NAMPT Inhibitor GMX1778 Enhances the Efficacy of ^{177}Lu-DOTATATE Treatment of Neuroendocrine Tumors

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Neuroendocrine tumors (NETs) can be treated by peptide receptor radionuclide therapy using radiolabeled somatostatin analogs. However, the efficacy of such treatment is low and needs to be optimized. Our study evaluated the potential radiosensitizing effects of inhibition of nicotineamide phosphoribosyltransferase on ^{177}Lu-DOTATATE treatment in a NET model. **Methods:** Nude mice xenografted with the human NET cell line GOT1 were treated with semiefficient doses of ^{177}Lu-DOTATATE (7.5 MBq, intravenously) or the nicotineamide phosphoribosyltransferase inhibitor GMX1778 (100 mg/kg/wk, orally). **Results:** Median time to tumor progression (tumor volume larger than at day 0) was 3 d for controls, 7 d for single-dose GMX1778, 28 d for single-dose ^{177}Lu-DOTATATE, 35 d for 3 weekly doses of GMX1778, and 98 d for combined treatment with ^{177}Lu-DOTATATE and GMX1778 × 1. After ^{177}Lu-DOTATATE and 3 weekly doses of GMX1778, none of the tumors progressed within 120 d. **Conclusion:** GMX1778 enhances the efficacy of ^{177}Lu-DOTATATE treatment and induces a prolonged antitumor response.

Key Words: neuroendocrine tumor (NET); ^{177}Lu-DOTATATE; peptide receptor radionuclide therapy; NAMPT inhibitor; GMX1778

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Neuroendocrine tumor (NETs) express high levels of somatostatin receptors (SSTRs), enabling the use of somatostatin analogs for both imaging and therapeutic purposes. Peptide receptor radionuclide therapy with radiolabeled somatostatin analogs is used in select cases to treat nonresectable NETs, resulting in symptomatic improvement, enhanced quality of life, and prolonged survival. Because of dose-limiting hematotoxicity and nephrotoxicity, the cure rate is low (*1*), and optimization of this treatment modality is needed. We have previously shown that the xenografted intestinal NET cell line GOT1 can be effectively treated with ^{177}Lu-DOTATATE. The antitumor effect was dose-dependent: administration of high doses (30 MBq or more) resulted in total tumor eradication, whereas lower doses (7.5 MBq; semiefficient) resulted in 50% tumor reduction followed by progression after 2 wk (Supplemental Fig. 1; supplemental materials are available at http://jnm.snmjournals.org) (*2*). We have also demonstrated a strong antitumor effect of the pyridyl cyanoguanidine GMX1778 (formerly CHS 828) on GOT1 cells both in vitro and in vivo. A weekly oral dose of 250 mg/kg/w completely eradicated the tumors within 3 wk, without any adverse effects. A lower dose (100 mg/kg/w) resulted in halted tumor growth, but no tumor regression (Supplemental Fig. 2) (*3*). It has been shown that GMX1778 inhibits nicotineamide phosphoribosyltransferase (NAMPT), an enzyme involved in nicotinamide-adenine-dinucleotide (NAD^+) metabolism (*4*). Radiotherapy causes DNA damage, which in turn induces activation of poly(adenosine diphosphate [ADP]-ribose) polymerase 1 (PARP-1) and consumption of NAD^+ (*5*). Inhibition of NAD^+ regeneration has been suggested as a radiosensitizing strategy (*6*).

The aim of this study was to investigate the potentially radiosensitizing effect of the NAMPT inhibitor GMX1778 on ^{177}Lu-DOTATATE treatment of NETs using a xenograft model.

MATERIALS AND METHODS

Animal Model and Xenografting

The xenograft model with the human small intestinal NET cell line GOT1 in nude mice has been described previously (*7*). In brief, small pieces (~1 mm) of excised tumor were transplanted subcutaneously to female BALB/c nude mice. All procedures were approved by the Ethical Committee for Animal Research at the University of Gothenburg.

Pharmaceuticals

GMX1778, (*N*-(6-chlorophenoxyhexyl)-*N′*-cyano-*N″*-4-pyridylguanidine), was formulated as a 20 mg/mL suspension in 2% carboxymethyl cellulose in 0.9% saline. The radiolabeling and quality control of ^{177}Lu-DOTATATE, with a specific activity of 30 MBq/μg, were performed as previously described (*2*).

Treatment with ^{177}Lu-DOTATATE and GMX1778

Animals were divided into 6 groups: controls (vehicle only, $n = 6$), ^{177}Lu-DOTATATE (7.5 MBq, $n = 10$), GMX1778 × 1 (single dose of 100 mg/kg, $n = 7$), GMX1778 × 3 (3 weekly doses of 100 mg/kg, $n = 5$), ^{177}Lu-DOTATATE (7.5 MBq) + GMX1778 × 1 ($n = 6$), and ^{177}Lu-DOTATATE (7.5 MBq) + GMX1778 × 3 ($n = 5$). ^{177}Lu-DOTATATE was injected in a tail vein, and GMX1778 was given by oral gavage. In the combined therapy groups, GMX1778 was given 1 h after ^{177}Lu-DOTATATE.

Animals were followed up to 17 wk and were killed when tumor weight exceeded 10% of body weight or body weight was reduced by

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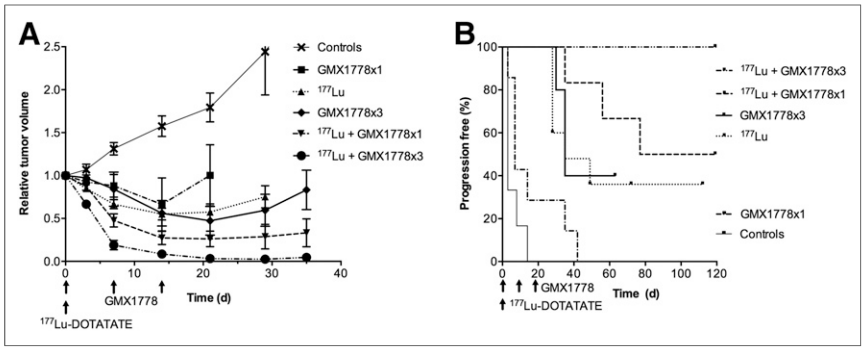

FIGURE 1. Antitumor effect of a single dose of ^{177}Lu-DOTATATE (7.5 MBq intravenously) or 1 or 3 weekly oral doses of NAMPT inhibitor GMX1778 (100 mg/kg/w) on xenografted small intestinal NET GOT1. (A) Relative individual tumor volumes normalized to start of treatment (day 0). Data are mean ± SEM. (B) Kaplan–Meier graph with time to progression—that is, tumor volume larger than at start of treatment (day 0). Censored data indicated as tick marks.

more than 10%. Animal weights and tumor sizes (longest diameter and the 2 perpendicular diameters measured by calipers) were monitored regularly. Tumor volumes were calculated by assuming spheroid tumor shapes ($V = 4\pi r_1 r_2 r_3/3$). The relative tumor volume at a given time point was defined as the tumor volume divided by the volume at day 0.

Kidney Uptake of ^{177}Lu-DOTATATE

Two groups of animals were given ^{177}Lu-DOTATATE (7.5 MBq, $n = 8$) or ^{177}Lu-DOTATATE (7.5 MBq) + GMX1778 × 1 (single dose of 100 mg/kg, $n = 7$), as described above. After 24 h animals were killed, the kidneys were weighed, and ^{177}Lu activity was measured using a γ-counter.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) of SSTR Subtype 2 (SSTR2) Messenger RNA

GOT1 cells were cultured as previously described (*3*). Cells were then treated with GMX1778 at 10 nM, 20 nM, or with vehicle control (0.2% dimethyl sulfoxide) for 1, 5, or 14 h. For each condition and time point, cells were seeded in duplicate and for each of these duplicates qPCR analysis was run in triplicate. The whole experiment was repeated 3 times. RNA extraction was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was synthesized using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Messenger RNA expression levels were analyzed using predesigned TaqMan Gene Expression Assay (Thermo Fisher Scientific): glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Hs02800695_m1), and *SSTR2* (Hs00990356_m1). The PCRs were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). For each RNA sample, the levels of *SSTR2* messenger RNA expression are given relative to those of the 2 housekeeping genes *GAPDH* and *HPRT1*.

Immunohistochemical Analysis of SSTR2 Protein

GOT1 tumors were analyzed with respect to SSTR2 protein expression using immunohistochemistry. Sections from formalin-fixed, paraffin-embedded tumors were incubated with antibodies against SSTR2A (clone UMB1; cat no. ab134152 [Abcam]) followed by Dako EnVision FLEX+ system. Stained sections were evaluated with respect to staining intensity and the percentage of labeled tumor cells according to Körner et al. (*8*).

In Vitro Measurement of NAD^+

GOT1 cells were cultured as previously described (*3*). Cells were divided into 4 groups: controls, GMX1778 (10nM), external radiation, and GMX1778 (10nM) + external radiation. Culture medium was changed, containing GMX1778 at 10 or 0 nM. After 1-h incubation, the culture plates were cooled on ice and irradiated at 1 or 0 Gy, maintaining sterile conditions, and then further incubated at 37°C. Four replicates (each ~20 million cells) per condition and time point were harvested at 1, 5, and 14 h. Cells were pelleted, flash frozen, and submitted to Metabolon Inc. for analysis. Briefly, a liquid chromatography–mass spectrometry method was used, and NAD^+ amount was normalized to total protein amount of each sample.

Data Analysis

Kaplan–Meier data for tumor volumes were analyzed using Cox regression followed by pairwise comparisons between groups using SAS (version 9.3; SAS Institute). *P* values were adjusted for multiple comparisons by Holm–Bonferroni adjustment, and values less than 0.05 were considered significant. Differences in *SSTR2* messenger RNA were analyzed by the unpaired Student *t* test. Differences in SSTR2 protein expression were analyzed by the Mann–Whitney test.

RESULTS

Treatment with GMX1778 Increases Reduction of GOT1 Tumor Volume After ^{177}Lu-DOTATATE

All 33 treated animals did well, and only 1 animal in the control group had to be killed because of weight loss. The maximal tumor volume reduction after single semiefficient doses of ^{177}Lu-DOTATATE (7.5 MBq) or GMX1778 (100 mg/kg) was seen after 2 wk, when the average tumor volumes were reduced by 45% and 34%, respectively. Three weekly doses of GMX1778 resulted in a maximal tumor volume reduction of 53% at 3 wk. Combining a single dose of ^{177}Lu-DOTATATE and a single dose of GMX1778 resulted in a more pronounced antitumor effect, with a maximal reduction of 73% at 3 wk. Combining ^{177}Lu-DOTATATE and 3 weekly doses of GMX1778 caused a maximal tumor volume reduction of 97% at 4 wk. One of 5 tumors was eradicated and had not recurred by the end of the experiment at 17 wk (Fig. 1A).

Combined Treatment with GMX1778 and ^{177}Lu-DOTATATE Induces Prolonged Antitumor Response in GOT1-Bearing Mice

A single dose of GMX1778 delayed tumor growth marginally, and the median time to progression was 7 d, compared with 3 d for controls. One dose of ^{177}Lu-DOTATATE or 3 weekly doses of GMX1778 further delayed tumor growth, and the median time to progression was 35 d. Combining ^{177}Lu-DOTATATE and a single dose of GMX1778 further increased median time to progression to 98 d. Combining ^{177}Lu-DOTATATE and 3 weekly doses of GMX1778 eradicated 1 of the tumors, whereas the remaining 4 tumors eventually regrew although none progressed—that is, exceeded the initial volume—by the end of the experiment at 17 wk (Fig. 1B). Statistical analysis revealed that time to tumor

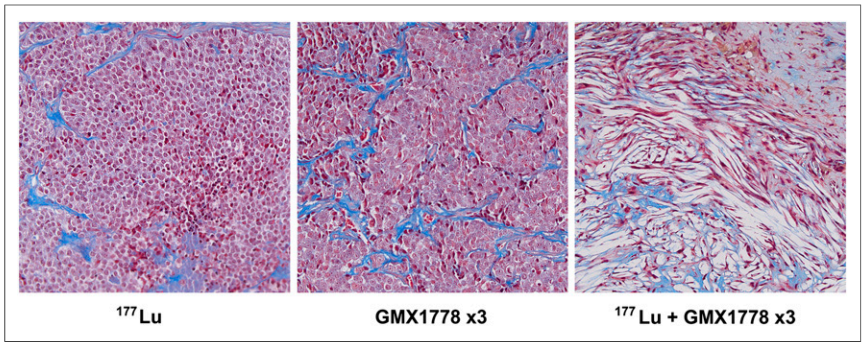

FIGURE 2. Micrographs of xenografted GOT1 tumors after treatment with ^{177}Lu-DOTATATE (7.5 MBq intravenously), 3 weekly oral doses of NAMPT inhibitor GMX1778 (100 mg/kg/w), or combined treatment with ^{177}Lu-DOTATATE (7.5 MBq intravenously) and 3 weekly oral doses of GMX1778 (100 mg/kg/w). Minor regressive changes were observed after monotherapies. Complete regression of tumors was observed only in a few tumors after combined treatment. Masson-trichrome stain.

progression for all treatment groups, except a single dose of GMX1778, was significantly different from controls. Time to tumor progression for the combination of ^{177}Lu-DOTATATE and 3 weekly doses of GMX1778 differed significantly from single doses of ^{177}Lu-DOTATATE or GMX1778. Histologic examination of xenografted tumors at the end of experiments verified the neuroendocrine phenotype. All treated tumors showed signs of regressive changes (Fig. 2).

GMX1778 Treatment Does Not Affect Uptake of ^{177}Lu-DOTATATE in Kidneys

With clinical ^{177}Lu-DOTATATE treatment, the kidneys are the main organs at risk and the kidney uptake is dose limiting. GMX-treated animals had a mean uptake of 90% of control animals, which was a nonsignificant difference according to the Student *t* test (data not shown).

GMX1778 Treatment Does Not Affect Expression of SSTR2 in GOT1 Cells and GOT1 Tumors

SSTR2 is often overexpressed in small intestinal NETs and is the most important receptor for ^{177}Lu-DOTATATE uptake and antitumor effect. A possible mechanism for the GMX1778 radiosensitizing effect could thus be upregulation of SSTR2. However, we found no alteration of *SSTR2* gene expression in cultured GOT1 cells after GMX1778 treatment for 1, 5, or 14 h (Supplemental Fig. 3A). Furthermore, immunohistochemical analysis of GOT1 tumors did not reveal any differences in SSTR2-labeling intensity or the percentage of labeled tumors in GMX1778-treated animals compared with control animals (Supplemental Figs. 3B and 4).

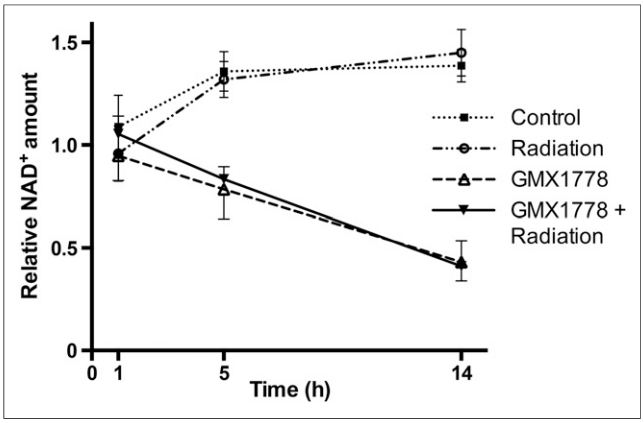

FIGURE 3. Relative NAD^+ concentrations in cultured GOT1 cells. Cells were incubated up to 14 h with or without GMX1778 (10 nM) and received or did not receive 1.0 Gy of external radiation at the start of the incubation (time = 0). Error bars = SDs.

GMX1778 Treatment Reduces Amount of NAD^+ in GOT1 Cells

Cultured GOT1 cells were incubated with GMX1778, 10 Nm, or irradiated with 1 Gy. These doses of GMX1778 and external radiation had a small cytotoxic effect visible after several days but no effect on cell viability within the first 24 h (data not shown). GMX1778 had a clear NAD^+-reducing effect already after 5 h and a more pronounced effect after 14 h (Fig. 3).

DISCUSSION

Clinical outcome of peptide receptor radionuclide therapy in NET patients may be improved by combination treatments. In a xenograft model with small cell carcinoma of the lung (NCI-H69), the combined treatment with ^{177}Lu-DOTATATE and carboplatin/etoposide resulted in a considerable tumor reduction compared with ^{177}Lu-DOTATATE or chemotherapy alone (*9*). In NET patients, radiolabeled somatostatin analogs have been combined with chemotherapy in small series with limited effects: 5-fluorouracil (*10,11*), its prodrug capecitabine (*12*), or both temozolomide and capecitabine (*13*).

An alternative way to increase the antitumor effect of peptide receptor radionuclide therapy might be the use of radiosensitizers, which act synergistically on DNA repair mechanisms to enhance tumor cell death (*14*). One such mechanism is the radiation-induced PARP-1 activation with subsequent NAD^+ depletion. The PARP-1–induced NAD^+ depletion is a radiation-induced cell death mechanism, which may be exploited to enhance the effect of radionuclide therapy. PARP-1 is a nuclear enzyme that, in response to DNA strand breaks, catalyzes the synthesis of ADP-ribose from the substrate NAD^+. The ADP-ribose polymers are incorporated into the damaged DNA, thus opening the condensed chromatin structures and making them more accessible to DNA repair enzymes (*5*). NAD^+ serves as a substrate during ADP-ribosylation, but is also a coenzyme involved in several redox reactions, including adenosine triphosphate generation. DNA damage induces PARP-1 activation and NAD^+ consumption. Massive DNA damage can result in NAD^+ depletion, which eventually causes depletion of adenosine triphosphate energy stores and cellular death (*15*). However, NAD^+ is normally resynthesized via the salvage pathway involving the rate-limiting enzyme NAMPT. GMX1778 inhibits NAMPT and can thereby, in the case of PARP-1 activation, reduce NAD^+ to lethally low levels (Fig. 4) (*4*).

After the identification of its mechanism of action—inhibition of NAD^+ synthesis via NAMPT inhibition—GMX1777/1778 has been

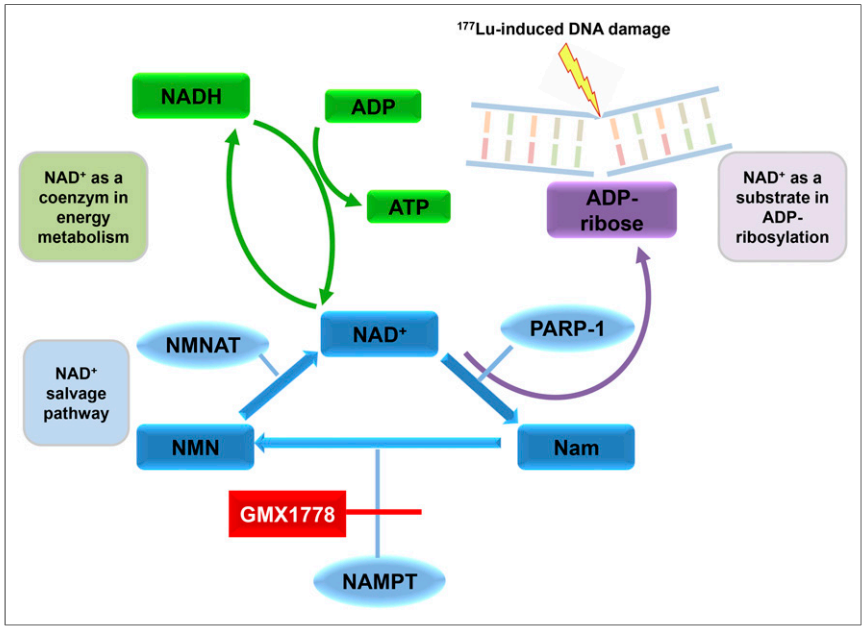

FIGURE 4. Hypothetic model of radiosensitizing effects of NAMPT inhibitor GMX1778. ^{177}Lu-DOTATATE–induced DNA damage activates PARP-1, which consumes NAD^+ for ADP-ribosylation of the damaged DNA. This cleavage of NAD^+ results in formation of Nam, which is used to regenerate NAD^+ via the NAD^+ salvage pathway. The first and rate-limiting enzyme in this pathway, NAMPT, is inhibited by GMX1778, thus preventing NAD^+ regeneration, resulting in NAD^+ depletion. ATP = adenosine triphosphate; NADH = nicotinamide adenine dinucleotide; Nam = nicotinamide; NMN = nicotinamide mononucleotide; NMNAT = NMN adenyltransferase.

shown to have a synergistic chemosensitizing effect, both in vitro and in vivo (*16*). Radiosensitizing effects of NAMPT inhibitors FK866 and GMX1777 have also been demonstrated experimentally in breast cancer and head and neck cancer models, respectively (*6,17*).

In this study, we have confirmed previous findings that semi-efficient doses of ^{177}Lu-DOTATATE (7.5 MBq) or GMX1778 (100 mg/kg/w) result in temporary halted tumor growth or moderate regression in nude mice xenografted with the small intestinal NET GOT1. When these treatment modalities were combined, the same doses of ^{177}Lu-DOTATATE and GMX1778 greatly enhanced the antitumor effect and resulted in complete or near-complete tumor regression in all animals. This is probably an example of mechanistic radiosensitization. We speculate that the ionizing radiation emitted by ^{177}Lu induces DNA damage, which activates PARP-1, which in turn consumes large amounts of NAD^+. The inhibition of the NAD^+ salvage pathway by GMX1778 decreases NAD^+ to lethally low levels. This is supported by our in vitro experiment showing that GMX1778, but not radiation alone, reduces NAD^+ levels already after a few hours. These findings are in agreement with evidence in the literature demonstrating that GMX1778 is an inhibitor of NAMPT and the NAD^+ salvage pathway. However, the exact mechanism of the enhanced antitumor effect of combined ^{177}Lu-DOTATATE and GMX1778 treatment remain to be elucidated.

GMX1778 (CHS828) or its prodrug GMX1777 (teglarinad chloride or EB1627) have been evaluated as monotherapy in a few phase I/II studies on solid tumors, without any antitumor effects (*18,19*). GMX1778 plasma levels of about 10 μM have been achieved in humans (*19*), which is of the same order of magnitude as plasma levels with antitumor effects in mouse tumor models (*20,21*). The doses used in those animal experiments are about the same as the doses used in this work.

CONCLUSION

Although NAMPT inhibition as monotherapy does not seem to be clinically successful, it may have a potential as a radiosensitizer in combination with ^{177}Lu-DOTATATE for the treatment of NETs.

DISCLOSURE

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