The PARP inhibitor ABT-888 synergizes irinotecan treatment of colon cancer cell lines

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Summary

Poly [ADP-ribose] polymerase-1 (PARP-1) localizes rapidly to sites of DNA damage and has been associated with various repair mechanisms including base excision repair (BER) and homologous recombination/non-homologous end joining (HRR/NHEJ). PARP-1 acts by adding poly-ADP ribose side chains to target proteins (PARylation) altering molecular interactions and functions. Recently small molecule inhibitors of PARP-1 have been shown to have significant clinical potential and third generation PARP inhibitors are currently being investigated in clinical trials. These drugs alone or in combination with radio/chemotherapy have resulted in meaningful patient responses and an increase in survival in metastatic breast cancer cases bearing BRCA-deficient or triple negative tumors and BRCA-deficient ovarian cancer patients. ABT-888, a potent PARP-1 inhibitor, sensitizes many cancer cells in-vitro and in-vivo to temozolomide. As such, we hypothesized that colon cancers would be sensitized to the DNA damaging chemotherapeutic agents, oxaliplatin and irinotecan, by ABT-888. Using colon cancer cell lines significant synergy was observed between ABT-888 and irinotecan at concentrations of ABT-888 as low as 0.125 μ M. The level of synergy observed correlated with the degree of PARP1 inhibition as measured biochemically in cell lysates. ABT-888 at concentrations of 0.5–4 μ M resulted in synergy with oxaliplatin. Furthermore, 24 h post treatment combinations of ABT-888/irinotecan generally resulted in increased G2/M cell cycle arrest and increased levels of DNA damage, followed by increased levels of apoptosis 48 h post treatment. In conclusion this study suggests that ABT-888 may be a clinically effective adjuvant to current colon cancer therapies that include the use of irinotecan and/or oxaliplatin.

Keywords

PARP; PARP inhibitor; VE-821; Irinotecan; SN38; Colon cancer

Introduction

Metastatic colon cancer related mortality remains high due to the development of drug resistance over the course of treatment [1]. Current chemotherapeutic regimens for the

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Conflict of interest The authors declare they have no conflict of interest.

treatment of metastatic colon cancer include the DNA damaging drugs, oxaliplatin or irinotecan, in combination with the nucleotide analog 5FU [1]. Although these treatments are initially effective in many patients, all patients eventually become resistant. A major cause of this resistance is increased levels of DNA repair [2–5]. As such an effective strategy to overcome chemotherapy resistance may be the use of small molecule inhibitors of DNA repair networks. We and others have used this strategy to show that inhibition of DNA-PK, a critical modulator of NHEJ, effectively synergizes with irinotecan to kill colon cancer cells and doxorubicin in killing breast cancer cells [6–9]. The present work focuses on a different inhibitor, 2-((R)-2-Methylpyrrolidin-2-yl)-1H-benz-imidazole-4-carboxamide (ABT-888), an Abbott laboratories lead compound, that interacts reversibly with PARP1 and 2 inhibiting the formation of poly-ADP ribose side chains [10–13]. PARP1 is a potential target to synergize DNA damaging drugs because of its' important role in the modulation of DNA damage repair. Specifically, PARP1 has been associated with the repair of single strand breaks. It is speculated that inhibition of PARP1 leads to increased single strand breaks in DNA and if left unrepaired that these eventually lead to the formation of increased double strand breaks and in turn increased levels of cell death [14–22]. Currently, as many as 55 clinical trials are in progress using ABT-888 in combination with chemotherapy against a variety of cancers [18, 23, 24]. Prominent among these are clinical studies treating breast cancer patients with ABT-888 in combination with temozolimide. In addition to being a potent inhibitor of PARP-1 and PARP-2, ABT-888 has good oral bioavailability, can cross the blood–brain barrier, and has been shown to potentiate a variety of DNA damaging anti-cancer therapies including: temozolimide, platinum agents, cyclophosphamide, and radiation in syngeneic and xenograft tumor models [10, 11, 13].

Due to the clinical potential of ABT-888, we hypothesized that concurrent treatment of colon cancer cell lines with oxaliplatin or SN38 (the active metabolite of irinotecan) and ABT-888 would synergize the DNA damaging and cytotoxic effects of these drugs. These studies were performed in chemotherapy resistant (p53 mutated) and sensitive (p53 wild type) colon cancer cell lines to demonstrate efficacy in different genetic backgrounds.

Materials and methods

Cell culture and reagents

HCT-116 and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection and were maintained at 37 °C in 5 % CO₂ in RPMI or McCoy's medium supplemented with 10 % fetal bovine serum and penicillin/streptomycin. Chemicals and reagents were obtained from Sigma-Aldrich or Invitrogen. ABT-888 was kindly provided by Abbott Laboratories.

Sulforhodamine (SRB) cytotoxicity assays

SRB assays were performed according to the method of Vichai et al. 2006 [25]. In this assay, SRB stain binds to basic amino acid moieties under mildly acidic conditions facilitating total protein quantification and by implication cell density determination. The assay is amenable to high throughput screening, is linear over a 20 fold range of cell numbers and has sensitivity similar to fluorescence based assays making it an ideal tool for cytotoxicity

studies. Briefly, cells were seeded at low density (final density within the linear range of the assay) in 96 well culture dishes and incubated overnight. Cells were subsequently treated with oxaliplatin or SN38 alone, the PARP inhibitor, ABT-888, alone or combinations of oxaliplatin or SN38 and ABT-888 (concentrations indicated in results) as previously described [7, 8]. Five days post drug treatment cells were fixed with trichloroacetic acid (10 %), stained with SRB, and analyzed for percent growth on a 96 well plate reader. Efficacies of the various drug treatments were determined by calculating 50 % inhibitory concentrations (IC₅₀) and synergy values. Synergy values (I values) were calculated using the equation of Berenbaum [26] as previously used in our laboratory [6–8]. Using this equation, I values significantly less than 1 indicate synergy, equal to 1 indicate additive behavior, and greater than 1 indicate inhibitory drug interactions. Each experiment was comprised of triplicate drug treatments and experiments were repeated at least 5 times.

Flow cytometric analyses

These experiments were performed as described [6–8]. Briefly, cells were treated with drugs as described in the results section and analyzed for cell cycle distribution (stained with 5 µg/mL 7AAD and 0.2 mg/mL RNase A) or γH2AX (anti-phospho-Ser139, Upstate, Lake Placid NY). The mean fluorescence intensity was measured to determine levels of phosphorylation of histone H2AX. Alexa488 conjugated secondary antibodies were used to detect the antigens of interest.

PARP inhibition assay

Levels of PARP inhibition were measured using the Universal PARP assay from Trevigen inc. Cells were plated in 6 well cluster plates at a density of 5×10^5 cells per well. After 24 h, cells were treated with SN38 (IC₅₀ level) alone or in combination with various concentrations of ABT-888. Cells were harvested 1 h and 24 h post drug treatment by scraping and suspending in the culture medium. After spinning at 2,000 rpm for 5 min, medium was removed and the resulting pellet suspended in 100 µL of lysis buffer. After homogenization and incubating on ice for 15 min lysates were centrifuged for 5 min at 13,000 rpm. The resulting supernatant was removed and assayed for protein content. The Trevigen PARP assay was immediately performed on all samples using aliquots with equivalent protein content.

Western blot analysis

Western blot analyses were performed to determine the relative amount of PARP in each of the treated samples [27]. PARP protein levels were normalized to actin and subsequently used to normalize levels of PARP activity to protein levels.

Apoptosis assay

Levels of apoptotic cell death were evaluated by monitoring drug treated cultures for membrane permeabilization and Annexin V content using flow cytometry as described [27]. Cells were treated with the stated drug combinations and concentrations for 24 or 48 h and assayed using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturers protocol.

Statistical analysis

There were at least 5 replicates for all SRB experiments and at least 3 replicates for all other experiments. Means were calculated and compared using Students *T*-test analysis (significance determined at $p < 0.05$) employing Graphpad incorporated's "Quickclacs" software.

Results

The ability of ABT-888 to synergize the effect of the anti-cancer agents, SN38 or oxaliplatin, was determined using the SRB assay (Tables 1 and 2). In brief the combined treatment of ABT-888 and SN38 significantly decreased IC₅₀ values of SN38 at ABT-888 concentrations as low as 250 nM compared to SN38 alone in HCT-116 cells. In a similar fashion, ABT-888 concentrations as low as 125 nM, resulted in significant decreases of IC₅₀ values (39 % reduction) in HT29 cells. ABT-888 was also effective in combination with oxaliplatin but required concentrations of 1 to 2 μ M to produce significant decreases in the IC₅₀ values of oxaliplatin for HCT-116 and HT-29 cell lines. Significantly, *I* values for ABT-888 and SN38 were less than 1 even at concentrations of 0.125 μ M indicating a strong synergistic effect.

PARP activity assays and PARP protein expression

PARP activity was measured in HCT-116 cell lysates grown for 24 h in the presence or absence of 10nM SN38 and increasing concentrations of ABT-888 (Fig. 1a). PARP activity was significantly increased over basal levels by the addition of SN38 (2.2 fold increase). In contrast, addition of ABT-888 resulted in a concentration dependent decrease in PARP activity. In the presence of SN38 in combination with ABT-888, PARP activity was not significantly higher than with the equivalent concentration of ABT-888 alone. To demonstrate that this change in activity was not due to altered protein expression, western blot analysis was performed on samples treated for 1 h or 24 h with 0.5 μ M ABT-888, 10 nM SN38 or combinations of these two (Fig. 1b). Levels of total PARP protein normalized to actin were consistent from sample to sample. However, activity measured in the same protein samples and normalized to total PARP protein demonstrated the highest PARP activity in samples treated with SN38 alone (Fig. 1c). PARP activity was significantly reduced in samples treated with SN38 in combination with ABT-888 (>4 fold at 24 h).

To understand the mechanism underlying the drug effects on colon cancer cells, levels of γ H2AX were measured in both HCT-116 and HT-29 cells treated with oxaliplatin or SN38 alone or in combination with ABT-888. γ H2AX, an indicator of DNA double strand breaks, was significantly increased in cells treated with SN38 or SN38 in combination with ABT-888 (Fig. 2) ($p < 0.05$). The highest levels of γ H2AX were detected in the presence of SN38 combined with ABT-888. An increase in γ H2AX was observed with oxaliplatin or oxaliplatin in combination with ABT-888 but these differences were not significant.

Cell cycle analysis was also determined (Fig. 3). At a 24 h time point no change in cell cycle progression was observed for either HCT-116 or HT-29 cells treated with ABT-888, oxaliplatin or the combinations of ABT-888 with oxaliplatin. In contrast, treatment with SN38 alone caused significant G2M arrest in both cell lines and this effect was increased by

the addition of ABT-888, being most prominent in p53 wild type HCT-116 cells. To demonstrate that DNA damage observed at 24 h was not due to DNA degradation associated with induction of apoptosis, Annexin V expression was analyzed at both 24 and 48 h. Although little apoptosis was detected at the 24 h time point, a significant increase in apoptosis was observed at 48 h in the presence of SN38 or SN38 plus ABT-888 for both cell lines (Fig. 4) indicating that γ H2AX preceded drug-induced-apoptosis.

Discussion

Ideally, DNA repair inhibitors will improve the therapeutic index of chemotherapeutic drugs by targeting cancer cells over healthy cells. The PARP inhibitor, ABT-888, has significant potential in this regard [28]. Typically, cancer cells have one or more mutations affecting DNA repair pathways and as such there is the possibility to exploit synthetic lethality using drugs that target DNA repair [29–31]. The efficacy of this approach has been demonstrated in BRCA1/2 deficient breast cancers (mutations affecting HRR) where PARP inhibitors including ABT-888 were shown to improve treatment with temozolomide [16, 32–38]. Interestingly, it was shown using mono-therapy that the a PARP inhibitor, AGO14669, increased double strand breaks in all cells but primarily effected those with deficiencies in HRR that were unable to repair the damage [39]. Here we show that treatment with ABT-888, at concentrations well below clinically achievable levels [10, 12, 13], sensitized both p53 wild type and mutant colon cancer cells lines to SN38 induced death in vitro. SN38, the active metabolite of irinotecan, is a potent topoisomerase I (topo I) inhibitor that through its' interaction inhibits the separation of topo I from the DNA strand and can result in the formation of DSBs [40, 41]. In healthy cells, this damage can be rapidly repaired via the major DNA damage repair pathways, HR and NHEJ. Here, sensitization was achieved in both cell lines tested although more pronounced in the p53 inactive HT-29 cells. This synergy was associated with increased DNA damage and was also reflected in dramatic cell cycle arrest in both cell lines. G2/M arrest observed with SN38 treatment alone was enhanced most prominently by the addition of ABT-888 in p53 functional HCT-116 cells. Other investigators have shown that a major effect of SN38 treatment is G2/M arrest [42]. In HT-29 cells, G2/M arrest was almost complete with SN38 treatment alone suggesting that in the face of DNA damage cells arrest in G2/M to facilitate repair and that although functional p53 is not required for this arrest, it is needed for cells to leave G2/M efficiently. It is likely SN38 treated p53 inactive HT29 cells were unable to progress in the cell cycle due to p53 inactivity [43]. In contrast, the inability to progress in the cell cycle might in part explain the higher level of resistance to SN38 of these cells, with active replication forks being required for the creation of DSBs. Alternatively, a lack of functional p53 in the face of overwhelming DNA damage might confer resistance by inhibiting apoptotic signaling. These data suggest that the presence of active p53 facilitates cell cycle progression in the presence of DNA damage and that this progression is inhibited by ABT-888. One possible explanation for this is that p53 is PARylated in response to PARP activation preventing nuclear export. Thus, it is possible that the presence of the PARP inhibitor abrogates p53 PARylation preventing normal functionality. PARP has a number of diverse cellular functions including: regulation of cell survival and death pathways, transcription regulation, telomere cohesion, mitotic spindle formation, intracellular energy metabolism and trafficking of signaling proteins such

as p53, p63 and NF κ B. In this context PARylation prevents interaction of these molecules with exportin-1 (XPO-1), a mediator of nuclear export, and as such inhibits normal trafficking to the cytoplasm [44]. Our results are in keeping with that of others that showed the PARP inhibitor, veliparib, synergized the topoisomerase I poisons topotecan and camptothecin [31]. Like many of the PARP inhibitors (including ABT-888) veliparib is an NAD⁺ analogue small molecule inhibitor of PARP and may function by preventing cross PAR-ylation (PARylation of PARP by PARP) and subsequent release of PARP from DNA damage sites. As such DNA bound PARP would induce cell death by interfering with transcription and the progress of replication forks [31]. Furthermore, it has been shown that ABT-888 potentiates temozolomide, platinum based drugs, cyclophosphamide and radiation [10]. Here we report a synergistic effect for combined treatment with oxaliplatin and ABT-888 but at higher concentrations of ABT-888 than with SN38. The platinum analogues function by forming complex DNA adducts including intrastrand and interstrand crosslinks (ICLs) [4]. Repair of these lesions is mediated by multiple DNA repair systems. The synergy seen with ABT-888 and oxaliplatin suggests that inhibition of PARP results in inhibition of many DNA repair pathways and thus sensitizes cells to this agent [19].

Although much work with PARP inhibitors has focused on BRCA1/2 deficient cancers, other investigators have shown that other DNA repair phenotypes may be sensitive to PARP inhibitor therapy. For example, recently it was shown that ATM deficient tumors are effectively targeted with PARP inhibitors. This response was even more pronounced when tumors were deficient in both ATM and p53 [45]. PARP inhibition has also been shown to result in extreme sensitization to methyl methane sulfonate (MMS) in mouse embryonic fibroblasts (MEFs). Here, PARP inhibition was accompanied by accumulation of S-phase cells that required ATR signaling. This study showed interaction of PARP and ATR by co-IP that required active PARylation of ATR [46]. Finally, microarray analysis showed that the DDR pathways elicited by ABT-888 and topotecan are the G1/S checkpoint, ATM and p53 in p53 wild type cells and BRCA1/2 and ATR in p53 mutant cell lines. Topotecan alone caused induction of the G1/S checkpoint and the combination of drugs enhanced G2 arrest, apoptosis and cell death. Cell death was further enhanced by the checkpoint kinase inhibitor UCN-01 that abolished G2 arrest [47]. Our data indicate that levels of PARP expression were not significantly affected by drug treatments and, in spite of this, a large increase in PARP activity with SN38 treatment alone was observed. However, in the presence of ABT-888, PARP activity was dramatically reduced.

Most importantly, the results of a Phase I clinical trial of ABT-888 and irinotecan demonstrated that a full dose of irinotecan can be given with doses of ABT-888 that suppress PARP activity [48] suggesting that an increase in the therapeutic index will be seen with this combination in the treatment of patients with metastatic colon cancer.

Acknowledgments

We gratefully acknowledge Abbott laboratories for providing the PARP inhibitor ABT-888 used in this study.

Grant support This work was supported by research grants to Lawrence Panasci and Raquel Aloyz from the Canadian Institute of Health Research (CIHR) and CIHR and the Leukemia, Lymphoma Society (USA), respectively. David Davidson received salary support from the Quebec-Clinical Research Organization in Cancer (Q-CROC).

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Fig. 1.
a PARP activity in HCT-116 cells treated with increasing concentrations of ABT-888 alone (Basal) or in combination with 10 nM SN38 (24 h), **b** total PARP expression in HCT-116 cell culture at 1 and 24 h after treatment with 10nM SN38, 0.5 μ M ABT-888 or the combination of SN38 and ABT-888, **c** PARP activity normalized to total PARP expression in HCT-116 cells 1 and 24 h post treatment with 10nM SN38, 0.5 μ M ABT-888 or the combination of these drugs. (* = significant at p 0.01)

Fig. 2. γ H2AX expression (*double stand breaks*) in HCT-116 (**a**, **b**) or HT-29 (**c**, **d**) cells treated with the [IC₅₀] of oxaliplatin (**a**, **c**) or SN38 (**b**, **d**) alone or in combination with ABT-888 (0.5 μ M). Cells were fixed and stained with a γ H2AX specific antibody 24 h post drug treatment. Mean γ H2AX in HCT-116 (**e**) or HT-29 (**f**) cells 24 h post drug treatment. (* = significant at $p < 0.05$)

Fig. 3. Cell cycle analysis of HCT-116 and HT29 colon cancer cells 24 h post treatment with [IC₅₀] of oxaliplatin or SN38 alone or in combination with 0.5 μM ABT-888

Fig. 4. Annexin V apoptosis assay of HCT-116 (a) or HT-29 (b) cells treated with SN38 (10 nM (HCT-116), 20 nM (HT-29)) or SN38 in combination with ABT-888 (0.5 μ M) for 4 h, 24 h or 48 h. (* = significant at p 0.05)

Table 1

IC₅₀ values of colon cancer cell lines treated with SN38 alone or combined with the PARP inhibitor ABT-888 determined using the SRB assay

Cell line	[ABT-888] (μM)	SN38 (nM) IC ₅₀ ±SE	I value	P value IC ₅₀
HCT-116	0	11.9±0.4		
	4	6.3±0.3	0.63	0.007
	2	7.1±0.7	0.62	0.001
	1	7.3±1.2	0.65	0.001
	0.5	7.3±0.4	0.63	0.001
	0.25	8.5±0.8	0.73	0.03
	0.125	9.1±0.8	0.76	NS
HT-29	0	23.3±0.9		
	4	10.7±0.9	0.56	0.001
	2	12.1±1.2	0.64	0.001
	1	13.0±1.1	0.67	0.001
	0.5	13.4±0.7	0.64	0.001
	0.25	13.6±1.1	0.72	0.001
	0.125	14.1±1.7	0.57	0.001

Table 2

IC₅₀ values of colon cancer cell lines treated with Ox alone or combined with the PARP inhibitor ABT-888 determined using the SRB assay

Cell line	[ABT-888] (μM)	Oxali (μM) IC ₅₀ ±SE	I value	P value IC ₅₀
HCT-116	0	0.34±0.07		
	4	0.19±0.02	0.54	0.05
	2	0.19±0.01	0.55	0.05
	1	0.23±0.03	0.67	NS
	0.5	0.24±0.04	0.71	NS
HT-29	0	1.59±0.12		
	4	1.12±0.08	0.70	0.05
	2	1.16±0.07	0.72	0.05
	1	1.27±0.08	0.83	0.05
	0.5	1.30±0.06	0.81	NS