Failure of Iniparib to Inhibit Poly(ADP-Ribose) Polymerase

In Vitro

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Abstract

Purpose: Poly(ADP-ribose) polymerase (PARP) inhibitors are undergoing extensive clinical testing for their single-agent activity in homologous recombination (HR)-deficient tumors and ability to enhance the action of certain DNA-damaging agents. Compared with other PARP inhibitors in development, iniparib (4-iodo-3-nitrosobenzamide) is notable for its simple structure and the reported ability of its intracellular metabolite 4-iodo-3-nitrosobenzamide to covalently inhibit PARP1 under cell-free conditions. The present preclinical studies were conducted to compare the actions iniparib with the more extensively characterized PARP inhibitors olaparib and veliparib.

Experimental Design: The abilities of iniparib, olaparib, and veliparib to (i) selectively induce apoptosis or inhibit colony formation in HR-deficient cell lines, (ii) selectively sensitize HR-proficient cells to topoisomerase I poisons, or (iii) inhibit formation of poly(ADP-ribose) polymer (pADPr) in intact cells were compared.

Results: Consistent with earlier reports, olaparib and veliparib selectively induced apoptosis and inhibited colony formation in cells lacking BRCA2 or ATM. Moreover, like earlier generation PARP inhibitors, olaparib and veliparib sensitized cells to the topoisomerase I poisons camptothecin and topotecan. Finally, olaparib and veliparib inhibited formation of pADPr in intact cells. In contrast, iniparib exhibited little or no ability to selectively kill HR-deficient cells, sensitize cells to topoisomerase I poisons, or inhibit pADPr formation in situ. In further experiments, iniparib also failed to sensitize cells to cisplatin, gemicitabine, or paclitaxel.

Conclusions: While iniparib kills normal and neoplastic cells at high (>40 μmol/L) concentrations, its effects are unlikely to reflect PARP inhibition and should not be used to guide decisions about other PARP inhibitors. Clin Cancer Res; 18(6); 1655–62. ©2012 AACR.

Introduction

The concept of synthetic lethality centers on targeting 2 separate molecular pathways that are nonlethal when disrupted individually, but are lethal when inhibited simultaneously. This approach is increasingly being used to guide the development of targeted anticancer therapies (1, 2). For example, based on the observation that PARP inhibitors are selectively toxic to cells lacking homologous recombination (HR) proteins such as BRCA1, BRCA2, and ATM (3–5), PARP inhibitors are being tested in tumors harboring BRCA1 or BRCA2 mutations (2, 6, 7). Recent results have shown that the PARP inhibitor olaparib induces partial or complete remissions in 41% of advanced breast and 33% of recurrent ovarian cancers in BRCA1/2 mutation carriers (8, 9). In light of these promising results, there has been substantial effort to develop olaparib, veliparib, and a variety of other third-generation PARP inhibitors as anti-neoplastic agents (reviewed in refs. 2, 6, 7, 10, 11).

Among the PARP-directed agents currently under development, iniparib is the furthest along in clinical testing. A simple mimic of nicotinamide (Fig. 1), this agent was originally described as the prodrug of 4-iodo-3-nitrosobenzamide, an agent that covalently inhibits PARP1 by binding to its first zinc finger under cell-free conditions (12). Subsequent studies suggested that iniparib exhibits single-agent activity in triple-negative breast cancer lines and enhances the cytotoxicity of cisplatin and gemicitabine (13). A phase II clinical trial suggested that iniparib not only is well tolerated when administered with gemicitabine and carboplatin, but also increases the response rate of the gemicitabine/carboplatin regimen in patients with triple-negative breast cancer (14). A subsequent phase III trial, however, failed to reproduce these results, raising questions about the future of PARP inhibition as a therapeutic strategy (15).
PARP inhibitors are currently undergoing extensive clinical testing. Recent negative results with one putative PARP inhibitor, iniparib, have raised concerns about the usefulness of inhibiting PARP to treat cancer. The present study shows that iniparib fails to exhibit hallmark features of PARP inhibitors, including ability to selectively kill homologous recombination–deficient cells or inhibit synthesis of pADPr in intact cells. These results raise questions about the classification of iniparib as a PARP inhibitor and encourage caution in making decisions about the utility of PARP inhibitors based on clinical trials of iniparib.

Recent studies from our laboratory showed that veliparib and olaparib, which are active site-directed noncovalent PARP inhibitors, selectively kill HR-deficient ovarian and pancreatic cells by causing activation of the error-prone nonhomologous end-joining DNA repair pathway (16). In anticipation of experiments to determine whether covalent modification of PARP1 by 4-iodo-3-nitrosobenzamide kills in a similar fashion, we examined the cytotoxicity of iniparib in HR-deficient cells. Surprisingly, we observed that iniparib exhibited little selectivity for HR-deficient cells. Further studies failed to show the ability of iniparib to sensitize cells to topoisomerase I poisons or inhibit pADPr synthesis in situ, two other hallmarks of PARP inhibitors. Collectively, these studies argue against the possibility that iniparib is inducing cytotoxicity by inhibiting PARP.

Materials and Methods

Materials
Veliparib (ABT-888) was purchased from Enzo Life Sciences; olaparib (AZD2281), iniparib (BSI-201), and VE-821 were from ChemieTek; and camptothecin, gemcitabine, paclitaxel, propidium iodide (PI), etoposide, bovine serum albumin, and gelatin from Sigma-Aldrich. Topotecan was provided by the Drug Synthesis Branch of the National Cancer Institute (Bethesda, MD). AZD 7762 was a kind gift from the Cancer Institute (Bethesda, MD). AZD 7766 and GM16667 human fibroblast lines from the Coriell Institute (Camden, NJ) were cultured in DMEM with 100 μg/mL hygromycin. PEO1 and PEO4 cells (18) from F. Couch (Mayo Clinic) were cultured in DMEM containing 100 μmol/L nonessential amino acids and 10 μg/mL insulin. SKOV3 cells (V. Shridhar, Mayo Clinic) were cultured in McCoy’s 5A. All media contained 10% heat inactivated FBS, 40 units/mL penicillin G, 40 μg/mL streptomycin, and 1 mmol/L glutamine. Lines were genotyped shortly before acquisition and were reinitiated every 2 to 3 months from stocks that were cryopreserved immediately after receipt from the indicated sources.

Apoptosis assays
Cells plated at 5 × 10⁴ cells per 60-mm dish were allowed to adhere for 24 hours, then treated with veliparib, olaparib, or iniparib in 0.1% (v/v) dimethyl sulfoxide for 4 days (olaparib) or 6 days (veliparib, iniparib) based on preliminary time course experiments. At the end of treatment, adherent cells were recovered by trypsinization, combined with cells in the supernatant, sedimented at 150 × g for 10 minutes, washed in ice-cold calcium- and magnesium-free Dulbecco’s phosphate buffered saline (PBS), and fixed at 4°C by dropwise addition of ethanol to a final concentration of 50% (v/v). Cells were subsequently rehydrated in cold PBS, sedimented, resuspended in 300 μL 0.1% (w/v) sodium citrate containing 1 mg/mL RNase A, incubated for 15 minutes at 37°C, diluted with 300 μL 0.1% sodium citrate containing 100 μg/mL PI, incubated in the dark at 20°C for 15 minutes, and analyzed (20,000 events) on a FACS Canto II flow cytometer (Becton Dickinson) using excitation and emission wavelengths of 488 and 617 nm, respectively. After data were analyzed with Becton Dickinson CellQuest software, normalized apoptosis was calculated as (observed – baseline)/(100 – baseline) to correct for differences in basal apoptosis rates between cell lines. Results are representative of at least 3 independent experiments.

Colony-forming assays
Wild-type and Atm⁻/⁻ MEFs were plated at 500 cells per dish in 60-mm dishes containing medium A, allowed to adhere overnight, and treated with the indicated agents

Translational Relevance
PARP inhibitors are currently undergoing extensive clinical testing. Recent negative results with one putative PARP inhibitor, iniparib, have raised concerns about the usefulness of inhibiting PARP to treat cancer. The present study shows that iniparib fails to exhibit hallmark features of PARP inhibitors, including ability to selectively kill homologous recombination–deficient cells or inhibit synthesis of pADPr in intact cells. These results raise questions about the classification of iniparib as a PARP inhibitor and encourage caution in making decisions about the utility of PARP inhibitors based on clinical trials of iniparib.

Figure 1. Structures of nicotinamide and the 3 drugs used in this study.
Results

Inability of iniparib to selectively kill HR-deficient cells

Previous studies have identified important cellular effects of PARP inhibitors, including selective toxicity in HR-deficient cells (3–5, 16, 21, 22), synergistic cytotoxicity when combined with topoisomerase I poisons (20, 23–27), and ability to inhibit pADPr formation in cells with damaged DNA. In the present study, we compared 3 of the agents currently undergoing clinical testing (Fig. 1) in assays of these effects.

To compare the ability of these agents to selectively induce apoptosis in HR-deficient cells, BRCA2-deficient PEO1 human ovarian cancer cells and their BRCA2-revertant PEO4 counterparts (18, 21) were incubated with olaparib, veliparib, or iniparib, then stained with PI and subjected to flow cytometry. As depicted in Fig. 2A and summarized in Fig. 2B, PEO1 cells were much more sensitive to olaparib and veliparib than the PEO4 cells. In contrast, iniparib exhibited much less selectivity (Fig. 2A and B, right). Similar results were observed when the cells were stained with Hoechst 33258 and examined for apoptotic morphologic changes (Supplementary Fig. S1).

In further experiments, antiproliferative effects of the 3 agents were compared in colony-forming assays. This assay likewise showed that veliparib and olaparib exhibited selectivity for the BRCA2-deficient PEO1 cells (Fig. 2C, left and middle), whereas iniparib exhibited no selectivity (Fig. 2C, right).

To ensure that these observations were not unique to PEO1 and PEO4 cells, we also examined the effects of the 3 agents in ATM-deficient GM16666 and ATM-restored GM16667 fibroblasts. Once again, veliparib and olaparib exhibited selectivity for the HR-deficient cells (Fig. 3A and B, left and middle), whereas iniparib exhibited very little selectivity (Fig. 3A and B, right). Similar results were also observed in Atm−/− mouse fibroblasts compared with their wild-type counterparts (Fig. 3C).

Failure of iniparib to synergize with topoisomerase I poisons

Another hallmark of PARP inhibitors is their ability to synergize with topoisomerase I poisons (20, 23–27). To avoid the potential confounding effect of P-glycoprotein, which is constitutively expressed at low levels in rodent cells (28) and has been reported to affect uptake of topotecan (29–31), experiments in MEFs used camptothecin. At sub-micromolar concentrations that were themselves nontoxic, veliparib and olaparib enhanced the sensitivity of wild-type MEFs to camptothecin (Fig. 4A). In contrast, 100-fold higher iniparib concentrations, which were just at the point of inhibiting colony formation by themselves, had no discernible effect on camptothecin sensitivity (Fig. 4B). When topotecan, which is used to treat epithelial ovarian cancer (32, 33), was administered to SKOV3 cells, veliparib and olaparib likewise enhanced the cytotoxicity of the topoisomerase I poison whereas iniparib did not (Fig. 4C and data not shown).

Further effects of iniparib in combination

In view of the inability of iniparib to sensitize cells to topoisomerase I poisons, we also examined the ability of iniparib to sensitize SKOV3 cells to a number of other classes of agents with which it is being combined in the clinic (www.clinicaltrials.gov). In these experiments, iniparib failed to sensitize cells to cisplatin (Fig. 5A). In contrast, sensitization by the ATR inhibitor VE-821 was readily detected (Fig. 5B) as previously reported (34), indicating that sensitization by iniparib could have been observed if present. Likewise, iniparib failed to sensitize to gemcitabine (Fig. 5C) even though sensitization by the checkpoint kinase inhibitor AZD 7762 (35) was readily demonstrated (Fig. 5D). We also failed to observe sensitization of SKOV3 cells to paclitaxel (Fig. 5E). In contrast, iniparib slightly but reproducibly sensitized SKOV3 cells to etoposide (Fig. 5F).

Failure of iniparib to inhibit pADPr synthesis

In view of the limited selectively of iniparib for HR-deficient cells (Figs. 2 and 3) and inability of iniparib to sensitize to topoisomerase I poisons (Fig. 4), we examined the ability of iniparib to inhibit PARP in situ. For these studies polymer levels were determined by quantitative fluorescence microscopy, a method that is widely used and is more quantifiable than immunoblotting (27, 36). Treatment with veliparib or olaparib caused a dose-dependent decrease in DNA damage–induced pADPr levels (Fig. 6). In contrast, iniparib had no detectable effect on polymer formation in situ at concentrations up to 100 μmol/L.

Other cell lines were plated at 1,000 cells per plate (PEO1 and PEO4), 750 cells per plate (GM16666 and 16667), or 500 cells per plate (SKOV3) in their respective media, allowed to adhere for up to 14 hours, treated with the indicated agents for 48 hours (GM16666 and 16667) or continuously, stained with Coomassie Brilliant Blue, and scored for colony formation (≥50 cells) manually.

pADPr levels

Relative pADPr levels were assessed by quantitative fluorescence microscopy (modified from refs. 19, 20). In brief, SKOV3 cells grown on ethanol-washed coverslips were treated with the indicated agents for 4 hours before addition of 1 mmol/L methyl methanesulfonate (MMS) for 30 minutes to stimulate polymer formation. Following treatment, cells were fixed in −20°C methanol:acetone (70:30); incubated for 1 hour at 21°C in blocking buffer consisting of 1% (v/v) glycerol, 0.1% (w/v) gelatin from cold-water fish, 0.1% (w/v) bovine serum albumin, 5% (v/v) goat serum, and 0.4% (w/v) sodium azide in PBS; exposed to 96-10 anti-pADPr antiserum (1:500 dilution in blocking buffer) overnight at 4°C; washed; incubated for 1 hour with Alexa Fluor 568–conjugated goat anti-rabbit IgG (Invitrogen) in blocking buffer; washed; counterstained with 1 μg/mL Hoechst 33258 in PBS; and examined by confocal microscopy as described (20).
Previous studies have identified a number of biological properties of PARP inhibitors, including selective toxicity in HR-deficient cells, ability to synergize with topoisomerase I poisons, and, by definition, ability to inhibit pADPr synthesis in intact cells. Iniparib was originally described as a prodrug of a covalent PARP inhibitor (12, 37) and more recently as a small molecule cytotoxic with some PARP inhibitory activity (14). This agent displayed promising activity in combination with gemcitabine and cisplatin in phase II trials in triple-negative breast cancer (14) and ovarian cancer (38, 39) as well as anecdotal activity against BRCA2-deficient pancreatic cancer (40). Interestingly, iniparib has not been previously compared head-to-head with other PARP inhibitors in preclinical studies. Results of the present study, however, indicate that iniparib (in contrast to olaparib and veliparib) fails to exhibit much selectivity for HR-deficient cells (Figs. 2 and 3), fails to sensitize to topoisomerase I poisons (Fig. 4), and fails to inhibit pADPr synthesis in intact cells (Fig. 6). These results have important implications for current interpretations of iniparib clinical trials.

Earlier studies showed that PARP knockdown or PARP inhibition is selectively toxic to cells with HR defects (2, 6, 7). More recent studies have suggested that this selective toxicity stems from the ability of active site-directed noncovalent PARP inhibitors to tip the balance toward error-prone nonhomologous end-joining in HR-deficient cells (16). In anticipation of studies that would...
determine whether the intracellular metabolite 4-iodo-3-nitrosobenzamide, a putative covalent inhibitor of PARP1, acts in a similar fashion, we examined cells lacking BRCA2 (Fig. 2) or ATM (Fig. 3). In both cases, iniparib at concentrations exceeding 40 μmol/L killed cells but failed to show the anticipated selectivity for the HR-deficient cells. This lack of selectivity, which was demonstrated using assays for both apoptosis and colony formation, immediately distinguishes iniparib from other widely studied PARP inhibitors such as veliparib, olaparib, and MK-4827 (Figs. 2 and 3; ref. 20).

A wide variety of PARP inhibitors have also been shown to selectively enhance the cytotoxicity of topoisomerase I poisons (20, 23–27). This sensitization was readily detected with submicromolar concentrations of olaparib and veliparib (Fig. 4). In contrast, iniparib failed to sensitize multiple cell lines to either camptothecin or topotecan, again indicating a substantial difference in behavior compared with bona fide PARP inhibitors. Conversely, iniparib sensitized cells to etoposide (Fig. 5F), albeit modestly, whereas other PARP inhibitors failed to sensitize cells to this agent (20, 23, 41), again distinguishing iniparib from the PARP inhibitors.

Earlier studies indicated that iniparib can inhibit pADPr synthesis when glutathione levels in cells are depleted with buthionine sulfoximine (42). In contrast, we were unable to detect inhibition of pADPr synthesis in cells containing endogenous levels of glutathione (Fig. 6). This failure to
inhibit pADPr synthesis presumably accounts for the inability of iniparib to selectively kill HR-deficient cells or synergize with topoisomerase I poisons. Although iniparib does not seem to be exerting its effects through inhibition of pADPr synthesis, this agent clearly is cytotoxic to a variety of cell lines at concentrations above 40

Figure 4. Inability of iniparib to sensitize cells to topoisomerase I poisons. MEFs (A and B) or SKOV3 cells (C) were treated continuously with the indicated concentrations of camptothecin (A and B) or topotecan (C) in the presence of 100 nmol/L veliparib, 100 nmol/L olaparib, or 10 μmol/L iniparib, then examined for colony formation.

Figure 5. Effects of iniparib in combination with cisplatin, gemcitabine, paclitaxel, or etoposide. SKOV3 cells were treated continuously with the indicated concentrations of cisplatin (A), gemcitabine (C), paclitaxel (E), or etoposide (F) in the presence of diluent or 10 μmol/L iniparib and examined for colony formation. In panels B and D, the ATR inhibitor VE-821 (1 μmol/L), which is known to sensitize other cells to cisplatin (34), or the Chk1 inhibitor AZD 7762 (100 nmol/L), which is known to sensitize to gemcitabine (35), was substituted for iniparib as positive controls. Error bars in each panel, ±SD of triplicate samples.
Evaluation of Iniparib as a PARP Inhibitor

μmol/L (Figs. 2 and 3). While the mechanism of this cytotoxicity was not explored in the present study, structural similarity of iniparib to nicotinamide (Fig. 1) raises the possibility that the cytotoxic effects of iniparib reflect the inhibition of one or more enzymes that bind the nicotinamide derivative NAD⁺, including possibly glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or sirtuins (42), rather than primary effects on PARP.

In further experiments, the effect of combining iniparib with other chemotherapeutic agents was examined. Current or recently completed trials (www.clinicaltrials.gov) have paired iniparib with paclitaxel (triple-negative breast cancer), carboplatin + gemcitabine (triple-negative breast cancer, ovarian cancer, non−small cell lung cancer), or carboplatin + paclitaxel (uterine carcinosarcoma). Accordingly, we specifically focused on the effects of combining iniparib with these classes of agents. Iniparib failed to sensitize SKOV3 cells to cisplatin, gemcitabine, or paclitaxel (Fig. 5A, C, and E), whereas other enzyme inhibitors caused readily detectable sensitization (Fig. 5B and D). Even though carboplatin was not used in these experiments because of its lower solubility and potency in vitro, results with carboplatin would likely be similar to our observations with cisplatin because of the identical mechanism of action of these agents (43). It is possible that different results might be obtained in different cell lines with different genetic and epigenetic changes. Nonetheless, our observation that iniparib has limited impact on sensitivity of SKOV3 cells to platinum agents, taxanes or gemcitabine might be important for interpreting results of recently completed and ongoing clinical trials of iniparib in combination with these agents.

In view of the marked differences between iniparib and other PARP inhibitors described here, it is important that clinical results obtained with iniparib not be allowed to unduly influence development of bona fide PARP inhibitors. In particular, the recent disclosure that results of the phase III iniparib trial in triple-negative breast cancer were negative (15, 44) should not be interpreted to mean that bona fide PARP inhibitors will also fail to exhibit activity in this disease, as it is unlikely that iniparib inhibited PARP in this trial. In a similar fashion, if iniparib exhibits clinical activity in a different setting (38−40), these positive findings should also be extrapolated to bona fide PARP inhibitors extremely cautiously, if at all, because of the failure of iniparib to exhibit the properties of a PARP inhibitor as documented in the present study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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