# Preclinical development of the nicotinamide phosphoribosyl transferase inhibitor prodrug GMX1777

Pierre Beauparlant, Dominique Bédard, Cynthia Bernier, Helen Chan, Karine Gilbert, Daniel Goulet, Michel-Olivier Gratton, Manon Lavoie, Anne Roulston, Émilie Turcotte and Mark Watson

**GMX1778 was recently shown to function as a potent inhibitor of nicotinamide phosphoribosyl transferase. To translate the discovery of GMX1778 mechanism of action into optimal clinical use of its intravenously administered prodrug, GMX1777, the efficacy of GMX1777 was evaluated in xenograft models and the pharmacokinetic profile of GMX1778 and its effect on nicotinamide adenine dinucleotide cellular levels was measured by liquid chromatography/mass spectrometry. Consistent with the requirement for a prolonged exposure for cytotoxicity *in vitro*, a dose of 75 mg/kg of GMX1777 administered as two bolus intravenous injections in 1 day were not effective at reducing the growth of multiple myeloma (IM-9) tumors, whereas the same dose of GMX1777 administered over a 24 h intravenous infusion caused tumor regression in the IM-9 model, a small-cell lung cancer (SHP-77) model, and a colon carcinoma (HCT-116) model. A 72 h continuous intravenous infusion of GMX1777 was also effective in the IM-9 model, but was associated with a smaller therapeutic index. GMX1777 at a dose of 75 mg/kg administered over a 24 h intravenous infusion produced GMX1778 steady-state plasma levels of approximately 1 μg/ml and caused nicotinamide adenine dinucleotide levels to decrease significantly in tumors. Consistent with the GMX1778 mechanism of action, nicotinic acid protected mice treated with a lethal dose of GMX1777. These data support the design of an open-label, dose-escalation trial, in which patients with refractory solid tumors and lymphomas receive 24 h infusions of GMX1777 as a single agent in 3-week cycles. Furthermore, these results indicate that nicotinic acid is a potent antidote to treat GMX1777 overdose. *Anti-Cancer Drugs* 20:346–354 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Gemin X Pharmaceuticals Inc., Montréal, Quebec, Canada

Correspondence to Pierre Beauparlant, PhD, Gemin X Pharmaceuticals Inc., 3576 Avenue du Parc, Suite 4310, Montréal, QC H2X 2H7, Canada
Tel: +514 281 8989; fax: +514 281 8540; e-mail: Pbeauparlant@geminx.com

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## Introduction

GMX1777 (1-[2-(2-(2-(2-methoxyethoxy)-ethoxy)-ethoxy- carbonyloxymethyl]-4-[*N*′-cyano-*N*″-(6-(4-chlorophenoxy)-hexyl)-*N*-guanidino]-pyridinium chloride) (EB1627) is a prodrug of the cyanoguanidinopyridine GMX1778 (*N*-(6-(4-chlorophenoxy) hexyl)-*N*′-cyano-*N*″-(4-pyridyl) guanidine) (CHS828) [1]. The antitumor activity of GMX1778 was first reported several years ago [2], but it was recently discovered that GMX1778 functions as a potent inhibitor of the enzyme nicotinamide phosphoribosyl transferase (NAMPRT) (Watson *et al.*, unpublished observation and [3]). This essential enzyme for the synthesis of nicotinamide adenine dinucleotide ($NAD^+$) is upregulated in cancer cells. In mammals, $NAD^+$ is produced primarily through biochemical salvage pathways from two distinct precursors: nicotinamide and nicotinic acid [4]. The synthesis of $NAD^+$ from nicotinic acid is independent of NAMPRT and is not affected by GMX1778 (Watson *et al.*, unpublished observation). Compared with normal cells, cancer cells depend on a higher rate of $NAD^+$ synthesis to maintain the ADP-ribosylation activity required for their elevated demand for DNA repair, genome stability, and telomere maintenance. In addition, $NAD^+$ is required to support the high demand for adenosine triphosphate (ATP) synthesis that most cancer cells depend on.

*In vitro*, GMX1778 has cytotoxic activity in the low submicromolar range over a broad spectrum of solid tumor and hematological cell lines including small-cell lung cancer (SCLC), histiocytic lymphoma, and multiple myeloma [2,5,6]. In an in-vitro study of 156 primary cell cultures from hematological and solid tumors, GMX1778 displayed particularly high activity against tumor cells from chronic lymphocytic leukemia, acute leukemia, and high-grade lymphomas. In addition, GMX1778 was less cytotoxic to normal peripheral blood mononuclear cells than malignant hematological cells [7]. Oral administration of GMX1778 was shown to inhibit the growth of subcutaneous small-cell lung (NYH), breast (MCF-7L), neuroblastoma, and neuroendocrine (GOT1, GOT1, and BON) human tumors in mice [5,8,9].

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Orally administered GMX1778 was discontinued in clinical trials because of high variability in exposure levels, gastrointestinal toxicity, and dose-limiting myelosuppression [10,11]. Intravenous delivery of GMX1778 was the solution chosen to provide a prolonged and sustained exposure of tumor cells while reducing the variability in systemic exposure that was problematic for orally administered GMX1778. Unlike GMX1778, GMX1777 is freely soluble in water at up to 97 mg/ml, which permits its administration intravenously. The conversion of GMX1777 to GMX1778 is believed to occur through hydrolysis of the carbonate ester bond [1]. This reaction is catalyzed by esterases that are present in blood, tissues, and organs [12,13]. GMX1777 administered intraperitoneally was shown to inhibit the growth of subcutaneous SCLC (NYH) tumors in nude mice [1].

The purpose of this study was to translate the discovery of GMX1778 mechanism of action into optimal clinical use of its intravenously administered prodrug, GMX1777. Using in-vitro cell cultures, GMX1778 exposure requirements to affect $NAD^+$ cellular levels and to cause cytotoxicity were studied. The applicability of these findings was verified in xenograft models, in which the therapeutic indices associated with bolus administrations and continuous infusions of GMX1777 were compared. An analytical assay to measure $NAD^+$ levels in tumors and other tissues was used to measure the effect of GMX1778 on its molecular target *in vivo*. Finally, as suggested by GMX1778 mechanism of action, the protective effect of nicotinic acid from GMX1777 overdose was measured in mice.

## Materials and methods

### Chemicals and cell lines

GMX1777 and GMX1778 were provided by ProPharma Ltd (Glasgow, United Kingdom). Nicotinic acid was supplied by Sigma-Aldrich; (St Louis, Missouri, USA). DMS-273 were obtained from the European Collection of Cell Cultures, all other cell lines were obtained from the American Type Culture Collection.

### In-vitro cytotoxicity assay

Viability was assessed by measuring cellular ATP levels using the bioluminescent ViaLight-HS (Rockland, Maine, USA). Cell lysis was measured by flow cytometry, using propidium iodide staining. Cells were diluted in buffer containing 1.4 ng/ml of propidium iodide and analyzed by flow cytometry within 1 h.

### Nicotinic acid rescue of GMX1778 cytotoxicity

IM-9 cells were treated with 50 nmol/l GMX1778 for 24 h. At this point, one sample had GMX1778 replaced with media, one had 10 μmol/l nicotinic acid added, and one was maintained with GMX1778. Samples were removed at 24, 48, and 72 h and analyzed for cell viability, cell lysis, and $NAD^+$ as described.

### Animal models

The animal studies presented strictly complied with the requirements of the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. Mice were fed *ad libitum* with autoclavable rodent chow number 5075 (Charles River Laboratories, St Constant, Quebec, Canada) and water. On the basis of chow composition (98 mg nicotinic acid/kg) the estimated daily nicotinic acid intake is 20 mg/kg. Female Balb/c nude and CB17 severe combined immunodeficiency (SCID)/SCID mice 6–8 weeks of age were purchased from Charles River Laboratories. CB17 SCID/SCID female mice were injected subcutaneously with 100 μl of a suspension of HCT-116 ($8 \times 10^7$ cells/ml), DMS-273 ($8 \times 10^7$ cells/ml), IM-9 ($10 \times 10^7$ cells/ml), HT1080 ($1 \times 10^7$ cells/ml), HT29 ($1.5 \times 10^7$ cells/ml), or SW480 ($2 \times 10^7$ cells/ml) cells in phosphate buffered saline (PBS). Balb/c nude female mice were injected subcutaneously with 100 μl of a cell suspension of SW620 ($5/10^7$ cells/ml in PBS) or SHP-77 cells [$10 \times 10^7$ cells/ml 50% Matrigel (BD Biosciences, Mississauga, Canada) in PBS]. Each treatment group included eight to 10 mice, with the exception of groups bearing SHP-77 tumors, which were limited to five individuals. Body weight and tumor size were measured three times per week. Relative tumor size and volume were calculated as follows: length (mm) × [width (mm)]$^2$/2. Percent of control growth (%C) was calculated as described by Alley *et al.* [14]

### Drug treatment *in vivo*

Animals were treated with vehicle (either 0.9% NaCl or 20 mmol/l citrate buffer at pH 4.8) or with GMX1777 (20 mmol/l citrate buffer at pH 4.8). Bolus intravenous injections were administered through tail vein. Unless indicated otherwise, intravenous infusions were delivered from an external syringe pump (pump model 220 from Lomir Biomedical Inc., Quebec, Canada). As indicated for some experiments, 24 h infusions were administered using mini osmotic pumps (ALZET pump model 2001D from Durect Corporation, Cupertino, California, USA) implanted subcutaneously. In both cases, mice were connected to the pump through a catheter inserted in the right jugular vein. Catheters were implanted surgically under general anesthesia using either isoflurane gas (Baxter Corporation, Mississauga, Canada) or an intraperitoneal injection of 2,2,2-tribromoethanol (Sigma Aldrich, Oakville, Canada) at 375 mg/kg. For infusions using external syringe pumps, at the end of infusion, the catheter was cauterized near the neck of the animal and inserted under the skin of the neck and sutured.

### Pharmacokinetic study

CB17 SCID mice were treated with a 24 h infusion of GMX1777 at 25, 75, or 400 mg/kg. At different time

points from the beginning of the infusion (5 min, 4, 22, 27, 30, and 44 h), mice were anesthetised with 2% isoflurane and blood was collected by cardiac puncture as a terminal procedure. For each time point, blood was collected from five animals. Plasma (200 µl) was extracted with an equal volume of acetonitrile. High-performance liquid chromatography/mass spectrometry (MS) analysis was performed on the organic phase using a Waters Alliance System 2795 (Milford, Massachusetts, USA) connected to a Waters EMD 1000 (Milford). The compounds were separated over an YMC ODS-AQ 3 µm, 2.0 × 50 mm analytical column (Waters). The mobile phases consisted of 5 mmol/l ammonium formate in water with 0.1% formic acid (A) and of 5 mmol/l ammonium formate in methanol with 0.1% formic acid (B). The gradient maintained for 1 min at 52% (B), increased to 80% (B) over 4 min then to 100% (B) in 1 min and held for 3 min. Finally, the mobile phase was returned to 52% (B) in 1 min and the column reequilibrated for 3 min. The flow rate was 0.250 ml/min. The injection volume was 5 µl. The settings of the electrospray ionization source were [nomenclature as used in Empower (Waters, Milford, Massachusetts, USA) software]: capillary voltage, 4.00 kV; cone voltage, 30 V; desolvation gas, nitrogen, temperature, 300°C; and flow, 600 l/h; source temperature at 150°C. The monitored ions (positive mode) were GMX1777 (*m/z* 636.6), GMX1778 (*m/z* 372.6), and the internal standard, GMX1921 (*m/z* 289.5). Noncompartmental analysis was used to calculate the derived pharmacokinetic parameters of GMX1778 (WinNonlin Enterprise, version 5.1, Pharsight Corporation, Mountain View, California, USA).

### Nicotinamide adenine dinucleotide measurement in cells and tissues

Cells were harvested by trypsinization and centrifugation. Subcutaneous tumors were excised from the right flank of the mice and all necrotic and nontumor tissues were removed. Muscle tissue was harvested from the hind legs of each animal and excess fat was trimmed. Samples were weighed, frozen in liquid nitrogen or in dry ice and stored at −80°C. Tumor and muscle tissues were homogenized while frozen with a tissue pulverizer. Fifty milligrams of homogenized tissue was extracted. Each sample was prepared by adding a volume of distilled water equal to four times its weight. The extraction was performed by adding a volume of 1 N perchloric acid containing 1 nmol/l of 2-chloroadenosine (as internal standard) equal to five times the weight of the tissues. Samples were vortexed for 10 min, left on ice for 10 min then centrifuged at 13 000*g* for 10 min at 4°C. Two hundred and fifty microliters of the supernatant was transferred to a new tube and 100 µl of KOH, 1 mol/l containing 0.075 mmol/l of EDTA was added to reach pH 6. The solution was mixed, left on ice for 10 min then centrifuged at 13 000*g* for 10 min at 4°C. The supernatant was analyzed using an ACQUITY TQD [ultra-performance liquid chromatography (UPLC)/MS/MS] (Waters). The compounds were separated over an ACQUITY UPLC HSS T3 1.8 µm, 2.1 × 50 $mm^2$ (Waters). The mobile phases consisted of methanol with 0.1% formic acid (A) and water with 0.1% formic acid (B). The gradient began at 2% (A), increased to 40% (A) over 1 min, then increased to 65% (A) over 0.2 min and returned to 2% after 0.1 min to reequilibrate the column for 0.7 min. The flow rate was 0.6 ml/min and the injection volume was 2 µl. The settings of the electrospray ionization source were (nomenclature as used in Empower software): capillary voltage, 1.90 kV; extractor, 3 V; radio frequency, 0.10 V; source temperature, 150°C; desolvation gas, nitrogen, temperature, 450°C; and flow 800 l/h; cone gas flow, 50 l/h; collision gas, argon, flow 0.10 ml/min. The monitored ions (positive mode) were 664.18 (precursor ion) and 136.07 (product ion) for $NAD^+$, 302.00 and 134.00 for 2-chloroadenosine. Solutions of $NAD^+$ and 2-chloroadenosine (Sigma-Aldrich) in water were used as references. The UPLC/MS/MS method used for analysis of $NAD^+$ in plasma and tissues was validated by verification of specificity, linearity, precision, accuracy, recovery, dilution integrity, and absence of matrix effects. Briefly, the intraday precision and accuracy were satisfactory as indicated by a coefficient of variation of $\leq$ 7.6% and a bias of $\leq$ 14.5%. The interday precision and accuracy were characterized by coefficient of variation and bias values of $\leq$ 9.1% and $\leq$ 9.1%, respectively. The extraction efficiency of $NAD^+$ and internal standard was consistent and had recovery level of $\geq$ 86.3%. The standard solutions of $NAD^+$ and experimental samples were verified to be stable during in-process treatment and storage time before analysis.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows. (GraphPad Software, San Diego, California, USA). Significant differences in tumor growth in mice treated with GMX1777 from mice treated with vehicle only were determined by two-way analysis of variance. Significant differences in $NAD^+$ levels in tumor tissue and muscle tissue obtained from vehicle and GMX1777-treated animals were determined with a modified unpaired Student's *t*-test (two-tailed).

## Results

### Sustained nicotinamide adenine dinucleotide depletion requirement for cancer cell death

It was previously reported that at least 36 h of continuous exposure to GMX1778 are required to reach the 72 h half maximal inhibitory concentration in three different cell lines (U937, MDA-MB231, and RPMI 8226) as well as in peripheral blood mononuclear cells isolated from a patient with chronic lymphocytic leukemia [15]. The required exposure time to commit cells to death was confirmed in IM-9 multiple myeloma cells. Two assays were used to measure the effect of GMX1778: (i) cell viability as measured by intracellular ATP quantification and (ii) cell lysis as measured by propidium iodide

staining. As expected, a large increase in cytotoxicity was achieved by lengthening the exposure time from 24 to 48 h (Table 1) (Lonza, Rockland, Maine, USA). When IM-9 cells were exposed continuously to 50 nmol/l GMX1778 (about 2.5 times the 72 h half maximal inhibitory concentration), cellular $NAD^+$ levels dropped significantly within the first 24 h. However, if GMX1778 exposure was limited to 24 h, the $NAD^+$ levels returned to normal after 48 h and cell viability was not affected (Table 2). In IM-9 cells treated continuously with 50 nmol/l GMX1778 for 72 h, 10 µmol/l nicotinic acid added after the first 24 h increased cellular $NAD^+$ levels and protected cells from death. These results indicate that the reduction in $NAD^+$ levels induced by GMX1778 treatment leads to cell death only if this reduction is maintained for a prolonged period of time.

### Requirement for prolonged administration *in vivo*

GMX1777, the prodrug of GMX1778, can be administered intravenously. The duration of GMX1777 intravenous administration is expected to correlate with the duration of tumor exposure to GMX1778. To verify this assumption, three administration schedules were tested in mice bearing subcutaneous IM-9 multiple myeloma tumors: (i) two daily bolus injections, (ii) a 24 h infusion, and (iii) a 72 h infusion. A 24 h intravenous infusion of GMX1777 at a dose of 75 mg/kg induced a nearly complete regression of the tumors and a significant tumor growth delay (Fig. 1a). At 37.5 mg/kg, a 24 h intravenous infusion of GMX1777 reduced IM-9 tumor growth moderately, whereas at 18.75 mg/kg, a 24 h intravenous infusion of GMX1777 had no effect. Thus, we conclude that 75 mg/kg is the minimum effective dose of GMX1777 administered as a 24 h intravenous infusion in the IM-9 model. A dose of 75 mg/kg GMX1777 administered intravenously over 24 h also produced tumor regression in a SCLC (SHP-77) model and a colon carcinoma (HCT-116) model (Fig. 1c and d). By comparison, the same dose of GMX1777 administered in two bolus intravenous administrations (37.5 mg/kg each) in 1 day was ineffective in the IM-9 model (Fig. 1a). This lack of efficacy associated with the short bolus administrations can be explained by the requirement for prolonged and sustained exposure to the drug. In the IM-9 multiple myeloma tumor model, GMX1777 administered as a 72 h infusion was effective at inducing a nearly complete regression of the tumors and a significant tumor growth delay at 150 mg/kg GMX1777, but not at 75 mg/kg (Fig. 1b). To ensure that the difference in efficacy resulted from the duration of GMX1777 treatment, and not from the stress caused by the intravenous infusion, after GMX1777 infusion, infusion was continued with 0.9% NaCl so that all animals remain infused for a total duration of 120 h.

**Table 1 Effect of GMX1778 exposure time on IM-9 cell viability and lysis**

| | $IC_{50}$ (nmol/l) | |
|---|---|---|
| Drug exposure time (h) | Cell viability | Cell lysis |
| 8 | >300 | >300 |
| 24 | >300 | >300 |
| 48 | 20 | 29 |
| 72 | 25 | 21 |

Cells were seeded and exposed to a concentration range of GMX1778 for 8, 24, 48, or 72 h after which the cells were rinsed once and then provided with fresh media until viability was determined at 72 h after initial drug treatment. Cell viability was estimated by measuring ATP levels in triplicate using the Vialight assay and cell lysis was measured by fluorescence activated cell sorter analysis of 10 000 propidium iodide stained cells at 72 h after initial drug treatment. $IC_{50}$ values were determined by non-linear regression analysis of the concentration-effect curves.

### Therapeutic indices

The toxicity of GMX1777 24 and 72 h infusions was measured in CB17 SCID/SCID female mice. Each treatment group consisted of five animals. GMX1777 administered as a 24 h infusion was well tolerated at 500 mg/kg with no weight loss observed. However, one of the five animals died in each of the groups receiving 550 and 600 mg/kg. This experiment was repeated and no animals died in the group receiving 500 or 550 mg/kg, but three of five animals died in the group receiving 600 mg/kg. We concluded that 500 mg/kg GMX1777 is the maximum tolerated dose in a 24 h intravenous infusion in mice. The

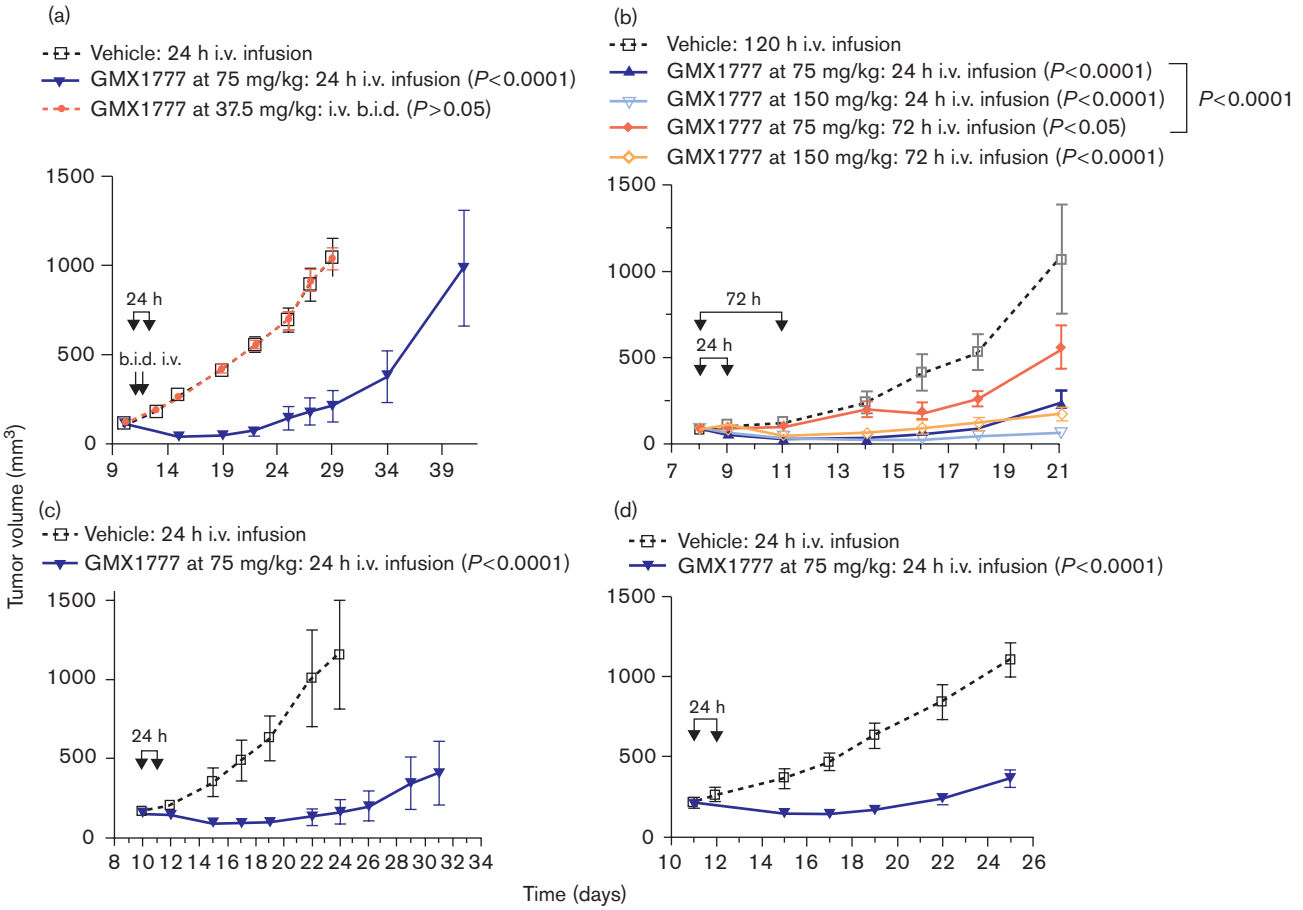
**Table 2 Effect of GMX1778 exposure time on $NAD^+$ levels and rescue by nicotinic acid**

| Treatment | | | Time points | | |
|---|---|---|---|---|---|
| Day 1 | Days 2 and 3 | Parameter (%) | 24 h | 48 h | 72 h |
| 50 nmol/l GMX1778 | 50 nmol/l GMX1778 | $NAD^+$ | 5.5 ± 0.5 | 1.1 ± 0.1 | 1.0 ± 0.3 |
| | | ATP levels | 70.4 ± 5.9 | 7.2 ± 3.0 | 1.9 ± 0.7 |
| | | Cell lysis | 8.0 ± 0.4 | 17.3 ± 1.0 | 78.1 ± 6.7 |
| 50 nmol/l GMX1778 | 50 nmol/l GMX1778 + 10 µmol/l nicotinic acid | $NAD^+$ | 5.5 ± 0.5 | 10.0 ± 2.8 | 16.8 ± 5.7 |
| | | ATP levels | 70.4 ± 5.9 | 66.6 ± 15.4 | 79.6 ± 4.4 |
| | | Cell lysis | 8.0 ± 0.4 | 11.7 ± 0.1 | 17.5 ± 0.7 |
| 50 nmol/l GMX1778 | | $NAD^+$ | 5.5 ± 0.5 | 90.2 ± 15.4 | 111.8 ± 15.4 |
| | | ATP levels | 70.4 ± 5.9 | 103.2 ± 27.4 | 105.2 ± 16.9 |
| | | Cell lysis | 8.0 ± 0.4 | 9.8 ± 2.6 | 13.7 ± 0.4 |

Nicotinamide adenine dinucleotide ($NAD^+$) levels, ATP levels, and cell lysis were measured as described in Materials and methods. IM-9 cells were treated 50 nmol/l GMX1778 or dimethyl sulfoxide (DMSO, control). $NAD^+$ values were normalized to protein amount. Samples were taken at times 0, 24, 48, and 72 h and the data are presented as percentage of the time-matched DMSO (control) with standard deviation.

$IC_{50}$, half maximal inhibitory concentration.

**Fig. 1**




Impact of intravenous (i.v.) administration duration on GMX1777 antitumor activity in mouse tumor models. Graphs represent tumor volume over time from the day of implantation. Error bars represent standard error of the mean. (a) CB17 severe combined immunodeficiency (SCID)/SCID female mice bearing IM-9 multiple myeloma tumors were treated with two bolus i.v. injections of GMX1777 at 37.5 mg/kg administered on the same day (total dose of 75 mg/kg) or with a 24 h i.v. infusion of vehicle (20 mmol/l citrate buffer pH 4.8) or GMX1777 at 75 mg/kg. Infusions were delivered using implanted osmotic pumps. (b) CB17 SCID/SCID female mice bearing subcutaneous IM-9 multiple myeloma tumors were treated with vehicle (0.9% NaCl infused i.v. over 120 h) or GMX1777 at 75 mg/kg or 150 mg/kg. GMX1777 treatments were administered either as a 24 or a 72 h continuous i.v. infusion followed by a 96 or a 48 h infusion of vehicle, respectively. (c) Balb/c nude female mice bearing human small-cell lung cancer SHP-77 tumors were treated with vehicle or 75 mg/kg GMX1777 administered as a 24 h continuous i.v. infusion. Infusions were delivered using implanted osmotic pumps. (d) CB17 SCID/SCID female mice bearing subcutaneous human colon carcinoma HCT-116 tumors were treated with vehicle or 75 mg/kg GMX1777 administered as a 24 h continuous i.v. infusion. b.i.d., twice daily.

toxicity of GMX1777 administered as a 72 h infusion was comparable: two out of five mice died after treatment at either 750 or 1000 mg/kg. One of five mice also died after treatment with a 72 h intravenous infusion of GMX1777 at 500 mg/kg. However, this death was not attributed to GMX1777 treatment but to the anesthesia with isoflurane gas performed to connect mice to the infusion apparatus: this animal showed distress from the beginning of the treatment. Thus, the maximum tolerated dose of GMX1777 seems to be the same (i.e. 500 mg/kg) whether it is administered as a 24 or a 72 h infusion.

Given that a 24 h intravenous infusion of GMX1777 at 75 mg/kg was effective in the IM-9 multiple myeloma, SHP-77 SCLC, and HCT-116 colon carcinoma models, the therapeutic index of GMX1777 administered as a 24 h intravenous infusion is approximately 6. A 72 h intravenous infusion of GMX1777 is also effective in the IM-9 human multiple myeloma xenograft model, but the associated therapeutic index is smaller than when GMX1777 is administered as a 24 h infusion.

### GMX1778 pharmacokinetics

The plasma levels of GMX1778 in mice after 22 h of continuous infusion of GMX1777 at 75 mg/kg were found to be approximately 1 μg/ml as measured by high-performance liquid chromatography /MS. Pharmacokinetic studies of GMX1777 and GMX1778 during and after a 24 h intravenous infusion of GMX1777 showed that GMX1777 was quickly converted to GMX1778 in plasma

**Table 3 Derived pharmacokinetic parameters for GMX1778 plasma levels during and after a 24 h intravenous infusion of GMX1777 at 25, 75, or 400 mg/kg**

| Dose (mg/kg) | $C_{max}$ (μg/ml) | $AUC_{24}$ (h × μg/ml) | $AUC_{last}$ (h × μg/ml) | $t_{1/2}$ (h) |
|---|---|---|---|---|
| 25 | 0.47 | 8.46 | 8.88 | 2.55 |
| 75 | 0.95 | 16.86 | 17.63 | 7.13 |
| 400 | 13.01 | 232.36 | 239.43 | 11.71 |

The area under the curve (AUC) from time zero to infinity (AUC) was determined as $AUC_{0-tz} + C_z/\lambda_z$, where $AUC_{0-tz}$ is the AUC from time zero to the last detectable concentration ($C_z$). The maximum observed plasma concentration ($C_{max}$) was obtained by comparing the concentrations from time zero to the last sampling point.

with a half-life ($t_{1/2}$) of GMX1777 less than 0.7 h. GMX1778 exhibited nonlinear pharmacokinetics across the dose range 25–400 mg/kg, presumably because of a rate-limiting elimination at the high dose, 400 mg/kg (Table 3). In agreement with the requirement for prolonged administration *in vivo* the half-life of GMX1778 was less than 8 h at the effective dose of 75 mg/kg.

### Antidotal use of nicotinic acid *in vivo*

If GMX1778 toxicity *in vivo* is a consequence of NAMPRT inhibition, nicotinic acid may potentially be used to treat patients who accidentally receive an excessively toxic dose of GMX1777. In humans, nicotinic acid is used as a hair and skin-conditioning agent and is also given orally to treat hypercholesteremia or related conditions. Two studies have been reported in which nicotinic acid was administered by intravenous infusion. Both studies used the same infusion rate: 90 mg nicotinic acid/$m^2$/h [16,17]. The longest infusion administered was in the study of Landau *et al.* [17], in which normal individuals received a 4 h infusion for a total dose of 360 mg/$m^2$. Nicotinic acid was well tolerated and the effect consisted of a 75% decrease in plasma nonesterified fatty acid concentration and a statistically significant decrease in plasma glucose concentration. To test the antidotal potential of nicotinic acid at a dose shown to be safe in humans, mice were first treated with a 24 h intravenous infusion of GMX1777 at 650 mg/kg followed by an infusion of saline (0.9% NaCl) for 24 h. As expected, this GMX1777 dose was lethal in three out of five treated animals (Table 4). One mouse was found dead 2 days after the end of treatment (day 3) and two more mice were dead the subsequent day (day 4). Two more groups of mice were treated with the same dose of GMX1777, but in addition, they received either a 4 or a 24 h infusion of nicotinic acid at 90 mg/$m^2$/h just after the end of the GMX1777 treatment. In these groups, no death occurred and no drug-related symptoms were noted (Table 4). These results suggest that GMX1777 toxicity in mice is mainly target related, as a consequence of NAMPRT inhibition, and that nicotinic acid is a potent antidote to treat GMX1777 overdose.

GMX1777 antitumor activity in mice can also be attributed to inhibition of NAMPRT, as a 4 h infusion

**Table 4 Antidotal effect of nicotinic acid in mice treated with a lethal dose of GMX1777**

| | GMX1777 treatment | | Nicotinic acid treatment | | |
|---|---|---|---|---|---|
| Group | Dose (mg/kg) | Duration (h) | Dose (mg/$m^2$/h) | Duration (h) | Mortality |
| 1 | 650 | 24 | 0 | 0 | 3/5 |
| 2 | 650 | 24 | 90 | 4 | 0/4 |
| 3 | 650 | 24 | 90 | 24 | 0/5 |

Mice in each group received a 24 h infusion of GMX1777 at 650 mg/kg. GMX1777 treatment was followed by a 24 h infusion of saline (group 1); a 4 h infusion of nicotinic acid at a dose of 90 mg/$m^2$/h, followed by a 20 h infusion of saline (group 2); or a 24 h infusion of nicotinic acid at a dose of 90 mg/$m^2$/h (group 3). Mice were monitored for 18 additional days and mortalities were recorded.

of nicotinic acid at 90 mg/$m^2$/h completely abrogated the antitumor effect of a 24 h infusion of GMX1777 at 150 mg/kg on IM-9 multiple myeloma tumors (Fig. 2a). The neutralizing effect of nicotinic acid on antitumor effects was observed if nicotinic acid was administered immediately after the end of the 24 h GMX1777 infusion but not if nicotinic acid was administered 24 h after the end of infusion (Fig. 2b). These results suggest the possibility that nicotinic acid could be used to treat GMX1777 side effects that may persist more than 24 h after the end of GMX1777 24 h infusions without affecting GMX1777 antitumor activity.

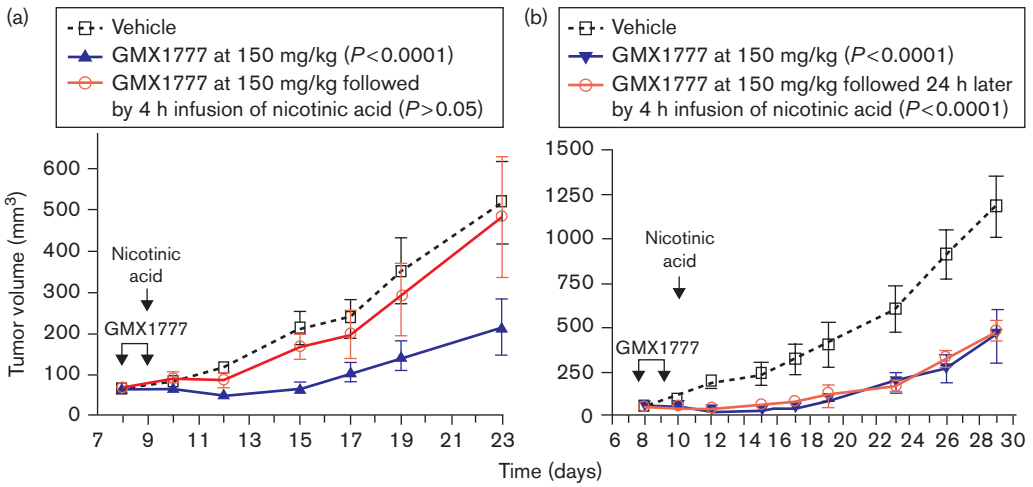
### Nicotinamide phosphoribosyl transferase inhibition *in vivo*

To confirm that GMX1777 antitumor activity *in vivo* results from NAMPRT inhibition and to provide a pharmacodynamic assay to identify effective doses of GMX1777 in future clinical trials, a robust analytical assay was developed to measure $NAD^+$ levels in tumors from treated animals. For this study, the SCLC tumor DMS-273 was selected because it is less necrotic than many tumors when grown subcutaneously in mice. Mice bearing human DMS-273 cells were treated with 24 h continuous intravenous infusion of either 0.9% NaCl or GMX1777 at 75 and 150 mg/kg. Tumor fragments and muscle tissue were collected at the end of treatment and relative $NAD^+$ levels were measured by the UPLC/MS/MS. Consistent with GMX1778 mechanism of action, $NAD^+$ levels decreased significantly in tumors at both doses of GMX1777, whereas $NAD^+$ levels in muscle tissue showed only a modest but significant decrease at the 150 mg/kg dose (Fig. 3). These data suggest that NAMPRT inhibition and dramatic $NAD^+$ depletion occurs in the DMS-273 tumor cells during a 24 h infusion with GMX1777 in a mouse xenograft model, whereas the normal mouse muscle tissue is minimally affected.

### Alternative administration schedule

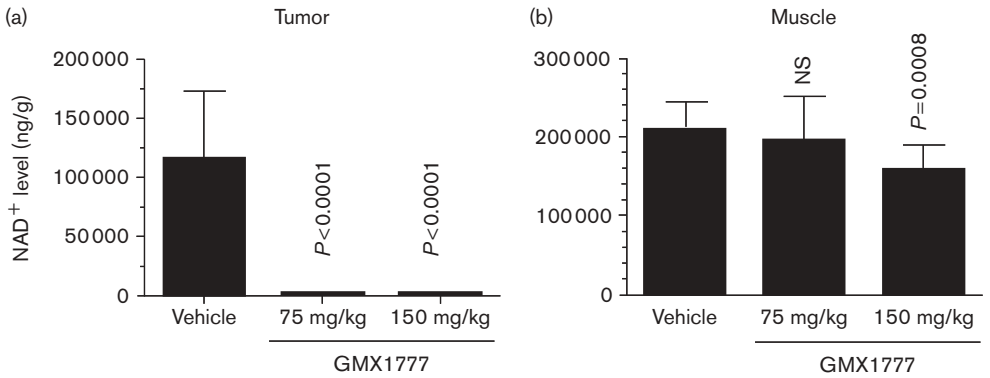
Additional preclinical research in mouse tumor models may further help to optimize the use of GMX1777 for the treatment of cancer or allow the identification of other clinical applications. As administering a 24 h intravenous infusion in mice requires special skills, we

**Fig. 2**




Effect of nicotinic acid on GMX1777 antitumor activity in the human multiple myeloma IM-9 xenograft model. CB17 severe combined immunodeficiency (SCID)/SCID female mice bearing IM-9 cell subcutaneous tumors (implanted on day 0) were treated with vehicle (0.9% NaCl infused intravenously over 104 h) or 150 mg/kg GMX1777 administered as a 24 h continuous intravenous infusion. (a) GMX1777 treatment was followed by an 80 h infusion of vehicle or a 4 h infusion of nicotinic acid at a dose of 90 mg/m$^2$/h, followed by a 76 h infusion of vehicle. (b) GMX1777 treatment was followed by an 80 h infusion of vehicle or a 24 h infusion of vehicle, followed by a 4 h infusion of nicotinic acid at a dose of 90 mg/m$^2$/h and then followed by a 52 h infusion of vehicle. Error bars represent standard error of the mean.

**Fig. 3**




Effect of GMX1777 delivered in a 24 h infusion on the nicotinamide adenine dinucleotide ($NAD^+$) content of human tumors and mouse muscle tissue in mice. CB17 severe combined immunodeficiency (SCID)/SCID female mice bearing DMS-273 tumors were treated with a 24 h infusion of vehicle or GMX1777 at 75 or 150 mg/kg. Tumor fragments and muscle tissue were collected at the end of infusion. Tumor (a) and (b) muscle tissue were weighed and $NAD^+$ levels were analyzed by ultra-performance liquid chromatography/mass spectrometry (MS)/MS. Error bars represent standard deviation.

undertook to identify an alternative administration. Repeated intramuscular administrations were selected, as they were likely to provide the required prolonged exposure to GMX1778. Five daily intramuscular administrations of GMX1777 were well tolerated at a dose up to 150 mg/kg. At 100 mg/kg/day using this administration schedule, GMX1777 induced a complete or nearly complete regression of subcutaneous human multiple myeloma (IM-9), colon carcinoma (HCT-116, HT29, SW480, and SW620), fibrosarcoma (HT1080), and SCLC (SHP-77 and DMS-273) tumors in SCID mice (Table 5). In addition to confirming the wide range of antitumor activity of GMX1777, these observations indicate that repeated intramuscular administrations are an acceptable alternative to 24 h infusion, which should facilitate exploratory preclinical studies on GMX1777.

## Discussion

The purpose of this study was to translate the discovery of GMX1778 mechanism of action into optimal clinical use of its intravenously administered prodrug, GMX1777. The 24 h infusion was the most effective administration

**Table 5 Activity of GMX1777 in multiple mouse xenograft tumor models**

| | | | Weight change (%) | | | Percent of control growth (%C) | | |
|---|---|---|---|---|---|---|---|---|
| Model | Drug | Death | Day 7 | Day 14 | Day 21 | Day 7 | Day 14 | Day 21 |
| IM-9 (multiple myeloma) | Cisplatin | 0/9 | −2 | −9 | −7 | 46 | 45 | 56 |
| | GMX1777 | 0/9 | −11 | −13 | −3 | −112 | −112 | −112 |
| HT1080 (fibrosarcoma) | Cisplatin | 0/10 | −3 | −10 | −14 | 39 | 48 | 76 |
| | GMX1777 | 0/10 | −7 | −11 | −6 | −61 | −4 | 18 |
| HT29 (colon carcinoma) | 5-Fluorouracil | 0/10 | −8 | −1 | 0 | 71 | 55 | 58 |
| | GMX1777 | 0/10 | −4 | −6 | 2 | −22 | 12 | 25 |
| SW480 (colon carcinoma) | 5-Fluorouracil | 0/10 | −14 | −10 | 2 | 38 | 39 | 48 |
| | GMX1777 | 0/10 | −14 | −13 | −4 | −101 | −104 | −104 |
| SW620 (colon carcinoma) | 5-Fluorouracil | 0/10 | −2 | 3 | 8 | 73 | 67 | 68 |
| | GMX1777 | 0/10 | −8 | −4 | 0 | −86 | −102 | −102 |
| HCT-116 (colon carcinoma) | 5-Fluorouracil | 0/10 | −22 | −8 | −7 | 33 | 55 | NA |
| | GMX1777 | 0/10 | −9 | −6 | 2 | −68 | −97 | −90 |
| SHP-77 (SCLC) | Paclitaxel | 0/10 | 1 | 2 | 5 | 59 | 116 | NA |
| | GMX1777 | 0/10 | −9 | −8 | −4 | −102 | −102 | −102 |
| DMS-273 (SCLC) | Paclitaxel | 0/10 | −4 | 2 | 2 | −13 | −93 | −94 |
| | GMX1777 | 0/10 | −9 | −7 | −6 | −81 | −89 | −43 |

Once tumors grew to an average volume of about 100 $mm^3$ or more, animals received five consecutive daily intramuscular (i.m.) injections GMX1777 at 100 mg/kg. Corresponding control animals received five daily i.m. administrations of vehicle (5% dextrose). Positive control animals received treatment with either cisplatin (3.5 mg/kg administered intraperitoneally every 2–3 days for a total of 5 treatment days), 5-fluorouracil [23 mg/kg administered intravenously (i.v.) daily for 5 days, or paclitaxel 8.5 mg/kg administered i.v. twice a day every 2 days for a total of 6 treatment days]. Weight change (%) and percent of control growth (%C) on day 7, 14, and 21 after the beginning of treatment and the number of death (number of dead animals/total number of animals) observed during the same period are presented. NA, not available because the animals in the control group had tumors exceeding 1500 $mm^3$ on average and had to be killed as per protocol.

schedule identified in mouse xenograft models, in agreement with maximal cytotoxic treatment durations of GMX1778 *in vitro*. These preclinical data support the design of an ongoing open-label, dose-escalation trial, in which patients with refractory solid tumors and lymphomas receive 24 h infusions of GMX1777 as a single agent in 3-week cycles.

Using a robust analytical assay, which may be a suitable pharmacodynamic assay to identify effective doses of GMX1777 in future clinical trials, it was possible to demonstrate that, in mice, $NAD^+$ levels are significantly reduced in subcutaneous DMS-273 xenograft tumors, but not in muscles. A possible explanation for this specificity is that cancer cells consume a higher proportion of their $NAD^+$ to maintain the ADP-ribosylation activity required for their elevated demand for DNA repair, genome stability, and telomere maintenance. As shown *in vitro*, depleting cellular $NAD^+$ does not immediately result in cell death. It is still uncertain whether the consequence of prolonged $NAD^+$ depletion is necrosis, apoptosis, or a combination of both. For instance, it has been shown that another NAMPRT inhibitor, FK866, could induce autophagy in cell culture [18,19]. Notably, in subcutaneous DMS-273 SCLC xenograft tumors an increased number of cleaved caspase-3 immunostained cells were found after three intramuscular injections of GMX1777 at 100 mg/kg (unpublished work) suggesting that GMX1778 can trigger apoptosis *in vivo*.

The elucidation of GMX1778 mechanism of action also permitted the identification of a potent antidote. By inhibiting NAMPRT, GMX1778 inhibits the synthesis of $NAD^+$ from nicotinamide, but has no effect on $NAD^+$ synthesis from nicotinic acid. Hence, administration of nicotinic acid protected mice from a lethal dose of GMX1777 and could be useful in a clinical setting in case of accidental overdose.

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