4 Gy) delivered after 4 h and monitored for 24 h. C, intracellular NAD+ and ATP levels and PI positivity in 20 nmol/L GMX1778-treated FaDu cells and monitored over 120 h. D, intracellular ATP levels in GM05757, C666-1, and FaDu cells after exposure to GMX1778 for 12 to 96 h. E, ATP levels in GM05757 fibroblasts versus FaDu cells after radiotherapy (4 Gy) alone, GMX1778 (20 nmol/L) alone, or GMX1778 plus radiotherapy (4 Gy) delivered 4 h post-drug. F, PARP activity in FaDu cells after vehicle, GMX1778 (20 nmol/L), radiotherapy alone (4 Gy), or GMX1778 plus radiotherapy (delivered 4 h post-drug). Samples were collected for PARP assessment 30 min post-treatment completion. Each experiment was conducted at least three times independently, in triplicate. Mean ± SE. *, P < 0.05; **, P < 0.01.
Fig. 3. Therapeutic efficacy of GMX1777 with or without radiotherapy in FaDu and C666-1 xenograft tumors. A, FaDu tumors were treated with vehicle, local tumor radiotherapy alone (4 Gy; days 2 and 5), GMX1777 alone (50 mg/kg/d, intramuscularly; days 1–5), or GMX1777 plus radiotherapy. Tumor plus leg diameter was measured at least thrice per week. B, total body weight (g) of FaDu tumor-bearing mice. C, as in A but for C666-1 tumors. D, body weight (g) for C666-1 tumor-bearing mice. E, intestinal crypt cell survival in FaDu tumor-bearing mice following treatment as in A. Surviving crypts were scored 3 d post-treatment completion based on three to four mice per treatment group. Crypt surviving fraction was calculated as the percentage of surviving crypts in the treatment versus vehicle control group. **, P < 0.001, statistically significant difference between indicated treatment groups.
%ID/g values in pretreated and post-treated mice, as per FaDu tumors. Furthermore, a high degree of intravariability in physiologic [18F]FDG uptake exists among normal tissues (e.g., myocardium), a well-known phenomenon in human and mice models, which is influenced by many factors, including fasting duration, body temperature, plasma glucose, free fatty acid, and insulin levels, at the time of scanning (27–29); thus, interpretation of such images for normal tissues should be undertaken with precaution.

**Discussion**

Metabolic reprogramming of human cancers is highly complex, represented by the iconic “Warburg” effect (30), wherein cancer cells upregulate glycolysis, which is inefficient in the production of ATP molecules yet enhances survival under conditions when oxygen tension is constantly fluctuating (3). Oxidative phosphorylation is an energy-efficient process wherein each glucose molecule will generate 38 ATP molecules; in contrast, during glycolysis, each

![Fig. 4. Ki-67 expression in FaDu (A–E) and C666-1 (F–J) tumors. Tumor-bearing mice were treated as described in Fig. 3: vehicle (A and F), radiotherapy alone (B and G), GMX1777 alone (C and H), or GMX1777 plus radiotherapy (D and I). Tumors were removed (two mice per group) 24 h post-treatment completion and then subjected to Ki-67 immunostaining. E and J, relative fold change in the proportion of Ki-67–expressing tumor cells, normalized to that of vehicle control. Scoring was conducted by counting the percentage of Ki-67–positive tumor cells in three high-power fields for each tumor. All data are plotted as mean ± SE fold change from four different xenograft tumors (12 high-power fields) for each treatment group. *, P < 0.05; **, P < 0.01.](image-url)
glucose molecule will only generate 2 ATP and 2 NADH (reduced form of NAD\(^+\)) molecules. NADH, in turn, is critically involved in redox reactions wherein NADH is oxidized to NAD\(^+\) and 2 electrons donated to the electron transport chain. NAD\(^+\) can be synthesized de novo from amino acids, such as tryptophan, or can be recycled through the salvage pathway using nicotinic acid, nicotinamide, and nicotinamide riboside, involving the key rate-limiting enzyme NAMPT, also known as pre-B-cell colony-enhancing factor-1, or visfatin (31). Given that this enzyme is overexpressed in human malignancies (9), and cancers have an increased demand for energy and metabolic turnover, many compounds have been developed, targeting different components of these complex pathways (5), including two different NAMPT inhibitors, which have shown anticancer properties (10, 11).

**Fig. 5.** CD31 expression in FaDu (A–E) and C666-1 (F–J) tumors. Tumor-bearing mice were treated as described in Fig. 3: vehicle (A and F), radiotherapy alone (B and G), GMX1777 alone (C and H), or GMX1777 plus radiotherapy (D and I). Tumors were removed (two mice per group) 24 h post-treatment completion and then subjected to CD31 immunostaining. E and J, relative fold change in the proportion of CD31 expression, normalized to vehicle-treated tumors. Scoring was conducted by counting the proportion of CD31 expression in three high-power fields for each tumor. All data are plotted as mean ± SE fold change from four different xenograft tumors (12 high-power fields) for each treatment group. *, \(P < 0.05\); **, \(P < 0.01\).
The NAMPT inhibitor examined in this work was GMX1777, a prodrug of GMX1778 (11), with documented cytotoxicity in several preclinical models such as lymphoma, multiple myeloma, and lung cancer (12, 13, 32, 33), through the induction of apoptosis and necrosis, associated with rapid reduction in intracellular NAD⁺ and ATP levels (12, 13, 33). GMX1777, a pyridyl cyanoguanidine compound, can be administered orally and has been subjected to a phase I evaluation in patients with solid tumors (34); a dose of 420 mg every 3 weeks was recommended for phase II testing. GMX1777 has shown synergy with etoposide (35) but has never been examined with ionizing radiation or in head and neck cancer models. Given the importance of NAD⁺ as a substrate for PARP and sirtuins (36), both involved in DNA repair, it followed that GMX1777 warranted evaluation in combination with radiotherapy.

Based on the clonogenic survival curves (Fig. 1E and F) and the Chou-Talalay combination index (Supplementary Fig. S1), it was clear that GMX1777 interacted with radiotherapy in a synergistic manner for all surviving levels. This synergy was recapitulated in vivo, where GMX1777...
plus radiotherapy significantly delayed tumor growth (Fig. 3A and C), in a more than additive manner. The mechanism of synergy was related in part to interference with DNA damage repair because PARP activity was significantly reduced when GMX1778 was combined with radiotherapy (Fig. 2F), corroborated by the significant increase in nuclear γ-H2AX foci (Supplementary Fig. S4). The most notable effect of GMX1777 treatment was the rapid decline in intracellular NAD⁺ down to 50% as early as 4 h after initial exposure, becoming undetectable by 24 h (Fig. 2B and C). Shortly thereafter, total cellular ATP also fell rapidly to only 20% by 48 h and to an undetectable level by 72 h, which in turn was associated with a significant increase in cell death, as measured by PI staining (Fig. 2C). Similar kinetics in the decline of NAD⁺ and ATP levels have been reported for lymphoma, multiple myeloma, and lung cancer cell lines with GMX1777 treatment (12, 13, 33), corroborating that perturbation of the NAD⁺ salvage pathway is responsible for its cytotoxicity.

Given the crucial involvement of NAMPT in NAD⁺ metabolism, the mechanisms by which GMX1777 contributes to genotoxicity might be attributable to two possible processes. Firstly, as a rate-limiting determinant for NAD⁺ production, GMX1777-mediated NAMPT inhibition will reduce intracellular NAD⁺, which in turn will decrease the activities of prosurvival enzymes that use NAD⁺ as a substrate, such as the PARP and sirtuins (6). Secondly, NAMPT inhibition will result in accumulation of intracellular nicotinamide, which is an endogenous inhibitor of several NAD⁺-dependent enzymes, including the same PARP and sirtuins (6), thereby further hindering DNA repair, resulting in cytotoxicity. Both processes may render GMX1777-treated tumors more vulnerable to the DNA-damaging effects of ionizing radiation, overwhelming the DNA repair capacity of the cell, resulting in the observed enhanced cell death (Figs. 1 and 3).

A similar but structurally distinct agent, FK866, which also targets NAMPT, has been examined in combination with radiotherapy and showed likewise reductions in NAD⁺ levels and bioenergetic status, observed using ¹H decoupled ³¹P magnetic resonance spectroscopy in a mouse mammary tumor model (14). FK866 was also examined in a phase 1 clinical trial for 24 patients with solid tumors, with 4 patients observed to have stable disease (37). Given the reduction in bioenergy status determined by magnetic resonance spectroscopy, we asked whether similar changes could be observed using [¹⁸F]FDG-PET/computed tomography images, which should reflect the state of tumor glucose metabolism (26, 38). In our work, the early response of head and neck cancer xenograft tumors to GMX1777 appeared to reflect overall sensitivity of the two head and neck cancer models in that the more sensitive FaDu tumors showed a greater reduction in [¹⁸F]FDG-PET uptake compared with the C666-1 tumors (Fig. 6; Tables 1 and 2). These data suggest that [¹⁸F]FDG-PET images might be able to provide an early indicator of tumor response to GMX1777; certainly, this modality has been suggested to be useful in predicting response of FaDu tumors to cisplatin (39).

An intriguing observation is the reduction of tumor vascularity in both head and neck cancer models, concordant with decreased tumor cell proliferation, as measured by Ki-67 staining (Figs. 4 and 5). Tumor vascularity was assayed because of the impressive delay in tumor growth observed with FaDu tumors, more than would have been predicted based just on the in vitro clonogenic survival curves (Fig. 1E and F), suggesting an additional tumor microenvironmental influence. The degree of tumor vascularity reduction, as evaluated by CD31 immunostaining, correlated with GMX1777 sensitivity, being more significantly reduced in the FaDu versus C666-1 tumors (Fig. 5). These novel observations would be consistent with the recently reported induction of vascular endothelial growth factor by visfatin (or NAMPT) in several normal tissue models such as HUVECs and chick chooroallantoic membranes (40, 41). Hence, agents that inhibit NAMPT would be expected to reduce tumor vessel growth presumably mediated by decreased vascular endothelial growth factor production.

In conclusion, a novel anticancer agent GMX1777, which targets the NAD⁺ salvage pathway, is shown to be highly efficacious when combined with radiotherapy for head and neck squamous cell cancer and nasopharyngeal cancer models, supporting the utilization of this combinatorial regimen as a novel therapeutic strategy for head and neck cancer. GMX1777 efficacy was mediated by a remarkable rapid decline in bioenergetic status of tumor cells, causing cell death, which is tumor selective, because normal human fibroblasts (Fig. 2) and tissues (Fig. 3) were minimally perturbed by such treatments. Synergy with radiotherapy was attributable to interference with DNA damage repair through the NAD⁺-PARP pathway, further

### Table 1. Relative maximum %ID/g of [¹⁸F]FDG uptake between vehicle control and GMX1777-treated (50 mg/kg/d, intramuscularly, for 2 d) FaDu tumors

<table>
<thead>
<tr>
<th></th>
<th>Maximum %ID/g</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>18.1</td>
<td>1</td>
</tr>
<tr>
<td>GMX1777</td>
<td>13.1</td>
<td>0.72</td>
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</tbody>
</table>

### Table 2. Relative maximum %ID/g of [¹⁸F]FDG uptake between vehicle control and GMX1777-treated C666-1 tumors

<table>
<thead>
<tr>
<th></th>
<th>Maximum %ID/g</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>21.3</td>
<td>1</td>
</tr>
<tr>
<td>GMX1777</td>
<td>20</td>
<td>0.94</td>
</tr>
</tbody>
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compromised by reduction in cellular ATP levels. In vivo efficacy was additionally contributed by inhibition of tumor vascularity and reduced tumor cell proliferation, with minimal toxicity observed in normal tissues. Finally, of particular interest is the potential ability of [18F]FDG-PET imaging to predict early response to this novel therapeutic strategy. Hence, GMX1777 has significant potential as a radiation sensitizer and definitely warrants clinical evaluations for patients with head and neck cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Efficacy of Combining GMX1777 with Radiation Therapy for Human Head and Neck Carcinoma

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