Iniparib Nonselectively Modifies Cysteine-Containing Proteins in Tumor Cells and Is Not a Bona Fide PARP Inhibitor


Abstract

Purpose: PARP inhibitors are being developed as therapeutic agents for cancer. More than six compounds have entered clinical trials. The majority of these compounds are β-nicotinamide adenine dinucleotide (NAD⁺)-competitive inhibitors. One exception is iniparib, which has been proposed to be a noncompetitive PARP inhibitor. In this study, we compare the biologic activities of two different structural classes of NAD⁺-competitive compounds with iniparib and its C-nitroso metabolite.

Experimental Design: Two chemical series of NAD⁺-competitive PARP inhibitors, iniparib and its C-nitroso metabolite, were analyzed in enzymatic and cellular assays. Viability assays were carried out in MDA-MB-436 (BRCA1-deficient) and DLD1/C0/C0 (BRCA2-deficient) cells together with BRCA-proficient MDA-MB-231 and DLD1/+. Capan-1 and B16F10 xenograft models were used to compare iniparib and veliparib in vivo. Mass spectrometry and the ³H-labeling method were used to monitor the covalent modification of proteins.

Results: All NAD⁺-competitive inhibitors show robust activity in a PARP cellular assay, strongly potentiate the activity of temozolomide, and elicit robust cell killing in BRCA-deficient tumor cells in vitro and in vivo. Cell killing was associated with an induction of DNA damage. In contrast, neither iniparib nor its C-nitroso metabolite inhibited PARP enzymatic or cellular activity, potentiated temozolomide, or showed activity in a BRCA-deficient setting. We find that the nitroso metabolite of iniparib forms adducts with many cysteine-containing proteins. Furthermore, both iniparib and its nitroso metabolite form protein adducts nonspecifically in tumor cells.

Conclusions: Iniparib nonselectively modifies cysteine-containing proteins in tumor cells, and the primary mechanism of action for iniparib is likely not via inhibition of PARP activity.

Introduction

PARP-1 is the founding member of a family of proteins that share a catalytic PARP homology domain and are characterized by their ability to poly (ADP-riboseyl)ate protein substrates (1–3). Of the 18 PARP family members identified to date (1, 2), PARP-1 and PARP-2 are unique in exhibiting stimulation of catalytic activity in response to DNA damage (4). Activation of PARP-1 and PARP-2 is an immediate eukaryotic cellular response to DNA damage induced by a variety of stimuli including ionizing radiation, alkylating agents, and oxidants (2). Activated PARP-1/2 bind to DNA strand breaks, and covalently attach poly (ADP-ribose) to nuclear proteins including PARP-1 itself, histones, and transcription factors (1). The enzymatic product of PARP, poly (ADP-ribose) [PAR], is generated from its substrate β-nicotinamide adenine dinucleotide (NAD⁺) and consists of linear and branched ADP-ribose units of variable size (5).

A number of PARP inhibitors have entered clinical trials over the last several years to investigate the effectiveness of these agents in various oncology settings (6). Most of these small-molecule inhibitors are based on a nicotinamide-like pharmacophore, due to their mechanism as competitive binders to the NAD⁺ pocket within PARP-1/2 (6). The more advanced of these compounds display single-digit nanomolar IC₅₀ values for both PARP-1 and PARP-2 (6). The preclinical activity of several of these compounds has been described in detail (7–10). As expected, these agents interfere with the base excision DNA repair (BER) process, leading to enhanced tumor...
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Translational Relevance

PARP inhibitors represent the most clinically advanced examples of agents that specifically target DNA repair for the treatment of cancer. Clinical proof-of-concept for this approach has been reported with the activity of some of these agents in BRCA-deficient breast and ovarian cancers. In addition, significant potential exists for the use of these agents in combination with DNA-damaging agents in a variety of tumor types. However, different potential mechanisms of action for inhibition of PARP have been reported among these compounds. In particular, iniparib is reported to inhibit PARP by interacting with the zinc finger DNA-binding domain of PARP-1, rather than competitively interacting with the NAD$^+$-binding site of PARP-1/2. In the present study, we characterize iniparib in enzymatic, cellular, and in vivo assays and show that this compound does not appear to be a bona fide PARP inhibitor. These results have significant translational relevance with regard to the interpretation of the clinical results with iniparib relative to that observed with other PARP inhibitors.

cell killing when administered in combination with various types of DNA-damaging agents (7–9, 11). In particular, PARP inhibitors have been shown to significantly enhance the activity of alkylating agents such as temozolomide in multiple tumor types, due to the importance of BER in repairing adducts formed by these agents (8, 11). In addition, preliminary evidence for clinical activity of this combination has been reported (12).

In addition, PARP inhibitors have been shown to have single-agent activity in tumors that are defective in double-strand break (DSB) repair. The basis for this "synthetic lethality" stems from the fact that inhibition of BER leads to an accumulation of single-strand DNA damage that, in turn, results in an elevated level of DSB damage. In the absence of functional DSB repair capacity, the accumulation of DSB damage results in cell death. The most notable example of this phenomenon is in tumor cells lacking BRCA1 or BRCA2. Preclinical studies with multiple NAD$^+$-competitive PARP inhibitors showed robust tumor cell killing, both in vitro and in vivo, in tumors lacking functional BRCA activity (13, 14). Furthermore, clinical proof-of-concept for this synthetic lethality has been established in phase II studies with olaparib where overall response rates of 33% and 41% were reported in BRCA-deficient ovarian and breast cancer, respectively (15, 16).

In contrast, 1 purported inhibitor is distinct from the other described compounds in both structural class and proposed mechanism of action. The benzamide, iniparib, is reported to be a produg whose C-nitroso metabolite, 4-iodo-3-nitrosobenzamide, selectively kills tumor cells by oxidizing the zinc finger of PARP-1 resulting in ejection of zinc and inhibition of PARP activity (17). Iniparib or its metabolites have been reported to show single-agent activity in certain tumor cell lines, although preclinical activity in BRCA-deficient lines has not been reported (17, 18). In addition, combination activity has been reported in vivo, most notably with platinum and nucleoside analogues in models of triple-negative breast cancer (19, 20). A phase II study examining the combination of iniparib with gemcitabine and carboplatin in the triple-negative setting produced a significant 3.5-month improvement in overall survival relative to the cytotoxic regimen alone (21). However, activity in the first-line setting was not recapitulated in a recently completed phase III study; however, it is noteworthy that this agent was well-tolerated and that the compound did appear to show some activity in the second- and third-line setting (22).

In the present study, we directly compared the biologic activities of 2 different structural classes of NAD$^+$-competitive PARP inhibitors with iniparib and its C-nitroso metabolite. All of the potent competitive inhibitors showed activity in a PARP-driven cellular assay, potentiated the activity of temozolomide, and elicited cell killing in BRCA-deficient tumor cells, both in vitro and in vivo. Cell killing was associated with induction of DNA damage. In contrast, neither iniparib nor its C-nitroso metabolite inhibited PARP enzymatic or cellular activity, potentiated temozolomide, or showed activity in a BRCA-deficient setting. We find that the nitroso metabolite of iniparib is highly reactive, forming covalent interactions with many cysteine-containing proteins. Furthermore, both iniparib and its nitroso metabolite form protein adducts nonspecifically in tumor cells. These results indicate that the primary mechanism of action for iniparib is likely not via inhibition of PARP activity, which will have important implications for the clinical development of this compound.

Materials and Methods

Chemicals

Veliparib, cmpd A-C, iniparib, and iniparib-met were synthesized at Abbott Laboratories. Temozolomide was purchased from Dik Drug Co. All other chemicals were from Sigma.

Measurement of protein concentration

Protein concentrations were determined using the BCA method (Pierce).

Cell lines

DLD1 BRCA2$^{+/+}$ and DLD1 BRCA2$^{del11/del11}$ (designated as DLD$^{+/+}$ and DLD$^{+/+}$ cells, respectively) human isogenic cell lines were obtained from Horizon Discovery Ltd, Cambridge, UK and engineered using homologous recombination mediating recombinant adeno-associated virus (rAAV) gene-targeting vectors. MDA-MB-231, Capan-1, B16F10, and MDA-MB-436 cells were obtained from the American Type Culture Collection (ATCC).
PARP enzymatic assay

PARP-1 enzymatic assays were carried out for veliparib, cmpd-A, cmpd-B, cmpd-C, iniparib, and iniparib-met. PARP-2 assays were carried out for veliparib, cmpd-A, and cmpd-B. PARP enzymatic assays were conducted in buffer containing 50 mmol/L Tris pH 8.0, 1 mmol/L dithiothreitol (DTT), 1.5 μmol/L [1H]-NAD⁺ (1.6 μCi/mmol), 200 nmol/L biotinylated histone H1, 200 nmol/L DNA oligo (CACAATGTTGACATTCCCTTCGAGGTTAAAACC-TATGGAGAGGAATGGCAACTTTG), and 1 nmol/L PARP-1 or 4 nmol/L PARP-2 enzyme. Compounds were assayed as 11-point, 3-fold dilution series from 10 μmol/L to 170 μmol/L in 2% dimethyl sulfoxide (DMSO). Reactions were carried out in 100-μL volumes in white-well plates (Perkin Elmer). These reactions were terminated after 1 hour by the addition of 150 μL of 1.5 mmol/L benzamide (≥1,000-fold more than its IC₅₀). Aliquots of 170 μL of the stopped reaction mixtures were transferred to streptavidin Flash Plates, incubated for 16 hours, and counted using a TopCount microplate scintillation counter.

PARP cellular assay

C4-I cells (40,000 cells per well) were treated with veliparib, cmpd-A, cmpd-B, cmpd-C, and iniparib at 0, 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μmol/L for 30 minutes in a 96-well plate. PARP was then activated by damaging DNA with 1 mmol/L H₂O₂ for 10 minutes. The cells were then washed with ice-cold PBS once and fixed with pre-chilled MeOH/acetone (V/V: 7:3) at −20°C for 30 minutes. After air-drying, the plates were rehydrated with PBS and blocked with 5% non-fat dry milk in PBS–Tween-20 for 250 μL per well, respectively, in a 96-well plate and treated with temozolomide at 0, 0.003, 0.01, 0.03, 0.1, 0.3, or 1 mmol/L in the presence of 0, 0.005, 0.05, 0.5, or 5 μmol/L veliparib or iniparib for 5 days. After treatment, CellTiter-Glo was carried out according to the manufacturer’s instructions (Promega, Inc.).

γH2AX assay

DLD1+/+ and DLD1−/− cells (0.3 million per well) were treated with DMSO, 0.1 mmol/L temozolomide, 5 μmol/L veliparib, or 0.1 mmol/L temozolomide and 5 μmol/L veliparib for 30 minutes. Cells treated with DMSO or temozolomide alone were pretreated with DMSO for 30 minutes. Cells treated with veliparib alone or temozolomide/veliparib combination were pretreated with 5 μmol/L veliparib for 30 minutes. The cells were then washed and incubated in complete medium containing either DMSO (for DMSO or temozolomide alone) or 5 μmol/L veliparib (for veliparib alone or temozolomide/veliparib combination) for 1 or 6 hour(s). Cells were harvested and fixed with 250 μL of BD Cytofix/Cytoperm solution (BD Biosciences) at room temperature for 20 minutes followed by washing 3 times with 1XBD Perm/Wash Solution (BD Biosciences). After blocking with 3% bovine serum albumin for 30 minutes, the cells were incubated with the following antibodies in Perm/Wash Solution with washes in between: mouse anti-γH2AX antibody (1:250; Upstate Biotechnologies) and Alexa 488 goat anti-mouse immunoglobulin G (IgG) (H+L)F(ab')2 fragment conjugate (1:200; Invitrogen). The DNA was immunostained with 50 μg/mL propidium iodide (PI) in staining buffer [PBS without Mg²⁺ or Ca²⁺, 1% heat-inactivated fetal calf serum (FCS), 0.09% (w/v) sodium azide, pH 7.4–7.6] for 10 minutes in the dark. Immuno-flow cytometry was carried out to quantify the γH2AX signal. Each data point is the average of 2 values.

Colony formation assay

DLD1 BRCA2+/– and DLD1 BRCA2+/+Δex11/Δex11 cells (200 cells per well) were seeded into 6-well plates. The cells were treated with veliparib and iniparib at 0, 0.001, 0.01, 0.1, 1, and 10 μmol/L for 11 days. The colonies were stained with Giemsa stain according to the manufacturer’s instructions (Sigma). The total area of colonies was scored by counting the colonies using the GelCount System.

In vitro PARP auto-modification assay

The PARP-1 auto-modification assay was adapted from a method reported previously using the same double-stranded DNA sequence obtained from Integrated DNA Technologies (23). Briefly, PARP-1 was incubated with DMSO, 110 μmol/L iniparib, 110 μmol/L iniparib-met, or 110 μmol/L cmpd-B for 10 minutes followed by addition of double-stranded DNA for 20 minutes. NAD⁺ was subsequently added to the reaction mixture for an additional 15 minutes. All steps were carried out at room temperature and the reaction was halted by the addition of 90 mmol/L EDTA and Laemmli reducing sample buffer. Final concentrations of amide (BSO) for 5 days. DLD1+/+ and DLD1−− cells (0.3 million per well) were plated at a density of 1,000 cells per well in a 96-well plate and treated with temozolomide at 0, 0.003, 0.01, 0.03, 0.1, 0.3, or 1 mmol/L in the presence of 0, 0.005, 0.05, 0.5, or 5 μmol/L veliparib or iniparib for 5 days. After treatment, CellTiter-Glo was carried out according to the manufacturer’s instructions (Promega, Inc.).
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PARP-1, compound, DNA, and NAD+ were: 740 nmol/L, 110 µmol/L, 7 µmol/L, 5.5 mmol/L, respectively. An aliquot of 10 µL of the reaction mixture was loaded onto an SDS-PAGE gel. Results were visualized by Coomassie staining.

Recombinant protein expression

The cloning, expression, and purification of PARP-1 zinc finger domain-1 was carried out as described previously (24). The DNA sequence encoding amino acids 223 to 324 of Human La antigen was cloned into pAG5 vector with a C-terminal his-tag and expressed in BL21(DE3)/plays (25). The protein was purified with a Ni-NTA column.

Detection of covalent compound addition by mass spectrometry

To monitor covalent additions of compound to protein, 1 µmol/L protein was incubated with 200 µmol/L compound for 2 hours at room temperature in the presence of 50 mmol/L HEPES and 100 mmol/L KCl. Intact protein molecular weights were measured by mass spectrometry. Liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI-MS) was carried out using an Agilent 6510 QTOF MS coupled to an Agilent 1100 capillary HPLC loading pump and a 1200 nano-HPLC gradient pump with a Chip Cube (40 nL enrichment column, 43 mm x 75 µm analytical column; Zorbas 300SB-C8). The samples were loaded with 0.1% formic acid in 4.5% acetonitrile with a flow rate of 4 µL/min. The gradient nano-pump used composition A (0.1% formic acid in 97% water and 3% acetonitrile) and composition B (0.1% formic acid in 97% acetonitrile) with a flow rate of 400 nL/min. The nanopump gradient was set to 3% to 75% B for 2 minutes, held at 75% B for 5 minutes, 75% to 3% B for 5.5 minutes, and held at 3% B for 8 minutes. Data were acquired with MassHunter B.02.00 and deconvoluted with MassHunter B.04.00.

Capan-1 xenograft tumor study

For this study, C.B.-17 severe combined immunodeficient mice (SCID) female mice were obtained from Charles River. Mouse body weight at initiation of therapy was ~21 g. Capan-1 brei was prepared by homogenizing 30 grams of tumor tissue with 30 mL media. Matrigel was added to the mixture (1:1) to complete the brei. An inoculation volume of 0.2 mL was injected into the right hind flank of the C.B.-17 SCID female mice on day 0. Single-agent therapy was administered per os, twice a day × 14 days for veliparib and intraperitoneal (i.p.), twice a day × 14 days for iniparib starting from day 25. Tumor volume was calculated twice weekly. Measurements of the length (L) and width (W) of the tumor were taken via electronic caliper and the volume was calculated according to the following equation: V = L × W²/2 using Study Director version 1.7.39 (Studylog Systems, Inc.). The percent tumor growth inhibition (% TGI) was calculated using the formula 100 − % TGI/C (drug-treated/vehicle-treated tumor volume × 100). Data were analyzed using the Student t test for differences in T/C values (StatView; SAS Institute).

B16F10 xenograft tumor study

C57BL/6 female mice were obtained from Charles River. Mouse body weight at initiation of therapy was ~22 g. A total of 6 × 10⁴ viable B16F10 cells were inoculated subcutaneously into the right flank of female C57BL/6 mice on day 0. The injection volume was 0.1 mL and comprised a 1:1 mixture of Gibco Minimum Essential Medium (S-MEM) and Matrigel. Veliparib was administered per os twice a day × 5 days and iniparib was administered per os twice a day × 5 days starting from day 7. Temozolomide was administered per os every day × 5 days. Tumor volume was calculated 3 times weekly. Tumor volume calculation, % TGI calculation, and data analysis are the same as in the Capan-1 xenograft tumor study.

Synthesis of ³H-iniparib and ³H-iniparib-met

Iniparib was radiolabeled by catalytic tritium/hydrogen isotope exchange using tritium gas and Crabtree’s catalyst according to the method described by Hesk and colleagues (26).

Results

Compound activity in PARP enzymatic and cellular assays

The structures of the compounds used in this study are shown in Fig. 1A. Veliparib, cmpd-A, and cmpd-B are all benzimidazole carboxamide (benzimidazole)-based compounds and cmpd-C is a tetrahydropyridopyridazinone (pyridazinone). Iniparib and its C-nitroso metabolite (iniparib-met) are of the benzamide class. To evaluate the ability of each of these compounds to directly inhibit PARP enzymatic activity, PARP-1 and PARP-2 enzymatic assays were carried out using a DNA-dependent radiometric activity assay. The nature of this assay allows for detection of PARP inhibition by compounds that are NAD⁺ competitive as well as compounds such as iniparib that have been hypothesized to interact with the PARP-1 zinc finger. As shown in Table 1, the benzimidazole and pyridazinone inhibitors exhibit single-digit nanomolar concentrations of IC₅₀ for both PARP-1 and PARP-2, whereas neither iniparib nor iniparib-met showed any significant activity against PARP-1.

To examine PARP inhibition in a cellular context, we assayed the ability of compounds to inhibit PAR formation following H₂O₂ stimulation in C4-I cells. H₂O₂ treatment activates PARP-1 resulting in an elevated PAR signal to background that enables robust detection of PARP-1 inhibition in cells (27). Consistent with their potent enzymatic activities, veliparib and cmpds A–C showed robust, single-digit nanomolar EC₅₀ concentrations in the cellular assay. In contrast, neither iniparib nor iniparib-met were able to effectively inhibit PARP-1 enzymatic activity and iniparib was also inactive in the PARP cellular assay (Table 1).

One of the properties of PARP-1 is its ability to poly-(ADP-ribosyl)ate itself (23, 24). As another measure of the ability of compounds to inhibit PARP activity, we examined auto-ribosylation of purified PARP-1 in the presence of...
double-stranded DNA. In the presence of NAD+, the potent NAD+-competitive cmpd-B completely inhibited auto-ribosylation (Fig. 1B; visualized as a single band). In contrast, a smear was observed in samples treated with iniparib, iniparib-met, or the DMSO control, indicating that iniparib and iniparib-met were unable to inhibit PARP-1 auto-ribosylation to any measurable extent (Fig. 1B).

**Single-agent activity in BRCA-deficient tumor cells**

Inhibition of PARP activity, by any mechanism, should result in compromised BER activity that, in turn, leads to synthetic lethality in DSB repair–deficient cells (13, 14). To examine this, we used 2 breast cancer cell lines, MDA-MB-436 and MDA-MB-231, with different DSB repair capacity. MDA-MB-436 cells have a mutation in the splice donor site of exon 20 of BRCA1, resulting in defective transcripts (28), whereas MDA-MB-231 cells have wild-type BRCA function (29). In 9-day proliferation assays, veliparib, cmpd-A, and cmpd-B inhibited MDA-MB-436 cells with EC₅₀ of 96, 105, and 550 nmol/L, respectively (Fig. 2A), whereas iniparib and iniparib-met showed no significant effects on proliferation at concentrations as high as 10 μmol/L (Fig. 2A). None of the compounds showed significant antiproliferative activity in the BRCA wild-type MDA-MB-231 cell line at concentrations as high as 10 μmol/L (Fig. 2B).

Because glutathione transferase (GSH) activity can potentially deactivate the C-nitroso metabolite of iniparib, it has been reported that the cellular cytotoxicity of iniparib-met can be enhanced by co-treatment with the GSH inhibitor BSO (17). To determine if the cellular activity of iniparib and iniparib-met was attenuated by glutathione transferase activity, we examined the antiproliferative activity of these compounds in the presence or absence of BSO in MDA-MB-436 and MDA-MB-231 cells. In MDA-MB-436 cells, the addition of BSO to iniparib-met did not lead to any appreciable decrease in cell proliferation (Fig. 2D); however, a modest effect was observed when BSO was added to the cells treated with 10 μmol/L iniparib (Fig. 2C). The addition of BSO to iniparib and iniparib-met produced a more significant effect on proliferation in MDA-MB-231 cells; however, BSO alone also appeared to produce some cytotoxicity in these cells (Fig. 2E and F).

To more rigorously evaluate the activity of these compounds in a DSB repair–deficient setting, we used an isogenic pair of tumor cell lines in which 1 line (DLD1 BRCA2⁺/⁺) is functional for BRCA2 activity whereas the other (DLD1 BRCA2³⁰⁵¹⁵¹/³⁰⁵¹) is defective in BRCA2 activity (henceforth referred to as DLD1⁻/⁻ and DLD1⁻/⁻ cells, respectively; ref. 30). In colony formation assays, veliparib was found to inhibit proliferation of the BRCA2-defective DLD1⁻/⁻ cell line with an EC₅₀ of 0.04 μmol/L, whereas no effect was observed in the BRCA2 wild-type DLD1⁺/⁺ cell line (Fig. 2G). In contrast, iniparib had no effect on either cell line at concentrations of up to 10 μmol/L (Fig. 2H).

**Combination activity with temozolomide**

Because PARP-1/-2 plays critical roles in BER, inhibition of PARP-1/-2 should enhance the efficacy of cancer therapeutics that cause DNA damage requiring BER-mediated...
repair. Temozolomide is a monofunctional DNA alkylating agent that induces DNA damage requiring BER-mediated repair and is used for the treatment of melanoma and glioma. Numerous preclinical studies have shown the ability of PARP inhibitors to potentiate the activity of temozolomide in both in vitro and in vivo model settings (7, 8, 11). This activity is particularly pronounced when used in tumors defective in DSB repair (8, 31).

We, therefore, examined the ability of these compounds to enhance the activity of temozolomide in BRCA-defective MDA-MB-436 cells and a DLD1+/− and DLD1+/− isogenic pair as a means of exploring PARP inhibitor-mediated combination activity.

As shown in Fig. 3A–E, veliparib, cmpd-A, and cmpd-C were able to potentiate temozolomide (5.2-, 4.2-, and 2.4-fold, respectively) at concentrations as low as 50 nmol/L in MDA-MB-436 cells in 5-day proliferation assays, whereas iniparib and iniparib-met did not show any enhancement of temozolomide cytotoxicity when used at concentrations of up to 5 μmol/L.

In the DLD1 isogenic pair, veliparib was able to potentiate the activity of temozolomide in both BRCA2 wild-type and BRCA2 mutant cells, with the effect being more pronounced in the mutant cells, requiring a lower concentration of veliparib (50 nmol/L vs. 5 μmol/L; Fig. 3F and G). In contrast, no enhancement of temozolomide activity was observed when iniparib or iniparib-met was used in BRCA2 wild-type cells (Fig. 3H).

In conclusion, our findings suggest that PARP inhibitors can be effective in enhancing the activity of temozolomide in tumors with defects in DSB repair, particularly in BRCA-deficient cells. These results have important implications for the development of combination therapies in cancer treatment.
observed in either cell line with concentrations of iniparib as high as 5 μmol/L (Fig. 3H and I). In addition, we have examined both temozolomide–veliparib and temozolomide–iniparib in HR-competent cell lines including MDA-MB-231 and HCT116. In all cases, veliparib, but not iniparib, potentiated the toxicity of temozolomide (data not shown).

Induction of γH2AX is a widely used measure of DSB formation and we have shown previously that cell cytotoxicity is correlated with the induction of γH2AX signal in HCT116 cells treated with veliparib plus temozolomide (11, 32). We used this same approach to monitor the induction of DNA damage in the DLD1 BRCA2 isogenic pair. As expected, DLD1+/− cells have a higher background

Figure 3. Combination activity with temozolomide (TMZ). A–E, MDA-MB-436 cells (4,000 cells per well) were treated with indicated concentrations of temozolomide and the indicated concentrations of veliparib (A), cmpd-A (B), cmpd-C (C), iniparib (D), and iniparib–met (E) for 5 days. CellTiter-Glo assays were carried out as described in Materials and Methods.

F–I, DLD1+/+(F and H) or DLD1−/− cells (G and I) were plated into 96-well plates (1,000 cells per well) and treated with the indicated concentration of temozolomide and the indicated concentration of veliparib (F and G) or iniparib (H and I) for 5 days. CellTiter-Glo assay was carried out as described in Materials and Methods (▪, 0 μmol/L; ■, 0.005 μmol/L; ▲, 0.05 μmol/L; □, 0.5 μmol/L; ○, 5 μmol/L).
Treatment with veliparib alone did not induce a significant level of γH2AX signal than DLD1+/− because of the deficiency in DSB repair (Fig. 4A). When examined 1 hour after treatment, temozolomide or veliparib alone did not significantly induce γH2AX signal in either DLD1+/+ or DLD1−/− cells, whereas temozolomide/veliparib induced γH2AX signal in both lines (Fig. 4A). DLD1+/− cells were able to repair DSBs after a 6-hour recovery as indicated by a return of γH2AX signal to baseline levels (Fig. 4B). In contrast, γH2AX levels remained elevated in DLD1+/− cells up to 6 hours after treatment (Fig. 4B). This result indicates that the enhanced cell killing produced by the addition of veliparib to temozolomide is associated with increased DNA damage due to attenuated BER capacity. This effect is significantly more pronounced in cells that are also compromised for DSB repair activity. In this experiment, treatment with veliparib alone did not induce a significant γH2AX signal in DLD1−/− cells because of the fact that these cells were treated for only 1 hour. Treatment of DLD1+/− cells with 10 μmol/L veliparib for 72 hours resulted in a significant increase in γH2AX signal (22% vs. 5% for DMSO control). In contrast, no effect was observed with iniparib treatment under similar conditions (5% vs. 5% for DMSO control; data not shown).

Activity in xenograft tumor models

To extend our in vitro analyses of these compounds to the in vivo setting, we examined the activity of several compounds in 2 xenograft models that have been extensively characterized for their response to PARP inhibitors. Capan1 is a BRCA2-deficient human pancreatic cancer cell line in which NAD+/−-competitive PARP inhibitors, olaparib and AG014699, were reported to show significant single-agent activity in both tissue culture and tumor xenograft models (29, 33). When given orally at doses of 200 mg/kg/d (100 mg/kg/d, 24 daily) for 14 days, veliparib produced a significant 53% TGI at day 59 compared with the vehicle group in the capan-1 model (Fig. 5A). Various doses and schedules have been reported for the use of iniparib in preclinical models, most typically using a dose of 50 mg/kg/d given intraperitoneally on an intermittent dosing schedule (19, 20). Consistent with previous reports of rapid and extensive metabolism of iniparib in vivo, we were unable to detect any presence of the parent molecule in mouse pharmacokinetic studies (data not shown). In tolerability studies in C.B.-17 SCID mice, doses greater than 100 mg/kg/d, once daily, i.p., were not tolerated. In the capan-1 model, no significant tumor growth inhibition was observed when iniparib was given at the maximum tolerated dose of 100 mg/kg/d (i.p., 50 mg/kg/d, twice daily) for 14 days compared with the vehicle control (16% TGI at day 59; Fig. 5A).

We and other researchers have shown previously that PARP inhibitors can effectively potentiate the activity of temozolomide in various xenograft models (7, 8, 31, 34). In addition, the doses of veliparib that are sufficient to produce antitumor activity have been shown to be pharmacodynamically active, leading to significant inhibition of PAR levels in vivo (8, 31, 35, 36). Consistent with our previous reports, temozolomide monotherapy at 50 mg/kg/d (i.p., once daily for 5 days) produced significant tumor growth inhibition (45% TGI at day 14) when compared with the vehicle group (Fig. 5B). Veliparib, given orally at 25 mg/kg/d (12.5 mg/kg/d, twice daily) for 5 days, in combination with temozolomide, was able to significantly enhance tumor growth inhibition (58% TGI at day 14) relative to temozolomide monotherapy (Fig. 5B). Similar effects were observed for cmpd-A (61% TGI at day 15 at 30 mg/kg/d), cmpd-B (49% TGI at day 14 at 10 mg/kg/d), and cmpd-C (57% TGI at day 14 at 60 mg/kg/d; data not shown). In contrast, iniparib, given intraperitoneally at doses 100, 50, or 25 mg/kg/d (50, 25, or 12.5 mg/kg/d, twice daily) for 5 days in combination with temozolomide did not produce significant improvement in antitumor activity relative to temozolomide monotherapy (9, 9, and 0% TGI, respectively, at day 14; Fig. 5B).
The data described above indicate that neither iniparib nor its C-nitroso metabolite shows the biologic activity that would be expected from inhibition of PARP function. The structure of iniparib-met indicates that this molecule is highly reactive and likely to interact nonspecifically with many proteins containing cysteine residues. To test this, we incubated iniparib-met with several proteins containing cysteine residues. To test this, we incubated iniparib-met with several proteins containing cysteine residues. Although we cannot rule out that iniparib and iniparib-met react nonspecifically with many cysteine-containing proteins beyond PARP-1, this appears to be a nonspecific interaction because iniparib-met is equally capable of interacting with many other cysteine-containing proteins beyond PARP-1.

Iniparib and iniparib-met form nonspecific protein adducts in tumor cells

To confirm that iniparib and iniparib-met form protein adducts nonspecifically in cells, we incubated 3H-labeled iniparib and iniparib-met with MDA-MB-436 and MDA-MB-231 cells in the presence or absence of BSO. The cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. 3H-labeled proteins were visualized by fluorography. As shown in Fig. 7, both iniparib and iniparib-met were able to form multiple protein adducts in cells. BSO did not significantly enhance the protein adduct formation under the current experimental conditions for both iniparib and iniparib-met. Western blot analysis indicated that PARP-1 was present in the cell lysate and was not a major protein to form adducts with either iniparib or iniparib-met (Fig. 7). In addition, we observed that more protein adducts were formed for iniparib than iniparib-met, indicating that iniparib-met may not be the sole mechanistic mediator for iniparib activity in cells (Fig. 7). Together, our data indicate that iniparib and iniparib-met react nonspecifically with many cysteine-containing proteins. Although we cannot rule out that iniparib or iniparib-met interacts with PARP-1 in tumor cells, we were unable to detect a significant interaction in MDA-MB-436 or MDA-MB-231 tumor cells.

Discussion

Many established cancer therapies involve DNA-damaging chemotherapy or radiotherapy. PARP-1 and PARP-2 are nuclear enzymes that are activated by DNA damage and play a critical role in base excision repair (BER) that occurs in response to damage induced by many such therapeutic modalities. As such, inhibition of PARP represents an attractive approach for the treatment of cancer for use in combination with DNA-damaging agents and as a monotherapy in tumor settings deficient in complimentary DNA-repair processes (7, 8, 13, 14).

As an attractive therapeutic target, multiple compounds are being developed to modulate PARP activity. The
Figure 6. Iniparib-met reacts nonspecifically with proteins containing cysteine residues: 1 µmol/L of the indicated proteins were incubated with 200 µmol/L of the indicated compounds for 2 hours at room temperature in the presence of 50 mmol/L HEPES and 100 mmol/L KCl. Intact protein molecular weights were measured by mass spectrometry.
majority of such compounds are expected to act by an NAD\textsuperscript{\textast} -competitive mechanism targeting both PARP-1 and PARP-2. Iniparib is structurally and mechanistically distinct. Its active metabolites are purported to covalently interact with the zinc finger of PARP-1, disrupting its interaction with DNA, and, thereby, specifically targeting PARP-1 (17). However, regardless of the mechanism of action, inhibition of PARP-1 function should result in compromised BER activity with the accompanying biologic responses associated with disruption of this DNA-repair process.

At least 6 PARP compounds are in various stages of clinical development for oncology, with several other compounds in preclinical development (6). Promising preliminary clinical data has been reported in combination with temozolomide, topoisomerase inhibitors, and cytoxan in phase I studies (12, 37, 38). In addition, evidence for single-agent activity has been reported in BRCA-deficient tumors in phase I/II studies with both olaparib and MK4827 (15, 16, 39, 40). A phase II study with iniparib in triple-negative breast cancer indicated very promising activity in combination with gemcitabine and carboplatin (21). However, activity in the first-line setting could not be confirmed in a subsequent phase III study; although the compound was well tolerated and did appear to show some activity in the second- and third-line settings (22).

To investigate potential differences in fundamental mechanisms and, therefore, potential differences in clinical use for the different classes of PARP compounds, in this study, we characterize and compare their respective activities in multiple established PARP-specific assays. Specifically, we used a variety of enzymatic, cellular, and in vivo assays to compare the activity of 2 distinct structural classes of NAD\textsuperscript{\textast} -competitive PARP inhibitors with the benzamidazole, iniparib, and its C-nitroso metabolite, 4-iodo-3-nitrosobenzamide. The NAD\textsuperscript{\textast} -competitive benzamidazole- and pyridazinone-based compounds displayed the expected biologic activity associated with PARP inhibition including potent activity in established enzymatic and cellular PARP assays. In addition, these compounds induced synthetic lethality in tumor cells with both endogenous and exogenous BRCA mutations. However conversely, neither iniparib nor iniparib-met showed any of the expected biologic activity associated with inhibition of PARP.

Inhibition of PARP activity is known to enhance the activity of various classes of DNA-damaging agents (7, 8). Potentiation of alkylating agents such as temozolomide is perhaps the best-characterized example of this effect, with robust preclinical activity reported for numerous PARP inhibitors (8, 31) and preliminary evidence of clinical activity has also been reported (12, 41). Consistent with our previously published work in HCF-116 cells, we showed that the addition of veliparib significantly enhanced the activity of temozolomide in the DLD1–BRCA isogenic pair and that these antiproliferative effects were associated with sustained DNA damage in the presence of veliparib (Figs. 3 and 4). This effect was more pronounced in the BRCA-deficient tumor cell line compared with the BRCA wild-type line (Fig. 3). Consistent with this finding, recently reported phase I/II clinical data indicate that the combination of veliparib plus temozolomide is also active in BRCA-deficient breast cancer (41). In contrast, iniparib was unable to significantly potentiate the activity of temozolomide in either cell line when used at concentrations as high as 5 \textmu m\textsuperscript{-}L\textsuperscript{-}1 (Fig. 3). Moreover, no evidence of enhanced DNA damage, as indicated by increased \textgamma\textsuperscript{-}H2AX signal, was observed on treatment of cells with 5 \textmu m\textsuperscript{-}L\textsuperscript{-}1 iniparib in combination with temozolomide or 10 \textmu m\textsuperscript{-}L\textsuperscript{-}1 as single agent in MDA-MB-346 cells (data not shown). Together, these data indicate that iniparib is mechanistically distinct from NAD\textsuperscript{\textast} -competitive PARP inhibitors with regard to use in combination with DNA-damaging agents or in the context of BRCA deficiency.

To extend the analysis to an in vivo setting, we investigated the activity of these compounds in capan-1 and B16F10 xenograft models in which PARP inhibitor responses have been well characterized. Capan-1 is a human pancreatic cancer cell line with BRCA2 deficiency and the NAD\textsuperscript{\textast} -competitive PARP inhibitors olaparib and AG014699 have both been reported previously to show significant single-agent activity in this cell line (29, 33). Consistent with reported sensitivity to PARP inhibition, veliparib showed significant efficacy in the capan-1 model (Fig. 5). B16F10 murine melanoma represents a clinically relevant cancer
that is relatively resistant to most chemotherapeutics including temozolomide. Consistent with our previous data, veliparib and several other NAD⁺-competitive compounds significantly potentiated temozolomide efficacy in the B16F10 model (8). In contrast, iniparib did not show significant activity in either model (Fig. 5).

Taken together, our data indicate that iniparib is not a PARP inhibitor and the clinical activity of this compound is mechanistically distinct from that of the NAD⁺-competitive class of molecules. Indeed, iniparib has been hypothesized to interact with targets beyond PARP that may account for the reported activity of this compound (42–45). The reactive nature of the iniparib-met molecule indicates that nonspecific modification of cysteine-containing proteins is likely to occur with this compound. Accordingly, assessment of biochemical reactivity by mass spectrometry detected covalent compound addition not only to the zinc finger domain of PARP-1 but also to several other cysteine-containing proteins as well (Fig. 6). Moreover, this general reactivity also pertains to the cellular context where iniparib and iniparib-met were observed to form multiple protein adducts. Importantly, adducts of PARP-1 were not prominent in this study despite the purported mechanism of these compounds and the cellular abundance of PARP-1 protein (Fig. 7).

If iniparib does not functionally inhibit PARP activity in tumor cells, what is the explanation for the reported preclinical and clinical activity of this molecule? We propose that the reported activity of iniparib may result from nonselective modification of numerous proteins. The formation of such nonspecific adducts could alter the stability, activity, and/or localization of the respective proteins resulting in a cellular insult capable of inducing any of various cellular responses including, but not limited to, apoptosis, stress, cell-cycle perturbation, or DNA damage. It is plausible that such responses might sensitize the effected cells to cytotoxic therapy such as that observed with iniparib clinically. This nonspecific mechanism of action is supported by the ability of iniparib and iniparib-met to form protein adducts with several cysteine-containing proteins, both purified and in a cellular context. This type of mechanism is somewhat analogous to the S-nitrosylation of proteins by nitric oxide. In this signal transduction paradigm, specificity does not result from specific interactions between nitric oxide and target proteins but rather from localized production of nitric oxide by nitric oxide synthase followed by nonspecific nitrosylation of reactive cysteines in the vicinity (46).

Indeed, numerous proteins with reactive cysteines have been shown to be S-nitrosylated (46). Perhaps not coincidentally, 2 proteins that are modified by iniparib-met, PARP-1 and GAPDH, have been shown to be targets of S-nitrosylation (47).

These data have important implications for how these compounds are developed clinically. Although there are no current active clinical trials with iniparib in a specifically selected BRCA population, self-selection for BRCA carriers may be expected to occur in iniparib trials because of the widely understood activity of PARP inhibition in the BRCA mutant setting. Because iniparib appears to function primarily through a mechanism unrelated to PARP inhibition, trials including BRCA-deficient patients or combinations designed to exploit inhibition of BER activity may not represent the most effective clinical application. In addition, our data raise concerns about the use of exclusion criteria based on the classification of iniparib as a PARP inhibitor that may prevent patients who have been treated previously with iniparib to receive subsequent treatment with bona fide PARP inhibitors.

In summary, our results clearly show that iniparib acts through a distinct mechanism from classical NAD⁺-competitive PARP inhibitors such as veliparib. As such, it is likely that clinical use, including potential therapeutic combinations, responsive patient populations, and appropriate clinical biomarkers, will not be the same between these distinct drug classes. Therefore, care should be taken not to translate clinical findings across studies using NAD⁺-competitive PARP inhibitors with those using iniparib.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Xuesong Liu, Yan Shi, David X. Maag, et al.


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