ORIGINIAL ARTICLE

The PARP inhibitor ABT-888 potentiates dacarbazine-induced cell death in carcinoids

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Monoagent DNA-alkylating chemotherapies like dacarbazine are among a paucity of medical treatments for advanced carcinoid tumors, but are limited by host toxicity and intrinsic chemoresistance through the base excision repair (BER) pathway via poly (ADP-ribose) polymerase (PARP). Hence, inhibitors of PARP may potentiate DNA-damaging agents by blocking BER and DNA restoration. We show that the PARP inhibitor ABT-888 (Veliparib) enhances the cytotoxic effects of dacarbazine in carcinoids. Two human carcinoid cell lines (BON and H727) treated with a combination of ABT-888 and dacarbazine resulted in synergistic growth inhibition indicated by combination indices < 1 on the Chou-Talalay scale. ABT-888 administered prior to varying dacarbazine doses promoted the suppression of neuroendocrine biomarkers of malignancy, ASCL1 and chromogranin A, as shown by western analysis. Ataxia telangiectasia mitogen factor phosphorylation and p21Waf1/Cip1 activation, indicative of DNA damage, were increased by ABT-888 when combined with dacarbazine treatment, suggesting BER pathway attenuation by ABT-888. PE Annexin V/7-AAD staining and sorting revealed a profound induction of apoptosis following combination treatment, which was further confirmed by increased PARP cleavage. These results demonstrate that ABT-888 synergizes dacarbazine treatment in carcinoids. Therefore, ABT-888 may help treat carcinoids unresponsive or refractory to mainstay therapies.

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INTRODUCTION

Carcinoids are heterogeneous neuroendocrine tumors (NETs) that arise from the body’s enterochromaffin cells and vary in anatomical site. They are most commonly found in the gastrointestinal tract, with the small intestine being the site of highest occurrence, followed by those of the lung, comprising about 2% of all bronchopulmonary tract tumors.1,2 Carcinoids can occur either sporadically or as part of hereditary syndromes, but are collectively rare with an age-adjusted incidence of about 2–4 per 100,000 people.1,3,4 Interestingly, the incidence of small bowel and bronchopulmonary carcinoids appears to have increased over a recent five-decade period as per pan-SEER reports, possibly from improvements in surveillance or secondary to an evolving disease. A significant portion of carcinoids present with distant metastases of which about half have primaries of unknown origin.5 In fact, carcinoids are among the most prominent causes of isolated hepatic metastasis, second only to colorectal cancer.

Surgical resection can offer definitive cure in the absence of metastatic disease, although no current data exist supporting adjuvant systemic therapies. Cytoreductive resection may be considered when intent is palliative and complete resection is not possible. In such cases, chemotherapies such as somatostatin analogs have been demonstrated to abate hormonal symptoms and also improve the time to progression.5 Cytotoxic agents are typically reserved for tumors with high proliferative indices (Ki-67 ≥ 5%), historically involving combined 5-fluorouracil or doxorubicin with the alkylating agent streptozocin.6,7 An alternative alkylating agent known as dacarbazine along with its oral, less toxic formulation temozolomide has also shown moderate activity in advanced NETs in addition to melanoma and glioma.8–12 Notably, prospective studies of dacarbazine-inclusive polychemotherapy have demonstrated limited efficacy among carcinoids relative to other NETs.13–17 Dacarbazine exerts its effect by methylating the O6-guanine position of its target DNA, thereby causing mismatch repair and eventual cell death. More commonly, however, dacarbazine methylates the N7-guanine and N3-adenine position, comprising 70% and 9% of adducts, respectively, and which may be removed by the base excision repair (BER) pathway.18 Hence, through its ability to restore DNA to its normal state, robust BER activities have been associated with dacarbazine resistance.18,19

The BER pathway is carried out by the enzyme poly (ADP-ribose) polymerase (PARP), a nick-sensing enzyme that recruits BER complex proteins to double-stranded DNA break sites to initiate repair following base excision of, for instance, N7 and N3 adducts.20 Inhibitors of PARP have been thus developed with the intent of circumventing dacarbazine resistance by blocking BER and promoting N7 and N3 methylation-induced cell death.20,21 This approach has been successfully demonstrated in malignancies of pulmonary, colonic, glial and hematopoietic origin both in vivo and in vitro.22–27 A novel PARP inhibitor, ABT-888, also known by its trade name Veliparib, has been shown to potentiate DNA-damaging chemotherapies in advanced solid tumors.28–32 Importantly, studies have supported the use of ABT-888 in combination with alkylating agents like dacarbazine and its sister drug temozolomide across a spectrum of cancers including glioblastoma, leukemia, hepatocellular carcinoma and metastatic melanoma with recent corroboratory clinical investigations.33–43

Given the clinical refractoriness of carcinoids to dacarbazine-based therapies and mounting evidence regarding resistance

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mechanisms implicating DNA-damage responses, we sought to investigate the in vitro interaction between ABT-888 and dacarbazine in carcinoid cell lines.

MATERIALS AND METHODS

Cell culture

Human gastrointestinal carcinoid cells (BON) were gifted by Drs Courtney M. Townsend, Jr. of the University of Texas Medical Branch (Galveston, TX, USA) and B. Mark Evers of the University of Kentucky (Lexington, KY, USA). Human bronchopulmonary carcinoid (H727) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). BON and H727 cells were grown in DMEM/F-12 (Life Technologies, Grand Island, NY, USA) and RPMI/F-12 (Life Technologies, Grand Island, NY, USA), respectively, at 5% CO₂ and 37 °C atmosphere. The media were supplemented with 100 IU ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin (Life Technologies, Grand Island, NY, USA) and 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA). ABT-888 (Selleck Chemicals, Houston, TX, USA) and dacarbazine (Sigma-Aldrich) were stored in aliquots of 10 μm in dimethyl sulfoxide at –80 °C, and freshly thawed before use. Cells were plated at sub-confluency the day prior to treatment, and then incubated in fresh medium containing ABT-888 (0–10 μM) for 24 h, after which dacarbazine was added (0–1000 μM) for 2 additional days. Dimethyl sulfoxide concentrations were normalized across all treatment groups.

Western blotting

Total BON cell lysates following dacarbazine ± ABT-888 treatment were prepared and analyzed by western blotting as previously described.46 Each antibody was diluted as follows: 1:2000 for mammalian achaete-scute complex-like1 (ASCL1) (BD Pharmingen, San Diego, CA, USA), 1:1000 for chromogranin A (Zymed Laboratories, San Francisco, CA, USA), 1:10 000 for glyceraldehyde-3-phosphate dehydrogenase (Trengren, Gaithersburg, MD, USA), and 1:1000 for p21Waf/Cip1, cleaved poly (ADP-ribose) polymerase (PARP), phosphorylated ataxia telangiectasia mitogen factor (ATM), total ATM, and Survivin (Cell Signaling Technology, Beverly, MA, USA). Antibody signals were detected using Supersignal West Femto, Dura, or Pico (Pierce, ATM, and Survivin (Cell Signaling Technology, Beverly, MA, USA). Antibody signals were detected using Supersignal West Femto, Dura, or Pico (Pierce, ATM, and Survivin (Cell Signaling Technology, Beverly, MA, USA).

Cell viability

BON and H727 cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) rapid colorimetric assay. All cell lines were seeded in 96-well plates, and treatment groups were plated in sextuplicate. To assess viability following treatment, the media were replaced with 0.5 μl of 0.5 μg ml⁻¹ MTT in serum-free media for 3.5 h at 37 °C, followed by addition of 75 μl dimethyl sulfoxide before measuring optical densities. The remainder of our protocol was followed as previously described.35

PE annexin V/7-AAD staining

BON cells treated with dacarbazine (0–600 μM) ± ABT-888 (0–10 μM) were collected and incubated with PE Annexin V and 7-Aminoactinomycin D (7-AAD) fluorescence solutions (BD Pharmingen, San Jose, CA, USA) according to the manufacturer’s protocol. The FACSCalibur (BD Biosciences, San Jose, CA, USA) fluorescent-activated cell-sorting (FACS) instrument was used for quantitative fluorescent sorting, and FlowJo v10.0.8 (TreeStar Inc., Ashland, OR, USA) was used for subsequent analysis.

Statistical analyses

Student’s t-test was used to compare means between groups and all data are represented as mean ± s.e.m. Compusyn (Paramus, NJ, USA) was used to calculate combination indices (CI) following dacarbazine ± ABT-888 treatment as per the Chou-Talalay method for drug interactions. CI > 1, 1 or < 1 signify antagonistic, additive or synergistic interactions, respectively, between two treatments.46

RESULTS

ABT-888 and dacarbazine synergistically inhibit carcinoid cell proliferation

We set out to determine whether BON and H727 cells could be sensitized to dacarbazine with the addition of ABT-888. We observed that both BON and H727 proliferation was dose dependently suppressed following dacarbazine doses of up to 1000 μM, with half-maximal inhibitory concentration (IC₅₀) values of 218.2 and 268.6 μM, respectively. To determine the correct dose range for concomitant ABT-888 treatment, its monotherapeutic effects were first established using viability assays. ABT-888's IC₅₀ in BON and H727 exceeded 50 μM, with nearly 92% of cells still viable at 20 μM. We conservatively chose ABT-888 doses of 5 and 10 μM for combination treatment with dacarbazine, as these exerted minimal cytotoxic effect alone, and were within the range necessary, as previously reported, to inhibit its molecular targets and achieve combinatorial benefits.35,39,43 Co-treatment of BON cells with 5 μM ABT-888 potentiated dacarbazine-induced cytotoxicity as indicated by CI < 1 following 500 μM dacarbazine and above. When ABT-888 co-treatment was increased to 10 μM, cells appeared further sensitized to dacarbazine, with CIs falling < 1 following dacarbazine doses of 400 μM and above (Table 1). H727 cells responded similarly, with improved sensitization to dacarbazine at 10 μM ABT-888 relative to 5 μM. Doses of dacarbazine above 750 and 400 μM interacted synergistically with 5 and 10 μM ABT-888, respectively, signified by CI < 1 (Table 2). These curves are represented in Figures 1a and b.

| Table 1. Combination indices* (CI) following dacarbazine and ABT-888 treatment in BON |
|--------------------------------------|------------------|------------------|
| Dacarbazine (μM) | 5 μM ABT-888 | 10 μM ABT-888 |
| 10       | 1.43            | 2.08            |
| 25       | 1.60            | 2.08            |
| 50       | 1.44            | 1.23            |
| 100      | 1.11            | 1.12            |
| 200      | 1.12            | 1.04            |
| 300      | 1.37            | 1.08            |
| 400      | 1.26            | 0.75            |
| 500      | 0.74            | 0.48            |
| 750      | 0.52            | 0.39            |
| 1000     | 0.42            | 0.37            |

*Calculated based on the Chou-Talalay method (>1, antagonism, =1, additivity, <1, synergy).
Following ABT-888 co-treatment, CgA expression was reduced markedly further, and ASCL1 expression was nearly entirely diminished (Figure 1c). Collectively, these data indicate that ABT-888 potentiates the anticancer effects of dacarbazine on the neuroendocrine phenotype of carcinoids.

Combination ABT-888 and dacarbazine treatment promotes apoptosis

Through its mechanism of action, ABT-888 is purported to enhance apoptotic induction by impairing the mechanisms that mend dacarbazine-induced DNA damage. To assess the extent of apoptosis in response to ABT-888 and dacarbazine, PE Annexin V/7-AAD was used to probe BON cells following 72 h of treatment (as described in the Materials and Methods section). The percentage of cells in apoptosis (upper right quadrant), pre-apoptosis (lower right quadrant), necrosis (upper left quadrant) and those still viable (lower left quadrant) were quantified using flow cytometry. Experimental replicates were then averaged and are conveyed in the adjacent bar graph (Figure 2a). ABT-888 alone showed no appreciable increase in apoptosis. However, cells treated with ABT-888 and dacarbazine exhibited marked apoptotic induction relative to dacarbazine alone, almost approaching significance ($P = 0.07$). To corroborate this, the degree of PARP cleavage was assessed following combinatorial treatment. Figure 2b shows considerable induction of cleaved PARP expression in BON cells treated with increasing dacarbazine doses in the setting of ABT-888 co-treatment, whereas dacarbazine alone only produced modest PARP cleavage. Once again, ABT-888 alone had no effect. These data further suggest that ABT-888 sensitizes cells to dacarbazine cytotoxicity and apoptosis.

**Table 2. CIs* following dacarbazine and ABT-888 treatment in H727**

| Dacarbazine (μM) | 5 μM ABT-888 | CI | 10 μM ABT-888 | CI |
|------------------|------------|----|--------------|----|
| 10               | 2.49       |     | 2.59         |     |
| 25               | 1.32       |     | 3.48         |     |
| 50               | 0.96       |     | 1.41         |     |
| 100              | 1.12       |     | 1.19         |     |
| 200              | 1.22       |     | 1.08         |     |
| 300              | 1.25       |     | 1.05         |     |
| 400              | 1.19       |     | 0.96         |     |
| 500              | 1.25       |     | 0.88         |     |
| 750              | 0.61       |     | 0.78         |     |
| 1000             | 0.54       |     | 0.40         |     |

*Calculated based on the Chou–Talalay method (> 1, antagonism; = 1, additivity; < 1, synergy).

**Figure 1.** ABT-888 and dacarbazine synergistically inhibit cell growth in BON and H727 lines, while suppressing ASCL1 and chromogranin A (CgA). BON GI (a) carcinoid and H727 (b) pulmonary carcinoid cell lines were treated with ABT-888 (0–10 μM) for 24 h, after which dacarbazine was added (0–1000 μM) for 2 additional days. A 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay demonstrated dose-dependent reduction following dacarbazine treatment alone in both cell lines. Dacarbazine-induced cytotoxicity was potentiated by the addition of 5 μM ABT-888. With 10 μM ABT-888, both cell lines were further sensitized to dacarbazine treatment. Combination indices indicated a synergistic interaction between ABT-888 and dacarbazine in both BON and H727, falling below 1 at higher dacarbazine doses (Tables 1 and 2). Combining ABT-888 (0–10 μM) with dacarbazine (0–600 μM) treatment enhanced suppression of neuroendocrine biomarkers ASCL1 and CgA in BON cells, relative to dacarbazine’s effects alone (c).
G2/M-phase progression and thereby promotes cell replication. Altogether, these data indicate that DNA damage occurs secondary to ABT-888 and dacarbazine combination treatment, thus mediating apoptotic induction (Figure 3).

**DISCUSSION**

Despite recent advances in targeted therapies, there is limited consensus on standardized treatment approaches for metastatic carcinoid tumors. In the setting of distant spread, 5-year survival is only about 38.5% compared with 78.2% for local disease, based on late SEER data. Patients with disseminated bronchopulmonary carcinoids fare very poorly as well, with survival approaching only 15% after 5 years, as do those with atypical pulmonary carcinoids, with mortality of 25%. Therapeutic management of carcinoids is also made challenging given their long natural histories raising concerns for treatment toxicity. Carcinoids are known to be slow-growing, insidious cancers that follow a subtle yet malignant clinical course. Neuroendocrine in origin, they comprise cells packed with neurosecretory granules containing biogenic and vasoactive peptides including serotonin, histamine and gastrin, which upon release lead to the debilitating carcinoid syndrome. Other markers of neuroendocrine malignancy include CgA and the neuroendocrine-specific transcription factor ASCL1.

Cytotoxic therapies have been thought to have a role in the treatment of patients with locally advanced or metastatic tumors wherein surgical resection is not curative. In most carcinoid patients, agents like dacarbazine and its sister drug temozolomide have shown little if any benefit compared with the benefit in patients with other NETs. One particular study showed that only 1 of 14 carcinoid patients achieved objective response to temozolomide and thalidomide therapy. A subsequent study similarly revealed no improvement among carcinoid patients receiving temozolomide and bevacizumab, whereas 33% of pancreatic NET patients achieved tumor response. Temozolomide-based combination therapy was later associated with only a 2% partial or complete response among carcinoid patients compared with 34% of those with pancreatic NETs. Although other studies have associated monotherapy and combinatorial temozolomide regimens with up to 70% efficacy for pancreatic NETs, this approach has generally failed to show efficacy in carcinoids.

The mechanism of temozolomide and dacarbazine relies upon DNA methylation at the O6-guanine position causing DNA mismatch and subsequent apoptosis, sensitivity to its effects would improve in the setting of impaired O6-methylguanine DNA methyltransferase activity as demonstrated in patients with advanced glioblastomas and melanomas. The BER pathway has also been acknowledged as a major contributor to temozolomide resistance.

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**Figure 2.** ABT-888 potentiates dacarbazine-induced apoptosis. BON cells treated with a combination of ABT-888 (0–10 μM) and dacarbazine (0–400 μM) underwent Annexin V/7-AAD staining followed by phosphatidylserine exposure. Using the BD FACSCalibur™ instrument, cells were quantified using fluorescent activated cell sorting, demonstrating an induction of apoptotic populations following 200 and 400 μM of dacarbazine alone. With the addition of 10 μM ABT-888 to dacarbazine treatment, the percentage of apoptotic cells markedly increased. Within each pane, the upper right quadrant indicates late apoptotic cells (Annexin V-positive/7-AAD-positive), the lower right quadrant represents preapoptotic cells, the upper left quadrant represents cells positive for 7-AAD only, and the lower left quadrant represents viable cells (Annexin V-negative/7-AAD-negative). Data from three experimental replicates were averaged and are represented in the graph on the right (mean ± s.e.m.) (a). Apoptotic induction following the addition of 10 μM ABT-888 to dacarbazine (0–600 μM) treatment was also indicated by enhanced cleavage of the terminal apoptotic marker PARP (b).
in other cancer models, but its disruption in conjunction with temozolomide has been shown to sensitize cells to treatment. Pharmacological inhibition of BER by the PARP inhibitor ABT-888 has been demonstrated to enhance the antitumor effects of dacarbazine and temozolomide, and have advanced to phase I and II clinical trials for pediatric and adult gliomas, hepatocellular carcinoma and metastatic melanoma with mixed success. To improve carcinoid susceptibility to alkylating agent therapy for such candidate patients, we investigated the ability of the PARP inhibitor ABT-888 to enhance the activity of dacarbazine in carcinoids.

Our data show that in gastrointestinal and bronchopulmonary carcinoid cell lines, ABT-888 effectively sensitized cells to dacarbazine cytotoxicity, generating combination indices < 1 (Tables 1 and 2) signifying synergistic interaction, and reduced the expression of neuroendocrine biomarkers of malignancy in BON cells (Figure 1). In addition to potentiating apoptosis (Figure 2), ABT-888 co-treatment profoundly increased the levels of phosphorylated ATM and p21\textsuperscript{Waf1/Cip1}, indicating induction of the ATM-mediated DNA damage response pathway (Figure 3). Activation of ATM following DNA damage in response to PARP blockade is central to recruitment of DNA repair proteins, as has been illustrated following pharmacological inhibition of PARP. Other papers have also reported on PARP’s participation in the DNA-damage response triggering ATM phosphorylation and recruitment. Relevant to the interpretation of our findings, Tanaka et al. demonstrated that phospho-ATM is an accurate indicator of DNA damage following chemotherapy-induced apoptosis. Further aligned with our study, Liu et al. reported that the extent of cytotoxicity following ABT-888 and temozolomide treatment in several cancer lines was proportional to the degree of DNA damage, as represented by levels of ATM’s immediate target hH2AX. In addition, as a downstream target of p53 activation following ATM phosphorylation, p21\textsuperscript{Waf1/Cip1} is a key regulator of G2/S checkpoint passage by maintaining G2-phase arrest in the setting of cellular stress. Of note, its expression has been shown to be a direct function of DNA damage given its inextricable link to the ATM-mediated DNA repair process. Previous reports have confirmed that pharmacologic inhibitors of PARP, including ABT-888, cause a G2/S-phase arrest state during replicative stress, confirmed by an upregulation of p21\textsuperscript{Waf1/Cip1} (refs 63–65). Combined with the simultaneous suppression of the Inhibitor of Apoptosis gene Survivin alongside PARP cleavage, these results strongly suggest that ABT-888 optimizes dacarbazine-induced cytotoxicity at synergistic doses by inducing DNA damage and subsequent alteration in cell cycle kinetics, suggested by the ATM pathway activity, to promote cellular demise.

Common treatment strategies for managing locally advanced or metastatic carcinoids, although not standardized, have employed the use of alkylating agents such as streptozocin-based regimens that are FDA approved for these indications. However, given the prolonged performance status and indolent growth pattern of carcinoids, the routine use of such treatment approaches often raises toxicity concerns despite their purported therapeutic potential. Even the alternative alkylating agent temozolomide, which has been explored clinically for treating NETs, including in phase II trials, has resulted in limited therapeutic benefit in carcinoids specifically, as described in the above discussion. Clinical challenges surrounding temozolomide and similar cytotoxic drugs are reflected by a study by Ramirez et al., who, although endorsing survival benefits from temozolomide and capectabine therapy in metastatic NET patients, add that adverse reactions forced dose reductions in 24% of patients. Therefore, translating these treatments into effective and rational therapeutic regimens is challenging, particularly due to the long natural histories of carcinoids making formal treatment comparisons difficult to devise. Alternatively, the use of compounds designed to sensitize patients to lower doses of cytotoxic therapies may be of value in this setting. Inhibitors of DNA repair, such as ABT-888, designed to enhance the therapeutic indices of alkylating agents may offer an option to circumvent long-term toxicity and improve treatment tolerability. Because drug sensitivity has been linked to inherent impairments in the DNA damage response, it is likely, as our data suggest, that manipulating synthetic lethality through BER pathway inhibition may improve the treatment of carcinoids by targeting malignant cells with dysregulated repair mechanisms that confer resistance while sparing healthy cells. Moreover, we demonstrate that lone ABT-888 treatment has limited toxicity, further supporting its candidacy as a treatment adjunct with favorable toxicity. Initial human trials showed that only a single dose was necessary to achieve adequate plasma concentration necessary for effective PARP inhibition. Since 2007, the National Cancer Institute has evaluated ABT-888 in 88 clinical trials for the treatment of several cancer types including melanoma, gliomas, hepatocellular carcinoma and pulmonary and colorectal cancers. Select trials have combined ABT-888 and temozolomide therapy and, despite offering no clinical suggestion of synergy, have demonstrated excellent tolerability in both pediatric and adult populations. Several studies report modest antitumor activity, with some approaching significance, although their general failure to demonstrate favorable drug interaction may be attributed to factors like acquired resistance through BER pathway overexpression. Previous in vitro reports have revealed that enhanced DNA repair mechanisms and homologous recombination capacity in response to ABT-888 and temozolomide therapy may underlie a learned resistance to this regimen. Hence, the effects of combination therapy may be more profound when these intrinsic resistance mechanisms are inherently or therapeutically disabled. In addition, given the low number of recruited patients in some of these trials, their results may be considered exploratory rather than confirmatory.

In summary, ABT-888 potentiates dacarbazine-induced cytotoxicity in carcinoid cell lines, while altering the neuroendocrine phenotype. Hence, this therapeutic strategy may be a viable option for circumventing treatment refractoriness while controlling syndrome symptomatology. Given the current clinical characterization and use of ABT-888 and dacarbazine’s more tolerable form, temozolomide, these findings warrant further investigations into the clinical use of combinatorial treatment for the management of locally advanced and metastatic carcinoids.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
YS executed all the experiments with assistance from Harpreet Gill and Jon Blake Matsumura. Drs Sam Lubner and Herbert Chen guided the project’s trajectory and interpretation of findings.

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