NAMPT inhibitor GMX1778 enhances the efficacy of $^{177}$Lu-DOTATATE treatment of neuroendocrine tumors

Short running foot line: NAMPT inhibition radiosensitizes NET

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Abstract

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 evaluates the potential radiosensitizing effects of inhibition of nicotinamide phosphoribosyltransferase (NAMPT) on $^{177}$Lu-DOTATATE treatment in a NET model. **Methods:** Nude mice xenografted with the human NET cell line GOT1 were treated with semi-efficient doses of $^{177}$Lu-DOTATATE (7.5 MBq, i.v.) and/or the NAMPT inhibitor GMX1778 (100 mg/kg/week, p.o.). **Results:** Median time to tumor progression (tumor volume larger than at day 0) was 3 days for controls, 7 days for single dose GMX1778, 28 days for single dose $^{177}$Lu-DOTATATE, 35 days for 3 weekly doses of GMX1778 and 98 days for combined treatment with $^{177}$Lu-DOTATATE and GMX1778 x1. After $^{177}$Lu-DOTATATE and 3 weekly doses of GMX1778 none of the tumors progressed within 120 days. **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
**Introduction**

NETs express high levels of somatostatin receptors (SSTR), 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 non-resectable NETs, resulting in symptomatic improvement, enhanced quality of life and prolonged survival. Due to dose-limiting hematotoxicity and nephrotoxicity the cure rate is low (1), and optimization of this treatment modality is needed. We have previously shown that xenografted intestinal NET cell line GOT1 can be effectively treated with $^{177}$Lu-DOTA$^0$-Tyr3-octreotate ($^{177}$Lu-DOTATATE). The antitumor effect was dose-dependent: administration of high doses (30 MBq or more) resulted in total tumor eradication, while lower doses (7.5 MBq; “semi-efficient”) resulted in 50% tumor reduction followed by progression after 2 weeks (Supplemental Fig. 1) (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 weeks, without any adverse effects. A lower dose (100mg/kg/w) resulted in halted tumor growth, but no tumor regression (Supplemental Fig. 2) (3). It has been shown that GMX1778 inhibits NAMPT, an enzyme involved in nicotinamide-adenine-dinucleotide ($\text{NAD}^+$) metabolism (4). Radiotherapy causes DNA damage, which in turn induces activation...
of 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 human small intestinal NET cell line GOT1 in nude mice has been described previously (7). In brief, small pieces (about 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-DOTA$^0$-Tyr3-octreotate ($^{177}$Lu-DOTATATE), with specific activity of 30 MBq/μg, was 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), GMX1778x1 (single dose of 100 mg/kg, n=7), GMX1778x3 (3 weekly doses of 100 mg/kg, n=5), $^{177}$Lu-DOTATATE (7.5 MBq) + GMX1778x1 (n=6) and $^{177}$Lu-DOTATATE (7.5 MBq) + GMX1778x3 (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 one hour after $^{177}$Lu-DOTATATE.

Animals were followed up to 17 weeks and were killed when tumor weight exceeded 10% of body weight, or body weight was reduced by more than 10%. Animal weights and tumor sizes (longest diameter and the two 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) + GMX1778x1 (single dose of 100 mg/kg, n=7), as described above. After 24 hours animals were killed and the kidneys were weighed and $^{177}$Lu activity measured using a gamma counter.

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Quantitative Real-Time PCR (qPCR) of SSTR subtype 2 (SSTR2) mRNA

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 % DMSO) for 1, 5 or 14 hours. 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 three times. RNA extraction was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s protocol. cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). mRNA expression levels were analyzed using predesigned TaqMan Gene Expression Assay (Thermo Fisher Scientific): GAPDH (Hs99999905_m1), HPRT1 (Hs02800695_m1) and SSTR2 (Hs00990356_m1). The PCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). For each RNA sample, the levels of SSTR2 mRNA expression are given relative to those of the two 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; Abcam, Cat no ab134152)
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 nM or 0 nM. After 1 hour incubation the culture plates were cooled on ice and irradiated at 1 Gy or 0 Gy, maintaining sterile conditions, and then further incubated at 37°C. Four replicates (each about 20 million cells) per condition and time point were harvested at 1, 5 and 14 hours. 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 correction, and values <0.05 were
considered significant. Differences in SSTR2 mRNA were analyzed by unpaired Student’s t-test. Differences in SSTR2 protein expression were analyzed by Mann-Whitney test.

RESULTS

Treatment with GMX1778 increases the reduction of GOT1 tumor volume after ¹⁷⁷Lu-DOTATATE

All 33 treated animals did well and only one animal in the control group had to be killed due to weight loss. The maximal tumor volume reduction after single semi-efficient doses of ¹⁷⁷Lu-DOTATATE (7.5 MBq) or GMX1778 (100 mg/kg) was seen after 2 weeks, 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 weeks. Combining a single dose of ¹⁷⁷Lu-DOTATATE and a single dose of GMX1778 resulted in a more pronounced antitumor effect with a maximal reduction of 73% at 3 weeks. Combining ¹⁷⁷Lu-DOTATATE and 3 weekly doses of GMX1778 caused a maximal tumor volume reduction of 97% at 4 weeks. One of five tumors was eradicated and had not recurred by the end of the experiment at 17 weeks (Fig. 1A).
Combined treatment with GMX1778 and $^{177}$Lu-DOTATATE induces a prolonged antitumor response in GOT1 bearing mice

A single dose of GMX1778 delayed tumor growth marginally and the median time to progression was 7 days, compared with 3 days 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 days. Combining $^{177}$Lu-DOTATATE and a single dose of GMX1778 further increased median time to progression to 98 days. Combining $^{177}$Lu-DOTATATE and 3 weekly doses of GMX1778 eradicated one of the tumors, while the remaining 4 tumors eventually regrew although none progressed, i.e. exceeded the initial volume, by the end of the experiment at 17 weeks (Fig. 1B). Statistical analysis revealed that time to tumor progression for all treatment groups, except single dose of GMX1778, were 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. Histological 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 the 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 non-significant difference according to Students t-test (data not shown).

**GMX1778 treatment does not affect the 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 hours (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 Fig. 3B and 4).

**GMX1778 treatment reduces the amount of NAD$^+$ in GOT1 cells**

Cultured GOT1 cells were incubated with GMX1778, 10 nM and/or irradiated with 1 Gy. These doses of GMX1778 and external radiation have a small cytotoxic effect visible after several days, but no effect on cell viability within the first 24 hours (data not shown).
GMX1778 had a clear NAD⁺-reducing effect already after 5 hours and a more pronounced effect after 14 hours (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 ¹⁷⁷Lu-DOTATATE and carboplatin/etoposide resulted in a considerable tumor reduction compared with ¹⁷⁷Lu-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. Poly(adenosine diphosphate-ribose) polymerase 1 (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

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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 ATP generation. DNA damage induces PARP-1 activation and NAD$^+$ consumption. Massive DNA damage can result in NAD$^+$ depletion, which eventually causes depletion of ATP 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).

Following the identification of its mechanism of action – inhibition of NAD$^+$ synthesis via NAMPT inhibition – GMX1777/1778 has been 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) results 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 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 $\mu$M 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 radiosensitizer in combination with $^{177}$Lu-DOTATATE for the treatment of NETs.

**DISCLOSURE**

This study was supported by The Swedish Research Council, The Swedish Cancer Society, the Sahlgrenska Academy (ALF agreement), BioCARE – a National Strategic Research Program at the University of Gothenburg, I.&A. Lundberg Research Foundation, the Assar Gabrielsson Research Foundation, the King Gustav V Jubilee Clinic Cancer Research Foundation, Gunnar Nilsson’s Cancer Foundation, Serena Ehrenströms Foundation, and Sahlgrenska University Hospital Research Funds. No potential conflict of interest relevant to this article was reported.

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combination with capecitabine in seven patients with gastroenteropancreatic


FIGURE 1.

Antitumor effect of a single dose of $^{177}$Lu-DOTATATE (7.5 MBq i.v.) and/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 is given as means ± SEM. (B) Kaplan-Meier graph with time to progression, i.e. tumor volume larger than at start of treatment (day 0). Censored data indicated as tick marks.
FIGURE 2.

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

Relative NAD$^+$ concentrations in cultured GOT1 cells. Cells were incubated up to 14 hours 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).
FIGURE 4.

Hypothetical 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.
Nam = nicotinamide, NMN = nicotinamide mononucleotide, NAMPT = Nicotineamide phosphoribosyltransferase, NMNAT = NMN adenyltransferase, PARP = poly(ADP-ribose) polymerase.
Supplemental Figure 1
Dose-dependent antitumoral effect of $^{177}$Lu-DOTATATE on xenografted GOT1. Single dose of $^{177}$Lu-DOTATATE (7.5 - 120 MBq i.v.) to mice xenografted with human small intestinal NET GOT1. Mean relative tumor volumes (individual tumor volumes normalized to start of treatment, i.e. day 0), error bars = SEM. Reprinted with permission of Ref. 2.
Supplemental Figure 2

Antitumoral effect of NAMPT inhibition on xenografted GOT1. Weekly oral doses of NAMPT inhibitor GMX1778 (100 or 250 mg/kg/w) to mice xenografted with human small intestinal NET GOT1. Mean relative tumor volumes (individual tumor volumes normalized to start of treatment, i.e. day 0), error bars = SEM. Reprinted with permission of Ref 3.
Supplemental Figure 3

Expression of SSTR2 in GOT1 cells and in GOT1 tumors after treatment with GMX1778. (A) SSTR2 mRNA levels in GOT1 cells were measured by qPCR after treatment with GMX1778 at 10-20 nM for 1-14 hours. There was no significant alteration in SSTR2 mRNA in treated cells compared to controls. (B) SSTR2 protein in GOT1 tumors was analyzed by immunohistochemistry in mice given GMX1778 (100mg/kg) and killed after 7 days. There was no significant difference in the percentage of labeled tumor cells in GOT1 tumors from treated animals compared to controls.
Supplemental Figure 4

Expression of SSTR2 in GOT1 tumors after treatment with GMX1778. Animals were given a single dose of GMX1778 (100mg/kg) and killed after 7 days. SSTR2 receptor protein was visualized by immunohistochemistry. A strong membranous labeling of tumor cells was observed in both controls and treated animals. There was no difference in the percentage of labeled cells after treatment. 40x objective.
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