Published in final edited form as:

Clin Cancer Res. 2017 December 01; 23(23): 7301–7311. doi:10.1158/1078-0432.CCR-17-1121.

Matrix Screen Identifies Synergistic Combination of PARP Inhibitors and Nicotinamide Phosphoribosyltransferase (NAMPT) Inhibitors in Ewing Sarcoma

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Abstract

Purpose: Although many cancers are showing remarkable responses to targeted therapies, pediatric sarcomas, including Ewing sarcoma, remain recalcitrant. To broaden the therapeutic landscape, we explored the *in vitro* response of Ewing sarcoma cell lines against a large collection of investigational and approved drugs to identify candidate combinations.

Experimental Design: Drugs displaying activity as single agents were evaluated in combinatorial (matrix) format to identify highly active, synergistic drug combinations, and

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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combinations were subsequently validated in multiple cell lines using various agents from each class. Comprehensive metabolomic and proteomic profiling was performed to better understand the mechanism underlying the synergy. Xenograft experiments were performed to determine efficacy and *in vivo* mechanism.

Results: Several promising candidates emerged, including the combination of small-molecule PARP and nicotinamide phosphoribosyltransferase (NAMPT) inhibitors, a rational combination as NAMPT is block the rate-limiting enzyme in the production of nicotinamide adenine dinucleotide (NAD⁺), a necessary substrate of PARP. Mechanistic drivers of the synergistic cell killing phenotype of these combined drugs included depletion of NMN and NAD⁺, diminished PAR activity, increased DNA damage, and apoptosis. Combination PARPis and NAMPTis *in vivo* resulted in tumor regression, delayed disease progression, and increased survival.

Conclusions: These studies highlight the potential of these drugs as a possible therapeutic option in treating patients with Ewing sarcoma.

Introduction

Ewing sarcoma is an aggressive bone and soft-tissue malignancy predominantly affecting children and adolescents in the second decade of life. Despite significant advances in the understanding of the biology of this cancer, patients with relapsed, recurrent, or metastatic disease continue to have abysmal long-term survival rates of less than 20% (1–3). Further, for patients who survive their disease, damaging late effects from treatment with multiagent cytotoxic chemotherapy occur and result in substantial risk of early death and secondary malignancy (4, 5). Targeted therapies for patients with Ewing sarcoma are an active area of research, as they offer the possibility of efficacy with minimal toxicity. Many targeted agents have shown promise in the preclinical arena, only to fail in early clinical trials (6–8). Given this, there is growing interest in identifying rational therapeutic combinations that can overcome resistance and result in durable response (9).

A majority of Ewing sarcoma cases are the result of a translocation between chromosomes 11 and 22 resulting in the aberrant transcription factor EWS-FLI1 (10). Attempts to directly target EWS-FLI1 or identify therapeutic liabilities associated with it have yet to yield an effective therapeutic. Thus, explorations within the existing pharmacopeia for EWS-FLI1-driven drug sensitivities have proven an attractive strategy (11). Technological advances have allowed for rapid screening of multiple cancer cell lines versus large libraries of agents. Further, systematic screening of drug combinations offers a method to rapidly identify novel targets and assess synergistic potential of candidate agents (12). Seeking out therapies that not only show robust single-agent activity but also combine in a synergistic fashion is ideal, as synergistic combinations have the potential to offer both enhanced efficacy and a greater therapeutic index (13).

Previous reports using high-throughput screening methods have identified several intriguing Ewing sarcoma drug sensitivities. Garnett and colleagues showed that PARP inhibitors (PARPis) have surprising activity in Ewing sarcoma. PARP enzymes mediate DNA repair, and as Ewing sarcoma cell lines are frequently defective in DNA break repair, they are susceptible to PARP inhibition (14–16). Although preclinical *in vitro* models have yielded

promising results, single-agent activity of PARPis in preclinical *in vivo* models and early phase clinical trials in Ewing sarcoma have failed to demonstrate meaningful responses (17, 18). Nonetheless, in hopes of exploiting the therapeutic promise associated with PARPis, rational drug combinations have been explored with cytotoxic DNA-damaging agents and show some enhanced efficacy when combined with PARPis in the preclinical setting (19–24).

To function, PARPis require nicotinamide adenine dinucleotide (NAD⁺) as a necessary substrate (16). In tumor cells, enzymes in the *de novo* NAD⁺ synthetic pathway are frequently silenced, and NAD⁺ production is reliant on the salvage pathway in which the enzyme mediating the rate-limiting step is nicotinamide phosphoribosyltransferase (NAMPT; refs. 25–27). In multiple studies, NAMPTis have been shown to deplete NAD⁺, resulting in a loss of cell viability in a variety of cancer types (25, 26, 28–30). Given that Ewing sarcoma cells rely on functioning PARPis, that PARPis require NAD⁺, and that NAD⁺ production relies on NAMPT, there appears to be a rationale for combining these two classes of agents in Ewing sarcoma.

Here, we report on the results of a broad examination of four established Ewing sarcoma cell lines versus the MIPE library of investigational and approved drugs and the entry of highly active agents into a wide-ranging matrix examination to explore synergistic drug combinations. These studies revealed remarkable and surprising synergy between PARPis and NAMPTis in Ewing sarcoma, the activity of which was confirmed in separate *in vivo* Ewing sarcoma models. Detailed metabolomics and proteomic studies of this drug combination provided insight into the mechanistic underpinnings of the observed synergy.

Materials and Methods

High-throughput drug screen

Cell lines.—Ewing sarcoma cell lines TC32, TC71, and EW8 have been previously described (31). RDES cell line was obtained from the ATCC. Cells were maintained in RPMI growth medium (Life Technologies) with 10% FBS, heat-inactivated (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies), and 2 nmol/L L-glutamine (Life Technologies) at 37°C in standard incubator conditions.

Compounds.—The MIPE 4.0 library of approved and investigational drugs included 1,912 individual small molecules (32). It encompasses small-molecule modulators of over 400 specific gene targets, cellular pathways, or phenotypes. Within well-explored targets, there are multiple, redundant agents incorporated as a means to inform on the on-target nature of phenotype-to-mechanism data associations and to explore instances where a phenotype is the result of the specific polypharmacology of an individual drug.

Screening protocol.—The cell-based screening methods employed in this study were similar to those previously published (12, 33). Briefly, all four Ewing sarcoma lines were screened in single-agent format in 1,536-well plates with 500 cells per 5-µL well for inhibition of cell viability (assessed by measuring ATP levels with CellTiterGlo) after a 48-hour incubation with the MIPE 4.0 library of approved and investigational drugs. For both

single-agent and combination studies, data were normalized to intraplate DMSO (100% viability) and bortezomib (0% viability) controls. Signal was measured as median relative luminescence units on a ViewLux (Perkin-Elmer) reader. Efficacious compounds from single-agent screens were advanced to matrix combinations studies to assess additivity/synergy. Matrix blocks were dispensed using an acoustic dispenser (EDC Biosystems), and 48-hour CellTiterGlo or 8- and 16-hour CaspaseGlo readouts were utilized to inform on cell viability and apoptosis induction as described.

Mechanistic studies

Metabolomics studies.—Metabolomics outcomes were generated by Metabolon (<http://www.metabolon.com/>). TC71 cells were prepared from standard cultures following treatment with vehicle (DMSO), niraparib (5 $\mu\text{mol/L}$), daporinad (5 nmol/L), or the combination of both drugs for either 6 or 24 hours and flash frozen as packed pellets (between 50 and 100 μL). Five biological replicates were analyzed on Metabolon's global metabolomics platform informing on a diverse range of biochemicals characterized via UPLC-MS/MS outcomes referenced to internal standards. Methods for metabolite quantification, data normalization, statistical analysis, and quality control methods are given in the Supplementary Information. The full dataset is available in Supplementary Dataset S2.

AR immunoassay.—For cell-based experiments, cells were plated in RPMI growth medium overnight before drug treatments were applied. Niraparib treatments were applied for 4 hours; daporinad treatments were applied for 24 hours prior to harvest. At harvest, cells were washed twice with ice-cold PBS (Life Technologies), and then lysed with cell lysis buffer (Cell Signaling Technology) with phosphatase and protease inhibitors (Life Technologies).

For tissue-based experiments, interim tumors were harvested on day 4 of treatment. Approximately 20 mg of frozen tumor was resuspended in 0.5 mL Cell Extraction Buffer (Invitrogen) supplemented with protease inhibitor (Roche) and homogenized with a PRO200 homogenizer with a 5-mm probe (ProScientific) in an ice bath. Lysates were incubated on ice for 30 minutes prior to adding sodium dodecyl sulfate (Ambion) to a final concentration of 1%. Tubes were then boiled for 5 minutes to inhibit intrinsic enzyme activity and stabilize PAR. Lysates were clarified by centrifugation at $12,000\times g$ for 5 minutes at 2 to 8°C, and the cleared lysates were transferred to a new tube. Protein levels were determined with the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific Pierce) according to the manufacturer's instructions.

The validated chemiluminescent immunoassay for PAR using commercially available anti-PAR mouse monoclonal antibody (clone 10 H; Trevigen) has been described in detail (34, 35). Briefly, 100 μL of antibody at a concentration of 4 $\mu\text{g/mL}$ in PDA II Antibody Coating Buffer (Trevigen) was added to each well of a Pierce White Opaque 96-well plate (Thermo Scientific Pierce) and incubated at 37°C for 2 hours. Each well was blocked with 250 μL of Superblock (Thermo Scientific) at 37°C for 1 hour. Cell lysates containing 250 k cells/well from cultured cells or tumor lysates containing 0.5 and 2 $\mu\text{g/well}$ protein from mouse xenograft tumors were loaded into the plate and incubated at 4°C overnight (18 ± 2 hours).

Rabbit anti-PAR polyclonal detection antibody (Trevigen) at a concentration of 0.5 µg/mL diluted with 2% BSA (Sigma-Aldrich) in 1×PBS (Invitrogen) supplemented with 1 µL/mL normal mouse serum (Sigma-Aldrich) was added into each well and incubated at 25°C for 2 hours. Goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibody (KPL) at a final concentration of 1 µg/mL (1:1,000) diluted with 2% BSA in PBS supplemented with 1 µL/mL normal mouse serum was added and incubated at 25°C for 1 hour. A BioTek EL x405 automatic plate washer was used to wash plate between each incubation step. Note that 100 µL/well of fresh SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific) was added, and the plate was immediately read on a Tecan Infinite M200 plate reader (Tecan Systems). Eight standards from 7.8 to 1,000 pg/mL were loaded into the plate along with testing samples and used to calculate the PAR values for samples. Three assay controls (Low-C, Medium-C, and High-C) were included in each run plate to monitor assay consistency.

RPMA.—Proteomics and phosphoproteomics outcomes were generated by TheraNostics Health (<http://www.theranosticshealth.com/>). TC71 cells were prepared from standard cultures following treatment with vehicle (DMSO), niraparib (5 µmol/L), daporinad (5 nmol/L), or the combination of both drugs for either 6 or 24 hours. Protein lysates were prepared according to published guidelines. Duplicate samples were analyzed on TheraNostics Reverse Phase Protein Array platform informing on 120 selected protein analytes. Methods for total protein quantification and normalization, immunostaining, data analysis, and quality control methods are found in the Supplementary Information. The full dataset is available in Supplementary Dataset S3.

Western blotting.—Cells were plated in RPMI growth medium overnight. Niraparib and daporinad treatments were then applied for between 18 and 24 hours prior to harvest. At harvest, plates were immediately placed on ice. Cells were washed once with ice-cold PBS (Life Technologies), and then lysed with cell lysis buffer (Cell Signaling Technology) with phosphatase and protease inhibitors (Life Technologies).

Protein lysates (30 µg/lane), as determined by BCA protein assay (Life Technologies), were separated in 4% to 12% SDS-PAGE (Life Technologies) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dried milk in TBS (KPL)-Tween 20 (Sigma-Aldrich; 20 mM Tris-HCl, pH 7.5; 8 g/L of sodium chloride; 0.1% Tween 20). Blots were incubated with antibodies against total p38 MAPK, phospho p38 MAPK, total SAPK/JNK, and phospho-SAPK/JNK (Cell Signaling Technology) at a 1:1,000 dilution. Anti-beta actin antibody (Abcam) and GAPDH (Santa Cruz Biotechnology) were used as loading controls. Bands were visualized on a camera using West Femto and Pico ECL detection reagent (Life Technologies).

In vivo studies

Animal studies were performed in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee. Four- to 6-week-old female Fox Chase SCID-Beige mice (CB17.B6-Prkdcscid Lystbg/Crl) were purchased from Charles River Laboratories. Two million TC32 or TC71 cells were suspended in a solution of HBSS

(Thermo Fisher Scientific) and injected orthotopically into the gastrocnemius muscle in the left hind leg of each mouse. When tumors were palpable, mice were randomized into groups of 12 to receive vehicle, niraparib (50 mg/kg), GNE-618 (25 mg/kg), or both. Both drugs were given once daily by oral gavage.

Treatment began on day 11 after injection (average tumor size of TC32-bearing mice was 250 mm³; average tumor size of TC71-bearing mice was 500 mm³). Treatment was given for 5 consecutive days through day 15 after injection, followed by 5 days without treatment. On day 21, treatment resumed for 5 more consecutive days, through day 25.

Mice were maintained in a pathogen-free environment. Tumors were measured twice weekly with calipers. Mice were monitored by observation of overall health and weekly weights to determine drug tolerability. Tumor volume was calculated by the following formula: V (mm³) $(D \times d^2)/6 \times 3.14$, where D is the longest tumor axis and d is the shortest tumor axis. Tumors were harvested at midpoints and at study endpoint for biology studies.

Xenograft statistical analysis

Tumor volumes were compared between groups using a Wilcoxon rank-sum test at serial time points selected to be appropriate according to the data being presented in each plot. Measurements for mice that had already reached endpoint were carried forward until all mice in the group had reached endpoint or the experiment was terminated. The Mantel–Cox analysis was performed to compare survival of mice in the combination group to each of the treatment groups.

Results

Combined PARP and NAMPT inhibition is synergistic in Ewing sarcoma cell lines

Utilizing quantitative high-throughput screen, we tested the MIPE library of 1,912 agents against four distinct Ewing sarcoma cell lines (TC32, TC71, RDES, and EW8) using a 48-hour CellTiterGlo readout to inform on antiviability/proliferation effect of each agent. Full screen results are available via the PubChem database (AID # 1259257) and in Supplementary Dataset S1. From this effort, 679 agents with a range of primary mechanisms were judged to be active based upon achievement of class -1.1 , -1.2 , and -2.1 curves in all four cell lines (Fig. 1A; Supplementary Table S1; Supplementary Fig. S1A; see ref. 36). The majority of these active agents possessed good half maximal activity (log) concentration (LAC50) correlations, suggesting robust on-target activity as the driver of these agents' antiproliferative actions (Supplementary Fig. S1B and S1C). Multiple parameters were utilized to justify advancing agents that were deemed active into combination assessments. Included were mechanism of action assessments, potency and percent response, clinical status, and the promiscuity of the outcome relative to all MIPE screens performed to date. As such, approved drugs with unique mechanisms and highly potent effects were given priority. Further, agents that were widely active across all MIPE viability screens were deemed less interesting. For example, the activities of the PARPis niraparib and olaparib and the NAMPTis daporinad and GMX-1778 were judged to be sufficiently unique to Ewing sarcoma as to warrant further examination (Fig. 1B and C). From this collection, 66 agents

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were selected for a matrix experiment exploring five combined and uniquely chosen dose matrices and a DMSO control (i.e., a 6×6 checkerboard matrix experiment). This experiment resulted in 2,145 discrete 6×6 tests and was run in the TC71 cell line (all single-agent and matrix outcomes are available via <https://tripod.nih.gov/matrix-client/>). Utilizing the results of this pilot study, subsequent 6×6 tests were performed including an examination of 44 highly active agents which informed on 946 specific drug combinations (Fig. 1D). Combinations that displayed synergy at selected concentrations, as defined by multiple metrics including the Bliss independence model and Gaddum noninteractive model, were advanced into matrix experiments exploring nine combined and uniquely chosen dose matrices and a DMSO control (i.e., a 10×10 checkerboard matrix experiment). In addition to 48-hour CellTiterGlo readouts, many of the drug combinations advanced to 10×10 experiments were examined in 8- and 16-hour CaspaseGlo experiments to gain insight into the apoptotic nature of the cell response. Further, 10×10 experiments expanded beyond the TC71 cell line to include TC32, RDES, and EW8 to assure that all synergistic outcomes were consistent. In total, 3,952 6×6 and 920 10×10 experiments were performed. From these studies, several drug combinations with strong synergy at selected concentrations were noted (Supplementary Fig. S2). The combination of PARPis and NAMPTis was among the most intriguing discovered during the HTS effort, with the combination of niraparib (a NAMPTi) and daporinad (a NAMPT inhibitor) demonstrating strong delta Bliss values across multiple overlapping concentrations of both drugs (Fig. 1E).

To affirm that the synergy displayed from these screens was the result of the on-target pharmacology of each agent, we expanded our studies to incorporate additional PARPis and NAMPTis from divergent structural classes. In addition to niraparib, the PARPis olaparib and veliparib were included, as were additional NAMPTis GMX-1778 and GNE-618. The results of these studies demonstrated strong synergy for all PARPi/NAMPTi combinations (Supplementary Fig. S3). Importantly, these outcomes were not assay format-dependent or altered by the addition of common ROS-mitigating agents NAC (1 mmol/L) and Trolox (0.5 mmol/L; Supplementary Fig. S3). To investigate long-term survival of Ewing sarcoma cells treated with the combination of niraparib and daporinad, IncuCyte assays were performed and confirmed prolonged inhibition of cell growth out to 500 hours, after a single treatment (Supplementary Fig. S4). Based on the aforementioned interest in PARPis as a potential therapy for Ewing sarcoma, the combined efficacy and synergy of the PARP/NAMPT combination, and the convincing data suggesting on-target basis for the activity, this combination was taken forward for further study.

Mechanism of cell growth inhibition depends on depletion of NMN and NAD⁺

NAD⁺ is a critical metabolite that cells derive through *de novo* synthesis or via the NAD⁺ salvage pathway. In cancer, there is frequently an increased reliance on the NAD⁺ salvage pathway whereby NAMPT converts nicotinamide (NAM) to nicotinamide mononucleotide (NMN), which is then converted to NAD⁺ by nicotinamide mononucleotide adenylyltransferase (Fig. 2A). To gain insight into the global effects of PARP and NAMPT inhibition on Ewing sarcoma cells, we generated a metabolite profile informing on 463 biochemicals of known identity from cells treated with vehicle (DMSO), niraparib (5 μ mol/L), daporinad (5 nmol/L), or the combination of both drugs. Cells were collected after

an acute (6 hour) or prolonged (24 hour) exposure to all four treatment scenarios. These data highlighted drug effects on the urea cycle and glycolysis and revealed several oxidative stress signatures (Supplementary Fig. S5). Critically, this dataset captured the key biochemicals within the NAD⁺ salvage pathway [i.e., NAM, NMN, NAD⁺, and nicotinamide riboside (NR)]. The comparative levels of NMN and NAD⁺ following drug treatment demonstrated a decrease in the amount of NMN and NAD⁺ following daporinad treatment at both time points, suggesting that the salvage pathway is critical in maintaining NAD⁺ levels in Ewing sarcoma cells (Fig. 2B). In contrast, niraparib increased the amount of NMN and NAD⁺ present at both 6 and 24 hours. Because PARP enzymes utilize NAD⁺ as a necessary substrate, it follows that PARP inhibition would result in an increase in NAD⁺ and NMN. In cells receiving the combination, NAD⁺ was diminished and NMN was depleted at 24 hours, suggesting that the NAD⁺-depleting effect of daporinad was more predominant than the NAD⁺-increasing effect of niraparib following prolonged exposure to both drugs. Interestingly, although daporinad had little effect on NAM and NR levels, treatment with niraparib led to a remarkable drop in NAM levels at both time points while increasing NR levels. The mechanistic basis for these changes is unclear. The equilibrium dynamics of these interrelated metabolites are complex; however, the changes observed are generally consistent with the anticipated drug effects.

To demonstrate that inhibition of the production of NMN and subsequently NAD⁺ was the contributing factor to the NAMPTi-specific cell toxicity, we attempted to rescue TC71 cells from the effect of NAD⁺ depletion by adding NMN to the combination of niraparib and GNE-618 (Fig. 2C). Addition of 1 mmol/L of NMN completely abrogated the efficacy of single agent GNE-618 at the concentrations examined. Furthermore, the presence of NMN significantly shifted the dose response of niraparib, making the cells less sensitive. These data suggest that the cytotoxic effects of the NAMPTis are primarily due to the depletion of NMN and NAD and that these biochemicals also contribute to the antiproliferative activity of PARPis.

Both NAD⁺ and ATP are required biochemicals for creation of the poly ADP-ribose (PAR) complex by PARP. Owing to requisite need for NAD⁺, we hypothesized that depletion of NAD⁺ by NAMPT inhibition would inhibit PAR activity and that the combination of PARP inhibition with NAMPT inhibition would further decrease PAR activity (Fig. 2D). To assess this, an assay measuring PAR activity was performed in TC32 and TC71 cells (Fig. 2E). Cells treated with increasing levels of niraparib showed a dose-dependent decrease in PAR activity in TC32 cells, as expected. In TC71 cells, PAR activity was stably decreased to nearly the same level despite increasing niraparib doses, suggesting that certain cell lines may have a limit to the amount of PAR activity inhibition that can be achieved with a given PARPi. Strikingly, a low dose of daporinad (5 nmol/L) inhibited PAR activity by 80% to 95% in both cells lines. Further experiments with additional doses of daporinad demonstrated a dose-dependent response, with doses in the IC₅₀ range (0.5 nmol/L) inhibiting 20% to 50% of PAR activity, depending on the cell line, and higher doses resulting in greater inhibition (Supplementary Fig. S6). The combination of daporinad and niraparib together further decreased PAR activity, lending support to our hypothesis. A statistically significant difference was noted between PAR levels from cells treated with the combination and all other treatment groups.

Combination PARP and NAMPT inhibition induces DNA damage and apoptosis

To gain further insight into the synergistic nature of this drug combination, we employed a reverse phase protein microarray (RPMA)-based assessment of key cellular responses to the combination of niraparib and daporinad captured at 6 and 24 hours (Fig. 3A and B and Supplementary Fig. S7). Although the 6-hour time point showed little acute proteomic/phosphoproteomic response, the 24-hour time point indicated several changes. Among the top targets with signal increase were several markers of apoptosis including cleaved caspase 7, cleaved PARP, and cleaved caspase 3, which were significantly increased at 24 hours. Phosphorylated histone H2AX, a marker of DNA damage, also displayed a significant increase at 24 hours, underscoring the role of PARP inhibition in this drug combination. The stress-activated protein kinases SAPK/JNK and p38 MAPK were of particular interest as they both showed dramatic increases in protein phosphorylation in the presence of the drug combination at the 24-hour time point. Transient activation of these signaling elements has been associated with cell survival, whereas sustained activation of these proteins has been correlated with apoptotic cell death in other cancer types (37, 38). Western blot analysis confirmed these results (Fig. 3C and D).

Combination PARP and NAMPT inhibition results in tumor regression in multiple Ewing sarcoma xenograft models

Two of the four Ewing sarcoma cell lines used in the screen (TC32 and TC71) were selected for *in vivo* study based on their favorable growth kinetics as xenografts. Mice were randomized to receive treatment with either vehicle, niraparib (50 mg/kg), GNE-618 (25 mg/kg), or both. For both models, treatment began on day 11 after randomization when tumors averaged 250 mm³ for TC32 and 500 mm³ for TC71. All treatments were given once daily by oral gavage. Mice were treated for 5 consecutive days through day 15 after injection, followed by 5 days without treatment. On day 21, treatment resumed for 5 more consecutive days, through day 25. Following day 25, treatment was discontinued.

Dual inhibition of PARPs and NAMPTs in TC32 xenografts resulted in tumor regressions during the treatment period and a period of continued growth suppression beyond the end of treatment in both tumor types. Specifically, in TC32 xenografts, tumors were noted to regress through day 29. Thereafter, tumor growth was slowed with a statistically significant difference achieved in tumor sizes from days 25 ($P=0.0011$) through 42 ($P=0.0227$; Fig. 4A). Survival to endpoint (maximum diameter of 2 cm) was superior in the combination group ($P<0.0001$). Similarly, in TC71 xenografts, tumor regression was noted through day 25, with subsequently slowed tumor growth with a statistically significant difference achieved in tumor sizes from days 22 through 32 ($P<0.0001$), and superior survival in the combination group ($P<0.0001$; Fig. 4B). In both models, there was no effect on tumor growth with single-agent niraparib and only temporary tumor stabilization with single-agent GNE-618. Tolerability was excellent with no toxicity-related deaths or significant weight loss in treated mice (Supplementary Fig. S8).

Given that one criticism of xenograft models is that tumor burden is small at the time of initial treatment, an additional experiment was performed on TC71 tumor-bearing mice. In this experiment, treatment was delayed until TC71 tumors became extremely large (1,400

mm³) and was then administered for one 5-day period at the doses described above. As with the prior experiments, niraparib had no effect and GNE-618 resulted in temporary tumor stabilization. Remarkably, the combination still resulted in significant tumor regression in all mice treated, despite the large starting size, and regrowth of tumor was not noted until 9 days after the end of treatment (Supplementary Fig. S9).

To assess whether a similar pattern of PAR activity observed *in vitro* was present *in vivo*, tumor tissue was obtained from mice after 4 days of treatment with vehicle, niraparib, GNE-618, or both agents and evaluated for PAR activity. As was seen *in vitro*, both xenograft models showed incomplete inhibition of PAR activity with niraparib, which was further decreased with the addition of GNE-618, supporting the proposed mechanism of action (Fig. 4C).

Discussion

Combinatorial drug matrix screening offers an efficient means to identify novel therapies that display synergistic potential within an *in vitro* setting. Here, we utilized a matrix screen in Ewing sarcoma to identify several potential therapeutically intriguing drug combinations including a highly synergistic combination of PARPis and NAMPTis. Utilizing a multi-omics approach, we further uncovered that the DNA damage and apoptotic phenotypes associated with this combination are related to the depletion of NMN and NAD⁺, enhanced inhibition of PARP, and sustained activation of cellular stress pathways. Expanded evaluation of this combination in multiple Ewing sarcoma cell lines and with multiple PARP and NAMPTis validated the on-target nature of synergy and efficacy demonstrated by this drug combination. Multiple preclinical *in vivo* xenograft models further highlighted the potential of a PARPi and NAMPTi regimen in Ewing sarcoma.

Although targeted therapies remain a promising avenue of exploration for Ewing sarcoma, therapeutic resistance has limited the utility of many of these agents in the clinic. Thus, the discovery of rational therapeutic combinations will be necessary to achieve improved clinical efficacy. PARPis were initially believed to have great potential for Ewing sarcoma, as preclinical data from multiple groups revealed that Ewing sarcoma gene fusions were dependent on the activity of PARP1 and that cell lines expressing these fusions were exquisitely sensitive to PARP inhibition (11, 24). Despite these promising preclinical data, single-agent efficacy of PARPis could not be recapitulated in xenograft models of Ewing sarcoma (18, 21). Furthermore, results from early-phase clinical trials in Ewing sarcoma using olaparib indicated a lack of response (17). Hence, an effort to identify combination treatments has ensued (15). PARPis have been tested *in vitro* with DNA-damaging agents including temozolamide and irinotecan with encouraging results in Ewing sarcoma (19–21). *In vivo* studies using PARPis with camptothecins, temozolamide, and trabectedin in Ewing sarcoma have had mixed results (14, 19, 23). Although several clinical trials are ongoing, there are currently no published results describing PARPis in combination with DNA-damaging agents in Ewing sarcoma. However, results in other cancer types suggest that although there may be a potential benefit in overall survival with such a combination, dose-limiting toxicities, most prominently myelosuppression, have been a limiting factor requiring dose reductions (39–44). In addition, analysis of PAR levels in peripheral blood

monocyte cells relative to tumor cells has revealed differences in the degree and duration of PARPi-induced PAR depletion (43, 44). Our findings suggest that adding a NAMPTi to a PARPi will augment the reduction of PAR activity, which may allow for the use of lower doses of PARPis, while rendering them more clinically efficacious. Moreover, because the known toxicity profiles of PARPis and NAMPTis appear to be distinct, this may be a more tolerable combination for patients. Besides the aforementioned role of PARP in PAR generation for DNA repair, PARP is involved in a number of other cellular processes including transcriptional regulation, signal transduction, heat shock response, and the ER unfolded protein response pathway (45). At this time, the impact of coinhibition of NAMPT on these alternative functions of PARP is not clear, but is an interesting avenue for further study.

As we demonstrate herein, inhibition of NAMPT clearly alters PARP activity. However, limiting the NAD⁺ available to cancer cells modifies multiple cellular phenotypes. Rapidly proliferating cancer cells have altered metabolic needs including a rapid rate of NAD⁺ cycling relative to normal cells (29, 46). Sustained depletion of NAD⁺ in cancer cells can trigger apoptosis and autophagy in several cancer types (25, 28, 29). Mutz and colleagues have recently shown that Ewing sarcoma cells are extremely sensitive to NAMPT inhibition, as it results in NAD⁺ depletion and subsequent mitochondrial dysfunction and blockade of DNA synthesis (30). When used as single agents, NAMPTis resulted in cell death and loss of clonogenic growth. These findings are consistent with the data shown in this study.

Although the combination of PARP and NAMPT inhibition has been shown to moderately slow tumor growth with continuous treatment in a xenograft model of triple-negative breast cancer (TNBC), the *in vivo* results from our work resulted in marked tumor regressions and continued growth suppression after short-term treatment in several models. Notably, when treatment was delayed until tumors had become very large (average tumor size at treatment initiation of 1,400 mm³, compared with 20 mm³ in TNBC), the combination still demonstrated tumor shrinkage (47). Although our data confirm that NAD⁺ depletion via NAMPT inhibition sensitized cell lines to PARP inhibition, increased the therapeutic window of the PARPi, and enhanced γH2AX levels resulting in apoptotic cell death, our work further extended the understanding of the mechanism of this combination. Specifically, we demonstrated that the combination of PARP and NAMPT inhibition activated stress-activated protein kinases SAPK/JNK and p38 MAPK, and that PAR activity was significantly decreased following PARP and NAMPT inhibition *in vitro* and *in vivo*, which had not been previously shown. Given the current clinical status of PARPis, these data provide the rationale for testing this novel combination. Further, based on published data indicating that sensitivity to both PARP inhibition and NAMPT inhibition is likely related to the presence of EWS-FLI1 or EWS-ERG fusions, we predict that these effects may be broadly applicable to the majority of Ewing sarcoma, and suggest that combining PARPis and NAMPTis may be a promising therapeutic strategy for patients with Ewing sarcoma (11, 30).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support

This work was supported by grants from the Intramural Research Programs of NIH, the National Center for Advancing Translational Sciences, the National Cancer Institute, and the Center for Cancer Research.

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Translational Relevance

PARP inhibitors (PARPis) have emerged as an intriguing treatment strategy for patients with Ewing sarcoma, in part because EWS-FLI1 expression confers sensitivity to PARP inhibition. Unfortunately, PARPis in preclinical *in vivo* models and clinical trials in Ewing sarcoma have failed to demonstrate meaningful responses. Combining PARPis with other therapies, typically DNA-damaging agents, although more efficacious, increases toxicity, due to overlapping side effects. As PARP utilizes nicotinamide adenine dinucleotide (NAD⁺) as a necessary substrate, combining nicotinamide phosphoribosyltransferase (NAMPT) inhibitors (NAMPTis), which block the rate-limiting step in NAD⁺ production, with PARPis is a rational approach to enhancing PARP inhibition potentially without additive toxicity. We show that combining PARPis and NAMPTis resulted in robust synergy in *in vitro* models of Ewing sarcoma through decreased PAR activity, increased DNA damage and apoptosis, and that the combination retained efficacy in multiple *in vivo* models. These data suggest that combining PARPis with NAMPTis may be a promising strategy for Ewing sarcoma.

Figure 1.

HTS matrix drug screen identifies synergy between PARP and NAMPT inhibition in Ewing sarcoma. **A**, Cell survival responses to all MIPE 4.0 library agents (as judged by relative AUCs) binned per mechanistic classes (mechanistic superclasses are listed in order from top to bottom: transcriptional regulation, physiological homeostasis, other, metabolism, DNA repair, cell surface protein, cell signaling, cell growth, antimicrobial). The entire single-agent dataset has been submitted to the PubChem database (AID: 1259257) and is available in Supplementary Dataset S1. **B**, Violin plots representing data for two NAMPTis (daporinad, GMX-1778) and two PARPis (niraparib, olaparib) in four Ewing sarcoma cell lines (TC71, TC32, EW8, and RDES). Data represent the activity of these agents in Ewing sarcoma cell lines (pink) relative to 89 alternative cell lines (blue) in viability assays (48-hour CellTiterGlo) of the MIPE 4.0 small-molecule library of approved and investigational drugs (1,912 total agents). **C**, Complete dose-response curves for niraparib and daporinad in four

Ewing sarcoma cell lines (TC71, TC32, EW8, and RDES). **D**, A combination response plot representing 946 discrete drug synergy scores (as judged by the Excess HSA metric) from the primary 6×6 matrix screen. Examples of high-ranking drug synergies are imposed including the combination of the Bcl-xL/Bcl-2 inhibitor navitoclax in combination with the mTOR inhibitor AZD-8055 (rank #4) and niraparib and daporinad (rank # 116). **E**, 10×10 matrix plot for the combination of niraparib (0 to 10,000 nmol/L) and daporinad (0 to 50 nmol/L) in TC71 in both viability and Bliss format.

Figure 2.

Mechanism of activity depends on loss of NMN and NAD⁺. **A**, Schematic of the NAD⁺ salvage pathway showing site of action of NAMPT inhibition. **B**, Reproductions of the box-plot representations of the scaled intensity of specified biochemical levels within TC71 cells treated with vehicle (DMSO), niraparib (5 μmol/L), daporinad (5 nmol/L), or the combination of both drugs. Cells were collected after an acute (6 hour) or prolonged (24 hour) exposure to all four treatment scenarios. The full dataset is available in Supplementary Dataset S2. **C**, 10 × 10 matrix plots for the combination of niraparib (0 to 25,000 nmol/L) and GNE-618 (0 to 50 nmol/L) in TC71 with and without the addition of NMN (1 mM). **D**, Schematic of PARP-mediated DNA repair pathway showing sites of action of PARP inhibition and NAMPT inhibition. **E**, PAR levels in TC32 and TC71 cells with vehicle (DMSO), niraparib (5 μmol/L), daporinad (5 nmol/L), or the combination of both drugs.

Figure 3.

Exploration of the single-agent and combination consequences of PARP and NAMPT inhibition on Ewing sarcoma cells. **A**, Response plots of drug-induced changes to selected/captured analytes via a RPMA-based proteomics and phosphoproteomics examination. Data represent quantitation data (QMN CV) scores and selected signal changes are shown for key analytes following treatment of TC71 cells with a combination of niraparib (5 $\mu\text{mol/L}$) and daporinad (5 nmol/L) for 24 hours. The full dataset is available in Supplementary Dataset S3. **B**, A bar-chart representation of changing analyte levels for key proteins/phosphoproteins following treatment with niraparib (5 $\mu\text{mol/L}$) and daporinad (5 nmol/L) alone and in combination with data captured at 6 and 24 hours. **C**, Phospho-SAPK/JNK and phospho-p38 MAPK activity by Western blot in TC32 and TC71 cells treated with vehicle (DMSO), niraparib (5 $\mu\text{mol/L}$), daporinad (5 nmol/L), or the combination of both drugs for between 18 and 24 hours. **D**, Densitometry-based quantitation of Western blot bands.

Figure 4.

Dual PARP and NAMPT inhibition slows tumor growth and prolongs survival in Ewing sarcoma xenografts. Tumor volumes and Kaplan–Meier curves for **(A)** TC32-bearing mouse xenografts and **(B)** TC71-bearing mouse xenografts. Mice were treated with vehicle (blue), niraparib (pink), GNE-618 (green), or the combination (orange). **C**, PAR levels in TC32 and TC71 xenograft tumor samples treated with vehicle (DMSO), niraparib (50 mg/kg), GNE-618 (25 mg/kg), or the combination of both drugs. Tumors were harvested at day 4 of treatment.