Synergy between the NAMPT Inhibitor GMX1777(8) and Pemetrexed in Non–Small Cell Lung Cancer Cells Is Mediated by PARP Activation and Enhanced NAD Consumption

Manuel Chan1, Michel Gravel1, Alexandre Bramoullé1, Gaëlle Bridon2,3, Daina Avizonis2,3, Gordon C. Shore1, and Anne Roulston1

Abstract

GMX1778 and its prodrug GMX1777 represent a new class of cancer drugs that targets nicotinamide phosphoribosyltransferase (NAMPT) as a new strategy to interfere with biosynthesis of the key enzymatic cofactor NAD, which is critical for a number of cell functions, including DNA repair. Using a genome-wide synthetic lethal siRNA screen, we identified the folate pathway–related genes, deoxyuridine triphosphatase and dihydrofolate reductase, the silencing of which sensitized non–small cell lung carcinoma (NSCLC) cells to the cytotoxic effects of GMX. Pemetrexed is an inhibitor of dihydrofolate reductase currently used to treat patients with nonsquamous NSCLC. We found that combining pemetrexed with GMX1777 produced a synergistic therapeutic benefit in A549 and H1299 NSCLC cells in vitro and in a mouse A549 xenograft model of lung cancer. Pemetrexed is known to activate PARPs, thereby accelerating NAD consumption. Genetic or pharmacologic blockade of PARP activity inhibited this effect, impairing cell death by pemetrexed either alone or in combination with GMX1777. Conversely, inhibiting the base excision repair pathway accentuated NAD decline in response to GMX and the cytotoxicity of both agents either alone or in combination. These findings provide a mechanistic rationale for combining GMX1777 with pemetrexed as an effective new therapeutic strategy to treat nonsquamous NSCLC. Cancer Res; 74(21); 5948–54. ©2014 AACR.

Introduction

Interest in nicotinamide phosphoribosyltransferase (NAMPT) as a potential anticancer target stems from its involvement as the major and rate-limiting step in the biosynthesis of NAD+ (1, 2). Tumor cells have elevated NAMPT expression and are dependent on NAD+ and ATP to support the increased metabolic demands of rapid cell proliferation. As a cellular response to DNA damage, PARP is activated and consumes its substrate NAD+ for the poly-(ADP-ribosylation) (PAR) of proteins (3). In this way, it contributes to increased NAD+ demands to support the ongoing DNA repair mechanisms in cancer cells. GMX1778 (formerly CHS-828) is a competitive inhibitor of NAMPT whose potency for NAMPT in vitro and cell growth inhibition is in the low nanomolar range (4). GMX1777 is a soluble prodrug of GMX used for in vivo studies (5, 6). NAMPT inhibitors, including GMX1778 (GMX) and FK866, have shown preclinical activity alone and in combination with several therapeutic DNA-damaging agents, but the mechanism of synergy has not been clearly elucidated (7–10).

Here, we report a synthetic lethal screen that revealed dihydrofolate reductase (DHFR) and deoxyuridine triphosphatase (DUT) as genes that modulate cell sensitivity to GMX in A549 non–small cell lung carcinoma (NSCLC) cells. Pemetrexed (PTX) is a multitargeted antifolate that inhibits DHFR, thymidylate synthase, and glycaminamide ribonucleotide transferase, and is currently approved for treatment of nonsquamous NSCLC (11). We demonstrate that GMX synergizes with PTX in vitro and in the A549 lung adenocarcinoma xenograft model. The combination is most efficacious when PTX precedes GMX. The mechanism of synergy involves a PARP-1–mediated accelerated and sustained NAD+ decline that results in enhanced cytotoxicity.

Materials and Methods

Cell lines and compounds

A549, HeLa, Panc-1, NCI-H2030, and H1299 cells were obtained from ATCC (2004–2009) and maintained in RPMI-1640 (Hyclone) with recommended supplements. The cell lines were not authenticated since first acquisition. GMX was a gift from Gemin X Pharmaceuticals Inc.; GMX1777 was synthesized by NuChem Therapeutics. PTX was from Selleckchem...
(in vitro experiments) and LC Biosciences (in vivo experiments) and olaparib (AZD2281) from Selleckchem. Methoxyamine (MX), nicotinamide mononucleotide (NMN), and folic acid were from Sigma-Aldrich.

**Genome-wide synthetic lethal screen**

A549 cells were reverse transfected with 20 nmol/L of human On-Target Plus Smart pool genome-wide library (Dharmacon) using RNAiMax (Invitrogen) according to the manufacturer’s instructions in replicates of 12 (4 wells × 3 plates). Controls included siNT (D-001810-10-20), siPLK-1 (L-003290-00), and siGAPDH (L-004253-00) as nontargeting, transfection, and sensitizing siRNA pools respectively. Forty-eight hours after transfection, cells were treated with vehicle or a dose range of GMX in duplicate. Three days after treatment, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. See Supplementary Methods for data analysis.

**Transfections, antibodies, and Western blot analyses**

siRNAs (20 nmol/L) targeting DHFR (pool, L-008799; Dharmacon; individual S000299992; Qiagen), DUT (pool, L-010258; Dharmacon), and PARP-1 (S002622996; Qiagen) or NAMPT (L-004581; Dharmacon) were transfection into A549 cells using RNAiMax (Invitrogen) as above. Protein was extracted from cells 48 hours after transfection and detected by Western blotting using antibodies to DHFR (EPR5285; Abcam), DUT (AB137097; Abcam), PARP-1 (9542; Cell Signaling Technology), α-tubulin (DM1A; Sigma), γ-H2AX (05-636; Millipore), or PAR proteins (MAB3192; Millipore).

**Drug combination effects and viability assays**

Cells were treated for 72 hours for simultaneous treatments or 48 hours followed by 72 hours for sequential treatments. Serial dilutions of GMX and/or PTX were added to microtiter plates (final DMSO 0.2%). Cell viability was determined using CellTiter-Glo. The combination index was determined using CompuSyn v1.0 (Biosoft) according to the method of Chou and Talalay (12).

**Murine xenografts**

Female SCID hairless outbred (Crl:SHO-Prkdcsid Hrhr) mice (6–8-week-old, Charles River Laboratories) were injected subcutaneously with 10^7 A549 cells in a 1:1 PBS/HC Matrigel (BD) suspension. At a mean tumor volume of 100 mm^3, animals were administered PTX i.p. daily for 5 days (600 mg/kg/day in 0.9% saline) or GMX1777 i.m. daily for 5 days (150 mg/kg/day in 0.9% saline) or GMX1777 i.m. daily for 5 days (150 mg/kg/day in 0.9% saline). The synergy is more pronounced in both lines if PTX treatment preceded GMX treatment by 48 hours. The reverse experiment, knocking down NAMPT, also sensitizes to PTX treatment (Supplementary Fig. S2). In an A549 xenograft model, the administration of PTX (daily × 5) preceded 48 hours by GMX1777 treatment (daily × 5) results in a synergistic anti-tumor effect without significant toxicity (<10% weight loss) for any single agent or combination treatment group (Fig. 2C). This demonstrates the potential clinical impact of this novel drug combination.

Antifolates such as PTX as well as nonfunctional DUT leads to an imbalance in dUTP/dTTP nucleotide pools and dUTP misincorporation followed by activation of base excision repair (BER), which leads to single strand DNA damage, if unrepaired, results in double-strand DNA breaks (DSB; refs. 13, 14). Because PARP activation is involved in the BER and DSB repair pathways (15), we sought to examine the activation of PARP in response to PTX. Figure 3A demonstrates that robust protein PAR occurs 44 hours following PTX treatment that lasts until at least 72 hours. This demonstrates that PARP is activated and is consuming NAD+ at 48 hours following a sublethal dose (150 nmol/L) of PTX treatment. Of note, the total NAD+ decline following treatment with PTX is modest over a 72-hour treatment compared with the decline in NAD+ from either lethal (25 nmol/L) or sublethal (6 nmol/L) doses of GMX (Fig. 3B). Although NAD+ levels decline with similar kinetics with both lethal and sublethal doses of GMX, they can recover only at lethal and sublethal doses of GMX, they can recover only at...
nonlethal doses. This suggests that cell fate is determined by a reduction in NAD$^+$ beyond a threshold concentration and/or duration. In support of these hypotheses, GMX has a steep dose–response viability curve in vitro, suggesting that a threshold effect (4) and sustained exposure to GMX is required for maximal cytotoxic effect (5). We, therefore, reasoned that the combined effects of increased NAD$^+$ utilization through simultaneous PARP activation and inhibition of NAD$^+$ biosynthesis would be enhanced in PTX$^+$ GMX$^-$ treated cells. Indeed, when the two agents are combined in sequence, with PTX followed by GMX, the steady-state NAD$^+$ and NADP$^+$ decline is greater than additive (Fig. 3C) and the rate of NAD$^+$ decline is enhanced (Fig. 3D). Similar effects have been observed for the combination of FK866 with MNNG, or temozolomide (7, 16). Overall, the combination results in a more sustained NAD$^+$ inhibition, and notably, a more rapid impact on ATP decline and cytotoxic effect.

Nicotinamide mononucleotide (NMN), the product of the NAMPT enzyme, can bypass the activity of NAMPT inhibitors and rescue GMX-mediated cytotoxicity (4). NMN has been detected in plasma (17). Although NMN does not affect PTX cytotoxicity, it partially rescues cell death caused by the GMX alone or in combination with PTX (Supplementary Fig. S3A). Similarly, the addition of folate completely abolishes the cytotoxic effects of PTX and the PTX$^+$ GMX$^-$ combination (Supplementary Fig. S3B). Therefore, regulators of intracellular and extracellular NMN levels, as well as folate supplementation, could affect the efficacy of NAMPT inhibitor–containing therapies.

To determine if the PTX/GMX synergy is mediated through PARP, cells were treated with PTX and GMX in the presence or absence of olaparib, a small-molecule PARP inhibitor (Fig. 4A and B; ref. 18) or following siRNA knockdown of PARP-1 (Supplementary Fig. S4A and S4B). Treatment with olaparib was sufficient to inhibit PTX-induced PAR and NAD$^+$ depletion. It was also sufficient to partially restore NAD$^+$ levels in the PTX/GMX-treated cells above the threshold level such that...
viability was also restored from 22% to 68%. SiRNA knockdown of PARP-1 completely inhibited PTX-mediated PAR and partially restored viability to the PTX/GMX-treated cells (Supplementary Fig. S4A and S4B), demonstrating that the synergy is mediated in part through PARP-1. Of note, by eliminating the PARP substrate, NAD$^+$, GMX functionally mimics a PARP inhibitor and prevents PTX-induced PAR (Fig. 4A and Supplementary Fig. S4B); no PAR occurs following GMX treatment alone, as expected (Fig 4B and D). GMX does not prevent the induction of DSB by PTX as measured by γ-H2AX levels (Fig. 4B), and, thus, does not interfere with the DNA-damaging aspects of PTX cytotoxicity or the cell’s demand for NAD$^+$. Methoxyamine has been previously shown to enhance PTX-induced accumulation of DSBs and cytotoxicity (19). This suggests that a BER-inhibiting agent such as methoxyamine may enhance the cytotoxic effects of the GMX-PTX combination, both by increasing NAD$^+$ depletion and DNA damage and may be a useful addition to this combination strategy.

Figure 4C and D demonstrate that the BER inhibitor methoxyamine enhances the NAD$^+$/NADH-depleting effects of GMX and the GMX/PTX combination. Methoxyamine consequently enhances the cytotoxicity of PTX and GMX alone, as well as the combination of the two (Fig. 4D). Methoxyamine has been previously shown to enhance PTX-induced accumulation of DSBs and cytotoxicity (19). This suggests that a BER-inhibiting agent such as methoxyamine may enhance the cytotoxic effects of the GMX-PTX combination, both by increasing NAD$^+$ depletion and DNA damage and may be a useful addition to this combination strategy.

Figure 2. The combination of GMX and PTX is synergistic in vitro and in tumor xenografts. A, A549 and H1299 cells were treated simultaneously with PTX alone (red) or in combination with various doses of GMX (blue, green). B, A549 and H1299 cells were pretreated with PTX for 48 hours followed by addition of DMSO (red) or GMX (blue, green) for an additional 72 hours. Cell viability was determined 72 hours after GMX by CellTiter-Glo. IC$_{50}$ values were determined in Prism 5.0 and combination index (CI) values determined using CompuSyn v1.0. C, SCID hairless outbred mice harboring A549 xenografts were administered vehicles, PTX (5 daily doses) and/or GMX1777 (5 daily doses) starting 2 days following PTX dosing initiation. Error bars, mean ± SEM; n = 11 for all groups. #, animal sacrificed because of tumor ulceration or malaise. Tumor volumes (left) and body weight (right) were measured three times per week. Statistical differences in tumor volumes between the PTX + GMX treatment group and all other groups were determined by one-way analysis of variance (P < 0.05) Prism 5.0.
Figure 3. The combination of PTX and GMX causes PARP activation and enhanced NAD\(^+\) depletion beyond the NAD\(^+\) threshold for cell survival in A549 cells. A, cells were treated with 150 nmol/L PTX for the indicated times and the levels of PAR proteins measured by Western blotting. α-Tubulin was used as a loading control. B, cells were treated with the indicated concentrations of PTX or GMX. Levels of NAD\(^+/\)NADH, ATP, or intact viable cells relative to time-matched DMSO controls were measured using NAD\(^+/\)NADH-Glo, CellTiter-Glo, and CellTiter-Fluor assays, respectively (mean ± SD of duplicates). C, cells were treated with DMSO or 150 nmol/L PTX for 24 hours followed by treatment with DMSO or 12 nmol/L GMX for 48 hours. The intracellular levels of NAD\(^+\) and NADP\(^+\) were measured from cell extracts and by LC-MS/MS. NAD\(^+\) levels are expressed relative to protein content (mean ± SD of quadruplicate samples). D, cells were pretreated with DMSO or 150 nmol/L PTX for 48 hours before the addition of GMX at 4 or 8 nmol/L. NAD\(^+/\)NADH, ATP, and intact cells were measured as in B. t\(_{1/2}\) (h) for each treatment group was determined in Prism 5.0.
This is the first demonstration that NAMPT inhibitors synergize with the multitargeted antifolate PTX in vitro and in a xenograft model of NSCLC. The mechanism of synergy involves PTX-mediated activation of PARP-1, resulting in an accelerated and sustained NAD$^+$ and ATP decline followed by cell death. PARP-1 is thought to play a role in BER both as a
sensor and in the repair mechanism. Mortusewicz and colleagues demonstrated that initial PARP recruitment and activation generates PAR-modified binding sites on PARP-1 and on histones near the site of DNA damage, recruiting and activating additional PARP-1 for a second and enhanced wave of PARP-1 activation following irradiation DNA damage (20). This suggests why pretreatment with PTX may result in a more profound combination effect than when the two agents are added simultaneously or if GMX is added first. This work demonstrates the potential for the combination of NAMPT inhibitors with various PARP-activating therapeutic agents (in addition to PTX) in clinical settings and that the schedule of administration may influence the treatment efficacy.

Disclosure of Potential Conflicts of Interest

G.C. Shore is a Chief Scientific Officer in, reports receiving a commercial research grant from, and has ownership interest (including patents) in Therrlis Development Company. No potential conflicts of interest were disclosed by the other authors.

References

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