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Differential anti-proliferative activities of poly(ADP-ribose) polymerase (PARP) inhibitors in triple-negative breast cancer cells

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Abstract

Despite recent advances in the clinical evaluation of various poly(ADP-ribose) polymerase (PARP) inhibitors in triple-negative breast cancer (TNBC) patients, data defining potential anti-tumor mechanisms beyond PARP inhibition for these agents are lacking. To address this issue, we investigated the effects of four different PARP inhibitors (AG-014699, AZD-2281, ABT-888, and BSI-201) in three genetically distinct TNBC cell lines (MDA-MB-468, MDA-MB-231, and Cal-51). Assays of cell viability and colony formation and flow cytometric analysis were used to determine effects on cell growth and cell cycle progression. PARP-dependent and -independent signaling mechanisms of each PARP inhibitor were investigated by western blotting and shRNA approaches. Potential synergistic interactions between PARP inhibitors and cisplatin in suppressing TNBC cell viability were assessed. These PARP inhibitors exhibited differential anti-tumor activities, with the relative potencies of AG-014699 > AZD-2281 > ABT-888 > BSI-201. The higher potencies of AG-014699 and AZD-2281 were associated with their effects on G₂/M

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arrest and DNA damage as manifested by γ -H2AX formation and, for AG-014699, its unique ability to suppress Stat3 phosphorylation. Abilities of individual PARP inhibitors to sensitize TNBC cells to cisplatin varied to a great extent in a cell context- and cell line-specific manner. Differential activation of signaling pathways suggests that the PARP inhibitors currently in clinical trials have different anti-tumor mechanisms beyond PARP inhibition and these PARP-independent mechanisms warrant further investigation.

Keywords

Poly(ADP-ribose) polymerase; PARP inhibitors; Triple-negative breast cancer

Introduction

Triple-negative breast cancers (TNBC) lack estrogen (ER), progesterone, and HER2 receptors and occur more frequently in young women, especially African-American and BRCA1 mutation carriers [1,2]. In contrast to ER-positive or HER2-overexpressing cancers, the only available treatment for TNBC is chemotherapy. Although these cancers initially respond to neoadjuvant chemotherapy with higher pathological complete response (pCR) rates than luminal (ER-positive) cancers, women who fail to achieve pCR tend to recur early with distant metastases and poor survival [3]. Thus, additional relevant targets and effective systemic therapies remain to be defined in TNBC.

In light of the significant prevalence of BRCA mutations in TNBC patients [4], there is a growing interest in the interplay between loss of DNA repair function due to BRCA mutations and that due to the pharmacological inhibition of poly(ADP-ribose) polymerase (PARP), a key enzyme involved in single-strand DNA break repair [5], in TNBC tumors [6, 7]. The synthetic lethality that results from the combined loss of these two functions was first demonstrated by the ability of BRCA deficiency to sensitize tumor cells to PARP inhibition [8, 9], and by the favorable therapeutic index of the PARP inhibitor AZD-2281 (olaparib) in women with advanced breast cancer and BRCA1/2 mutations [10].

PARP inhibitors have been used in combination with various chemotherapeutic agents in TNBC and other solid tumors (review: [11]). The results of a randomized Phase II trial showed the addition of BSI-201 (iniparib) to gemcitabine and carboplatin significantly improved progression-free and overall survival relative to chemotherapy alone in women with metastatic TNBC [12]. These results, however, were followed by those from a larger randomized Phase III trial of exactly the same design, schedule, and drug doses as the randomized Phase II demonstrating that BSI-201 did not meet the pre-specified criteria for significance for the primary endpoints of progression-free and overall survival [13, 14].

Results from these BSI-201 trials highlight the need for a more complete understanding of the activities and mechanism(s) of action of PARP inhibitors. Accordingly, we investigated the in vitro efficacies and mechanisms of anti-tumor action of AG-014699 (rucaparib) [15], AZD-2281 (olaparib) [16], ABT-888 (veliparib) [17], and BSI-201 (iniparib) [18], in TNBC cell lines harboring different genetic abnormalities; specifically, MDA-MB-468 (PTEN-null, p53 mutant, BRCA1 wt), MDA-MB-231 (PTEN wt, p53 mutant, BRCA1 wt), and Cal-51

(PI3KCA mutant, p53 wt, BRCA1 wt) [19]. Our results provide evidence supporting the involvement of non-PARP targeting mechanisms in anti-tumor efficacy of some PARP inhibitors.

Materials and methods

Cell lines, culture, and reagents

MDA-MB-231 and MDA-MB-468 cells were obtained from the American Type Culture Collection (Manassas, VA), and Cal-51 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified CO₂ (5 %) incubator. The PARP inhibitors AG-014699 [15], AZD-2281 [16], and BSI-201 [20] were synthesized in the authors' laboratory according to published procedures, and ABT-888 was purchased from ENZO Life Sciences (Plymouth Meeting, PA). Cisplatin was purchased from NovaPlus (Novation, Irving, TX). Antibodies against the following proteins were used: p-Thr308-Akt, Akt, p-Thr202/Tyr204-ERKs, ERKs, p-Thr180/Tyr182-p38, p38, PARP, p-Tyr705-STAT3, STAT3, and PHLPP (Cell Signaling Technology, Danvers, MA); BRCA1 and p53 (Santa Cruz Biotechnology, Santa Cruz, CA); p-Ser139-H2AX (γ -H2AX) and H2AX (Millipore, Billerica, MA); β -actin (MP Biomedicals, Irvine, CA); flag (Sigma-Aldrich, St. Louis, MO); poly(ADP-ribose) (PAR, BD Biosciences, San Jose, CA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) and goat anti-mouse IgG-HRP conjugates were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biomatik, Wilmington, DE) assay as previously described [21]. Cells (5,000/well) were treated with PARP inhibitors for 72 h in the presence of 5 % FBS and incubated with MTT for 1 h.

Clonogenic assay

Cell survival was determined by the clonogenic assay [16]. Cells were treated for 14–21 days until colonies were visible. The colonies were fixed with 4 % formaldehyde (Sigma-Aldrich) and stained with crystal violet (5 mg/ml in 2 % ethanol, Sigma-Aldrich). Colonies containing more than 50 cells were counted. Cell survival is expressed as a percentage and was determined from the numbers of colonies present in the drug-treated groups relative to that in the vehicle-treated control group. Each drug concentration was assessed in triplicate, and the experiments were repeated at least twice.

Immunoblotting

Drug-treated cells were collected and then lysed in a buffer containing 1 % sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetic acid, 50 mM Tris-HCl (pH 8.1), and 1 % protease inhibitor mixture (Sigma-Aldrich). Lysates were sonicated and then centrifuged at 13,000×g for 10 min. Protein concentrations in the supernatants were determined (Micro BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL) and equal amounts of proteins

were resolved in a SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membrane was washed twice with Tris-buffered saline containing 0.1 % Tween-20 (TBST), blocked with TBST containing 5 % non-fat milk for 30 min, and then incubated with primary antibody (1:500–1:4,000 dilution) in TBST at 4 °C overnight. After washing with TBST, the membrane was incubated with goat anti-rabbit or anti-mouse IgG-HRP conjugates (1:5,000 dilution) for 1 h at room temperature. The immunoblots were visualized by enhanced chemiluminescence.

Flow cytometric analysis

Cells were seeded into 6 cm plates (1.5×10^5 cells/plate), incubated overnight in 10 % FBS-supplemented medium, and then treated with DMSO vehicle or 1 μ M PARP inhibitors in 5 % FBS-supplemented medium for 72 h. Cells were harvested after trypsinization, washed with PBS, fixed in ice-cold 70 % ethanol, and stained with DNA staining solution containing propidium iodide (80 μ g/ml), RNase A (100 μ g/ml), and Triton X-100 (0.1 %, v/v) in PBS. Cell cycle phase distributions were determined on a FACSort flow cytometer and analyzed using the ModFitLT V3.0 software program (BD Biosciences).

Transfection and generation of stable sublines

Transfections were achieved by electroporation using the Amaxa Nucleofector system (Lonza Biologics, Inc., Hopkinton, MA) according to manufacturer's instructions. To generate cells expressing constitutively active Stat3 (A662C, N664C), MDA-MB-231 cells were transfected with the Stat3-C Flag pRc/CMV plasmid (Addgene, Cambridge, MA). To generate BRCA1-deficient cells, the BRCA1-functional MDA-MB-231 cells were transfected with plasmids expressing a BRCA1 shRNA (AGAATAGGCTGAGGAGG AAGTCTTCTACC) or scrambled non-effective shRNA (Origene, Rockville, MD). To generate p53-deficient cells, the p53 wild-type Cal-51 cells were transfected with a p53 shRNA plasmid (shp53 pLKO.1 puro; Addgene) or the Non-Target shRNA Control Vector (CCGGCAACAAGATGAAGAG CACCAACTCAGTTGGTGCTCTTCATCTTGTTGTTTTT; Sigma-Aldrich). Puromycin (0.4 μ g/ml, Invitrogen) and G418 (800 μ g/ml, Invitrogen) were used to select clones stably expressing BRCA1 or p53 shRNA and constitutively active Stat3, respectively. Appropriate expression levels of BRCA1, p53, and constitutively active Stat3 were confirmed by immunoblotting.

Drug combination studies

Combinations of PARP inhibitors with cisplatin were evaluated in MDA-MB-468 cells using a non-constant ratio design. Cells were treated with AZD-2281 (0–10 μ M), AG-014699 (0–10 μ M), ABT-888 (0–20 μ M), BSI-201 (0–20 μ M) or cisplatin (0–1.5 μ M) alone or with combinations of cisplatin and each PARP inhibitor. After 72 h of treatment, cell viability was determined by MTT assays. Data were analyzed for synergistic effects using the median-effect method of Chou and Talalay [22] and combination index (CI) values were calculated using CompuSyn software (3.0.1, ComboSyn, Inc., Paramus, NJ). CI = 1 indicated additivity; CI < 1 indicated synergism, and CI > 1 indicated antagonism. Correlation coefficients of the median-effect plots of single-agent dose–effect data ranged from 0.89 to 0.99 and those of the combination dose–effect data ranged from 0.79 to 0.99.

Statistical analysis

Quantitative data from in vitro experiments are presented as mean $\pm$ SD. Differences between group means were analyzed for statistical significance using the Student's *t* test (two-tailed). Differences were considered significant at $P < 0.05$. All western blots are representative of two independent experiments.

Results

Differential anti-tumor effects of PARP inhibitors in TNBC cells

The suppressive effects of AG-014699, AZD-2281, ABT-888, and BSI-201 on cell growth were assessed by MTT and clonogenic assays in MDA-MB-468, MDA-MB-231, and Cal-51 cells (structures and IC₅₀ values for PARP inhibition, Fig. 1a). According to a recent cluster analysis classifying TNBC into six subtypes, these cell lines were classified as basal-like 1 (BL-1), mesenchymal stem-like (MSL), and mesenchymal (M) subtypes, respectively [23]. MTT assays revealed differential potencies among the four PARP inhibitors (Fig. 1b). AG-014699 exhibited the highest cytotoxicity with about equal potency across all three TNBC cell lines (Table 1). Compared to AG-014699, the other three drugs had lower IC₅₀ values with AZD-2281 > ABT-888 > BSI-201 in MDA-MB-231 and Cal-51 cells. The PTEN-null MDA-MB-468 and Cal-51 cells were more susceptible to the cytotoxic effects of AZD-2281, ABT-888, and BSI-201 than MDA-MB-231 cells. This finding is consistent with a previous report showing that the homologous recombination deficiency caused by loss of PTEN function sensitized tumor cells to AZD-2281 [24].

The clonogenic survival of the cell lines exhibited high degrees of sensitivity to drug-induced inhibition in a cell type-dependent manner (Fig. 1c; Table 1). AG-014699 and AZD-2281 were highly effective in decreasing survival with IC₅₀ values between 0.1 and 0.7 μ M in all cell lines with the exception of MDA-MB-231 treated with AZD-2281. In contrast, ABT-888 and BSI-201 had 10–100-fold lower anti-clonogenic activity than AG-014699 and AZD-2281.

The differential activity of the PARP inhibitors in suppressing clonogenic survival correlated with their respective abilities to induce G₂/M arrest and DNA damage in MDA-MB-468 cells (Table 2; Fig. 2). AG-014699, AZD-2281 and to lesser extent ABT-888 induced dose-dependent increases in the G₂/M cell population. In contrast, BSI-201 had no appreciable effect on the cell cycle distribution. AG-014699 and AZD-2281 induced γ -H2AX formation and PARP cleavage in a dose-dependent manner indicative of DNA damage and apoptosis, respectively (Fig. 2), which were not observed with ABT-888 or BSI-201. As revealed by western blot analyses of PAR synthesis, AG-014699, AZD-2281, and ABT-888 completely blocked PARP activity at 1 μ M (Fig. 2), but BSI-201 showed no appreciable activity in reducing PAR expression levels even at 10 μ M.

Differential effects of PARP inhibitors on various signaling markers

The differential effects of PARP inhibitors on the phosphorylation status of various signaling molecules, including Stat3, Akt, ERK1/2, and p38 are illustrated in Fig. 3. AG-014699 at low concentrations (2.5 μ M) decreased the phosphorylation levels of Stat3

in MDA-MB-468 and MDA-MB-231, but this was not noted in response to the other three PARP inhibitors examined or in Cal-51 cells that lack appreciable p-Stat3 expression (Fig. 3a). Ectopic expression of constitutively active Stat3 partially protected MDA-MB-468 cells against AG-014699-mediated suppression of clonogenic survival (IC_{50} , 0.4 vs. 0.1 μ M for pcDNA control, $P < 0.05$) (Fig. 3b), indicating that downregulation of Stat3 phosphorylation contributed to AG-014699's anti-proliferative effect. Moreover, AG-014699 and AZD-2281 dose-dependently increased the phosphorylation levels of Akt, especially at Ser473, and/or ERKs in MDA-MB-468 and Cal-51 cells (Fig. 3a). This phenomenon, however, was not noted with ABT-888 or BSI-201, suggesting that this is a drug-specific event.

AG-014699 and AZD-2281 downregulate the expression of the tumor suppressor PH domain leucine-rich repeat phosphatase (PHLPP)

Considering the pivotal roles of Akt and ERKs in the development of drug resistance and tumor progression in cancer cells [25], we investigated the mechanism of their activation in PARP inhibitor-treated cells. From a mechanistic perspective, this drug-induced Ser473-Akt phosphorylation might be attributable to the increased activities of the upstream phosphoinositide-dependent kinase (PDK) 2 or reduced activities of PHLPP, a Ser473-specific Akt phosphatase [26]. Because the identity of PDK2 remains unclear [27], we examined the effects of AG-014699 and AZD-2281 versus ABT-888 on PHLPP expression in MDA-MB-468 cells. As shown, AG-014699 and AZD-2281 exhibited a unique ability to suppress PHLPP expression (Fig. 4a), which inversely correlated with the observed increases in Ser473-Akt phosphorylation (Fig. 3a). In contrast, no appreciable changes in the expression level of PHLPP were noted in ABT-888-treated cells, which was consistent with the lack of effect of ABT-888 on Akt phosphorylation. Together, these findings support the mechanistic link between drug-induced Akt activation and PHLPP downregulation.

Moreover, evidence suggests that this Akt activation might underlie increased ERK phosphorylation in drug-treated cells as the effect of AG-014699 and AZD-2281 on ERK phosphorylation was abrogated by co-treatment with the PI3K inhibitor LY294002 to block Akt activation (Fig. 4b).

The effects of BRCA1 and p53 knockdowns

The ability of BRCA deficiency to sensitize cancer cells to PARP inhibition through synthetic lethality is well established [8, 9]. Here, we performed a direct comparison of the four PARP inhibitors for their effects on viability, clonogenic survival, and γ -H2AX formation in cells which were made BRCA 1-deficient by shRNA-mediated knockdown. As expected, reduced expression of BRCA1 sensitized cells to the suppressive effects of AG-014699, AZD-2281, and ABT-888 on both viability and clonogenic survival ($P < 0.05$; Supplementary Fig. 1b, c). This effect was accompanied by increased γ -H2AX formation suggesting that the observed sensitization could be attributable to increased DNA damage as a result of BRCA1 knockdown (Supplementary Fig. 1d). In contrast, while BRCA1-deficiency also sensitized cells to BSI-201-induced reduction in clonogenic survival, a concomitant increase in γ -H2AX formation was not evident.

To evaluate the p53-dependency of the anti-cancer activities of these PARP inhibitors, the influence of altered p53 expression on the activities of the four PARP inhibitors was assessed. Because ectopic overexpression of p53 in MDA-MB-468 and MDA-MB-231 cells led to extensive cell death (data not shown), shRNA-mediated knockdown of p53 in Cal-51 cells was used in this experiment. As shown, the loss of p53 expression in Cal-51 cells diminished their sensitivity to the suppressive effects of PARP inhibitors ($P < 0.05$; Fig. 5b, c). This decreased chemo-sensitivity was associated with reduced γ -H2AX accumulation in p53-deficient Cal-51 cells relative to parental cells in response to AG-014699 and AZD-2281 (Fig. 5d).

Sensitization of TNBC cells to cisplatin by PARP inhibitors

PARP inhibition has been shown to sensitize cancer cells to cisplatin [17, 28–30]. Here, we compared the effects of the four PARP inhibitors in combination with cisplatin on the viability of TNBC cells. Among the three TNBC cell lines, MDA-MB-468 cells were the most sensitive to cisplatin alone (Fig. 6a). Synergistic anti-proliferative effects ($CI < 1$) were observed in MDA-MB-468 cells treated with AZD-2281 or AG-014699 (at 2.5 and 5 μ M) in combination with cisplatin (at 0.1–1.5 μ M; Fig. 6b). These combination treatments were also associated with increased accumulation of γ -H2AX, relative to the single agent treatments (Fig. 6c). Although this synergistic effect on cell viability was also noted in cells co-treated with cisplatin and BSI-201 (at 10 and 20 μ M), this combination did not give rise to increases in γ -H2AX formation. In contrast, ABT-888, at concentrations as high as 10 and 20 μ M, in combination with cisplatin, showed neither a synergistic anti-proliferative effect ($CI > 1$) nor enhanced γ -H2AX formation.

Similarly, this synergistic interaction between cisplatin and AZD-2281 or AG-014699 (each at 5 μ M) was also noted in Cal-51 and MDA-MB-231 cells for viability ($CI < 1$) and γ -H2AX formation (Fig. 6d, e, respectively). In contrast, the combination of 10 μ M ABT-888 or BSI-201 with cisplatin (1–5 μ M) produced an additive or marginally synergistic suppressive effect (CI , 0.9–1) on the proliferation of these two TNBC cell lines. Similar to observations in MDA-MB-468 cells, these two PARP inhibitors did not increase cisplatin-induced DNA damage in Cal-51 and MDA-MB-231 cells.

Discussion

According to the NCI ClinicalTrials.gov homepage, at least six different PARP inhibitors are undergoing different phases of clinical trials in TNBC patients, either as single agents or in combination with DNA-damaging agents. However, despite rapid advances in the clinical development of PARP inhibitors, in vitro mechanistic information is lacking to provide insights into the complete mode of action of these drugs in vivo, which can be problematic, especially when disappointing results arise in the course of clinical trials as has happened in the case of the Phase III trial of gemcitabine and carboplatin with or without BSI-201 [13, 14].

To our knowledge, this is the first study to compare the effects of four PARP inhibitors side-by-side in three TNBC cell lines. The present study suggests the involvement of PARP-independent mechanisms in the anti-tumor efficacy of these PARP inhibitors. In addition,

with increasing recognition of subclasses of TNBC [22], the anti-tumor activity of a particular PARP inhibitor may depend, in part, on a more refined understanding of the molecular-genetic expression profiles of TNBC.

Several observations from the current study support the hypothesis that there may be PARP independent mechanisms. Despite the lack of any discernable effect on PAR synthesis in the three TNBC cell lines (Fig. 2), BSI-201 showed modest suppressive effects on the viability and clonogenic survival of TNBC cells, of which the underlying mechanism is unknown (Fig. 1). The pro-drug nature of BSI-201 may underlie the lack of inhibitory activity. BSI-201 (4-iodo-3-nitrobenzamide) requires metabolic activation to an unstable intermediate, 4-iodo-3-nitrosobenzamide [18, 20]. These findings suggest that metabolic activation of BSI-201 was insufficient in these cells and that the BSI-201-mediated anti-proliferative activity that was observed might be mediated through a PARP-independent mechanism.

A second example is AG-014699, which, among the PARP inhibitors tested, had the unique ability to inhibit Stat3 phosphorylation in a dose-dependent manner in MDA-MD-231 and MDA-MB-468 cells (Fig. 3a). Furthermore, the expression of constitutively active Stat3 in MDA-MB-468 cells provided a partial protection against the suppressive effect of AG-014699 on clonogenic survival (Fig. 3b). The precise mechanism of inhibition of Stat3 phosphorylation by AG-014699 is unknown, but Stat3 activation plays an important role in cell survival and resistance to apoptosis [31, 32].

AG-014699 and AZD-2281 stimulated the phosphorylation of Akt, especially at Ser473, in the PTEN-null MDA-MB-468 and Cal-51 cells, and that of ERK1/2 in MDA-MB-468 and the PTEN-positive MDA-MB-231 cells. Moreover, AZD-2281, and to a lesser extent AG-014699, facilitated the phosphorylation of the p38 stress kinase in a dose-dependent manner in MDA-MB-468 cells, which was less evident in the other two cell lines (Fig. 2). In light of the importance of Akt and ERKs in regulating cell survival and drug resistance, the therapeutic implication of the activation of these signaling kinases by PARP inhibitors warrants investigation. We obtained evidence that the drug-induced activation of Akt and ERK might be attributable to the reduced expression of PHLPP, a tumor suppressor, of which the investigation of the underlying mechanism is currently in progress.

As expected, silencing of BRCA1 expression sensitized cells to DNA damage and increased the level of γ -H2AX formation in cells exposed to AG-014699, AZD-2281, and ABT-888 [11, 28, 29]. It is interesting that BRCA silencing also enhanced the ability of BSI-201 to suppress the clonogenic survival, but not viability, of MDA-MB-231 cells, despite no apparent effect on γ -H2AX accumulation (Supplementary Fig. 1). Conversely, shRNA-mediated knockdown of p53 led to decreased sensitivity of Cal-51 cells to the suppressive effects of these four PARP inhibitors, suggesting that p53 functional status might serve as a biomarker for patient stratification in clinical trials. Moreover, our data indicate that this reduced chemosensitivity to AG-014699 and AZD-2281 might, in part, be attributable to reduced γ -H2AX formation in the p53-deficient cells (Fig. 5d). This finding is reminiscent of a previous report that loss of p53 function decreased oxaliplatin-induced γ -H2AX and cytotoxicity in HCT116 colorectal cancer cells [33].

It is generally believed that the combination of PARP inhibitors with platinum agents would achieve mechanistic synergy in TNBC independent of the functional status of BRCA1/2 [29]. However, the present study indicates that the ability of individual PARP inhibitors to sensitize TNBC cells to cisplatin varied to a great extent in a cell context-and cell line-specific manner (Fig. 6). For example, in the cisplatin-sensitive MDA-MB-468 cells, AZD-2281, and AG-014699 at 2.5 and 5 μ M synergized with cisplatin in suppressing cell viability, in part, by causing a greater extent of DNA damage (Fig. 6b, c). In contrast, neither synergy nor enhancement of γ -H2AX formation was noted with the ABT-888/cisplatin combination. In contrast, BSI-201 at 10 and 20 μ M, despite showing no appreciable effect on γ -H2AX formation alone or in combination with cisplatin, exhibited a synergistic anti-proliferative effect with cisplatin, presumably through a non-PARP targeting mechanism.

In summary, this examination of the anti-tumor activities of four clinically relevant PARP inhibitors in TNBC cells indicates the possible involvement of mechanisms of action beyond PARP inhibition. These findings raise a question with regard to the relative roles of PARP-dependent versus independent pathways in mediating the therapeutic effects of these agents in the clinical trial setting in addition to suggesting the response to a particular PARP inhibitor/chemotherapy depends in part of molecular-genetic characteristics of the TNBC cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ER	Estrogen receptor
ERKs	Extracellular signal related kinases
PAR	Poly(ADP-ribose)
PARP	Poly(ADP-ribose) polymerase
pCR	Pathological complete response
PHLPP	PH domain leucine-rich repeat phosphatase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TNBC	Triple-negative breast cancer

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Fig. 1.

Differential effects of PARP inhibitors on the viability and survival of TNBC cells. **a** Structures and reported IC_{50} values for PARP inhibition of AG-014699 [15], AZD-2281 [16], ABT-888 [17], and BSI-201 [18]. **b** MTT assays of the dose-dependent suppressive effects of AG-014699, AZD-2281, ABT-888, and BSI-201 on the viability of MDA-MB-468, MDA-MB-231, and Cal-51 cells in 5 % FBS-supplemented DMEM medium after 72 h of treatment. Mean $\pm$ SD ($n = 6$). **c** Clonogenic assays of the dose-dependent suppressive effects of AG-014699, AZD-2281, ABT-888, and BSI-201 on the survival of

MDA-MB-468, MDA-MB-231, and Cal-51 cells in 5 % FBS-supplemented DMEM medium after 14–21 days of treatment. Mean $\pm$ SD ($n = 3$)

Fig. 2. Differential effects of PARP inhibitors on PARP inhibition, DNA damage, and apoptosis in TNBC cells. Effects of AG-014699, AZD-2281, ABT-888, or BSI-201 on the levels of PAR, γ-H2AX, and PARP cleavage in MDA-MB-468, MDA-MB-231, and Cal-51 cells. Cells were treated with the indicated concentrations of PARP inhibitors in the presence of 5 % FBS-supplemented DMEM medium for 72 h

Fig. 3.

Differential effects of PARP inhibitors on the activation status of signaling effectors pertinent to cell proliferation and survival in TNBC cells, suggesting the heterogeneity in their modes of action, **a** Effects of AG-014699, AZD-2281, ABT-888, or BSI-201 on the phosphorylation status of Stat3, Akt, ERK, and p38 in MDA-MB-468, MDA-MB-231, and Cal-51 cells after 72 h of treatment, **b**Left, verification by western blotting of ectopic expression of Stat3 in MDA-MB-468 cells transfected with an empty pcDNA vector or plasmid encoding Flag-tagged constitutively active Stat3 (CA-Stat3). Right, effect of the

ectopic expression of CA-Stat3 versus the pcDNA vector on AG-014699-mediated suppression of clonogenic survival of MDA-MB-468 cells. Mean $\pm$ SD ($n = 6$).

* $P < 0.05$

Fig. 4. AG-014699- and AZD-2281-mediated activation of Akt and ERK might be attributable to downregulation of PHLPP expression in MDA-MB-468 cells, **a** Dose-dependent effects of AG-014699 and AZD-2281 versus ABT-888 on PHLPP expression after 72 h of treatment, **b** Effect of LY-294002 (10 μM) on AG-014699- and AZD-2281-mediated phosphorylation of Ser473-Akt and ERKs

Fig. 5.

Effect of shRNA-mediated silencing of p53 on the sensitivity of Cal-51 cells to the anti-proliferative effects of PARP inhibitors, **a** Verification by western blotting of shRNA-mediated knockdown of p53 expression in the stably transfected Cal-51 subclone. **b** Effects of p53 knockdown on the potencies of AG-014699, AZD-2281, ABT-888, and BSI-201 in suppressing the viability of Cal-51 cells after 72 h of treatment. Mean $\pm$ SD ($n = 6$). * $P < 0.05$. **c** Effects of p53 knockdown on the potencies of AG-014699, AZD-2281, ABT-888, and BSI-201 in suppressing the clonogenic survival of Cal-51 cells. Mean $\pm$ SD ($n = 3$). *

P<0.05. **d** Effects of p53 knockdown on the DNA damage response, as indicated by γ -H2AX formation, to AG-014699 and AZD-2281

Fig. 6. Sensitization of TNBC cells to cisplatin-mediated cell death and DNA damage by PARP inhibitors, **a** Differential susceptibility of MDA-MB-468 (468), MDA-MB-231 (231), and Cal-51 (51) cells to cisplatin-mediated suppression of cell viability after 72 h of treatment. Mean $\pm$ SD ($n = 6$). **b** Effects of AZD-2281, AG-014699, ABT-888, and BSI-201 on the sensitivity of MDA-MB-468 cells to cisplatin. Mean $\pm$ SD ($n = 6$). § CI > 1; CI = 1; * 1 > CI ≥ 0.5; ** CI < 0.5. **c** Western blot analysis of the effects of AZD-2281, AG-014699, ABT-888, and BSI-201 on the sensitivity of MDA-MB-468 cells to cisplatin-induced DNA

damage, as indicated by γ -H2AX formation, **d** Effects of AZD-2281, AG-014699, ABT-888, and BSI-201 on the sensitivity of Cal-51 and MDA-MB-231 cells to cisplatin. Mean $\pm$ SD ($n = 6$). **e** Western blot analysis of the effects of AZD-2281, AG-014699, ABT-888, and BSI-201 on the sensitivity of Cal-51 and MDA-MB-231 cells to cisplatin-induced DNA damage, as indicated by γ -H2AX formation

IC₅₀ values of the four PARP inhibitors in suppressing cell viability and clonogenic survival, as determined by MTT and colony formation assays, respectively, in three TNBC cell lines

Table 1

	IC ₅₀ (μM)					
	Cell viability			Clonogenic survival		
	MDA-MB-468	MDA-MB-231	Cal-51	MDA-MB-468	MDA-MB-231	Cal-51
AG-014699	9.7	13	8.6	0.1	0.7	0.5
AZD-2281	18	>100	9.5	0.2	4.5	0.4
ABT-888	98	>100	34	1.3	5.0	2.2
BSI-201	28	94	41	1.7	7.5	>10

Table 2

Differential effects of AG-014699, AZD-2281, ABT-888, and BSI-201, at 1 and 5 μ M, on cell cycle distribution in MDA-MB-468 cells

Treatment	Conc (μ M)	% Cells in phase ^a			
		Sub-G ₁	G ₁	S	G ₂ /M
DMSO	-	0.6 $\pm$ 0.2	60.2 $\pm$ 2.6	15.6 $\pm$ 0.7	23.3 $\pm$ 2.5
AG-014699	1	2.6 $\pm$ 0.8	38.2 $\pm$ 4.6	14.5 $\pm$ 1.5	43.9 $\pm$ 4.4 ^{***}
	5	4.7 $\pm$ 1.3	15.8 $\pm$ 0.8	12.0 $\pm$ 2.6	67.2 $\pm$ 3.8 ^{***}
AZD-2281	1	2.8 $\pm$ 0.9	46.0 $\pm$ 2.1	15.3 $\pm$ 1.1	35.5 $\pm$ 3.7 ^{**}
	5	5.3 $\pm$ 2.3	34.9 $\pm$ 1.2	17.7 $\pm$ 2.8	41.6 $\pm$ 4.9 ^{***}
ABT-888	1	1.4 $\pm$ 0.4	56.6 $\pm$ 0.7	14.9 $\pm$ 1.4	26.9 $\pm$ 2.2
	5	1.6 $\pm$ 0.5	54.6 $\pm$ 2.2	14.3 $\pm$ 2.1	29.5 $\pm$ 4.0 [*]
BSI-201	1	1.9 $\pm$ 1.7	57.6 $\pm$ 2.8	16.1 $\pm$ 2.6	24.2 $\pm$ 2.6
	5	2.2 $\pm$ 2.2	57.0 $\pm$ 1.2	15.3 $\pm$ 1.1	25.5 $\pm$ 3.6

^aValues, mean $\pm$ SD ($n = 4$)

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$