The PARP Inhibitor, ABT-888 Potentiates Temozolomide: Correlation with Drug Levels and Reduction in PARP Activity *In Vivo*

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Abstract. *ABT-888 is a potent, orally bioavailable PARP-1/-2 inhibitor shown to potentiate DNA damaging agents. The ability to potentiate temozolomide (TMZ) and develop a biological marker for PARP inhibition was evaluated in vivo. Doses/schedules that achieve TMZ potentiation in the B16F10 syngeneic melanoma model were utilized to develop an ELISA to detect a pharmacodynamic marker, ADP ribose polymers (pADPr), after ABT 888 treatment. ABT-888 enhanced TMZ antitumor activity, in a dose-proportional manner with no observed toxicity (44-75% tumor growth inhibition vs. TMZ monotherapy), but did not show single agent activity. Extended ABT-888 dosing schedules showed no advantage compared to simultaneous TMZ administration. Efficacy correlated with plasma/tumor drug concentrations. Intratumor drug levels correlated with a dose-proportional/time-dependent reduction in pADPr. Potentiation of TMZ activity by ABT-888 correlated with drug levels and inhibition of PARP activity in vivo. ABT-888 is in Phase 1 trials using a validated ELISA based on the assay developed here to assess pharmacological effect.*

Abbreviations: TMZ, temozolomide; PARP, poly(ADP-ribose) polymerase; pADPr, poly(ADP-ribose) polymer; % TGI, % tumor growth inhibition; SSB, single-strand breaks; DSB, double-strand breaks; BER, base excision repair; HR, homologous repair; MMR, mismatch repair; OGAT, O^6 methyguanine; PK/PD, pharmacokinetic/pharmacodynamic; mg/kg/d, milligrams per kilogram per day; *q.d.*, drug administered once-a-day; *b.i.d.*, drug administered twice-a-day, *i.e.* mg/kg/day *b.i.d.* dose is divided into two doses per day; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; IACUC, Internal Institutional Animal Care and Use Committee; NIH, National Institutes of Health; *s.c.*, subcutaneous; TBST, Tris buffered saline-0.05% Tween 20; γ-H2AX, phosphorylated H2AX; PBMCs, peripheral blood mononuclear cells.

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The poly(ADP-ribose) polymerase (PARP) family of enzymes are characterized by the ability to ADP ribosylate protein substrates (1, 2), implicated in many cellular processes (differentiation, gene regulation, protein degradation, replication, transcription) and overall maintenance of genomic stability. The roles of PARP in transcriptional regulation (3, 4), epigenetic modification (5-7) and angiogenesis (8) have recently been shown. PARP-1 is a highly conserved (92% homology between human and mouse) and abundant nuclear enzyme (consisting of an *N*-terminal DNA binding, automodification and *C*-terminal catalytic domains) that functions as a DNA damage sensor for both single- (SSB) and double-stranded (DSB) DNA breaks (2). PARP is critical for SSB repair by base excision repair (BER) and repair of these lesions can lead to radio- and chemoresistance (*e.g.* to alkylating agents) (9, 10). PARP's role in DSB repair by homologous repair (error-free) involving *BRCA 1/2* has also been reported (11, 12). PARP-2 is a similar nuclear protein important in DNA damage recognition (13-15) and despite its low capacity to ribosylate proteins, PARP-2 plays an important role in BER by homo- or heterodimerization with PARP-1 (15). Therefore, PARP-1 and PARP-2 are critical for the maintenance of genomic stability through the regulation of DNA repair mechanisms. Enhanced expression of PARP in tumor cells is reported as a survival mechanism for genotoxic stress (16-18), making inhibition of PARP an attractive cancer therapeutic target mechanism (2).

Since PARP is essential in SSB repair, inhibition of PARP activity leading to impaired of DNA damage repair can sensitize cells to DNA damaging agents/cytotoxic therapies as demonstrated in preclinical studies *in vitro* and *in vivo*

(19-26). In addition, the role of PARP in DSB repair (homologous recombination mediated by *BRCA1/2* and other DNA repair genes) correlated with single agent sensitivity of deficient cells to some PARP inhibitors (11, 12, 27). While the early generation inhibitors possessed nonspecificity and low micromolar potencies, several more specific agents with nanomolar potencies are currently being investigated in clinical trials (28).

ABT-888 is a new PARP inhibitor with excellent potency (K_i 5.2 and 2.9 nmol/l, PARP-1/2), oral bioavailability and potentiates multiple DNA damaging agents including: cisplatin, carboplatin, cyclophosphamide, irinotecan, radiation and temozolomide (TMZ), as we have previously described in various xenograft/syngeneic preclinical models (25). The alkylating agent TMZ has shown clinical activity in glioma and melanoma (29, 30). Since therapies for these indications are disappointing, a potentiating agent for TMZ holds promise. In this study, the potentiation of TMZ efficacy by ABT-888 was further evaluated using the B16F10 syngeneic melanoma model (25), showing robust ABT-888 activity in a consistent, well-characterized and relevant model. Optimal dosing schedules to achieve maximum potentiation of efficacy were examined using various schedules of ABT-888 relative to the five-day TMZ clinical schedule of 200 mg/m^2, *p.o.*, *q.d.*x5 (31). Determining the optimal efficacious dose of ABT-888 may prove challenging since ABT-888 does not demonstrate single agent antitumor activity, therefore, biological markers that demonstrate PARP inhibition *in vivo* may facilitate assessment of pharmacological activity. Using the optimized schedule in B16F10 model, we sought to identify a pharmacokinetic/pharmacodynamic (PK/PD) biological marker and develop an assay for PARP activity in preparation for a Phase 0 clinical trial (32).

Materials and Methods

Compound. Enantiomerically pure ABT-888 was synthesized by Abbott Cancer Research and Process Chemistry. The synthesis and cell-based evaluation are published elsewhere (26, 33).

Cell lines for in vivo studies. B16F10 syngeneic murine melanoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to their recommendations without antibiotics and routinely tested for *Mycoplasma*. Cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), maintained at 37˚C in a humidified atmosphere equilibrated with 5% CO_2, 95% air and used between passages 3-7 when in log phase for tumor cell inoculation.

In vivo studies. Mice were obtained from Charles River Laboratories (Wilmington, MA, USA) at 5-6 weeks of age and used for studies when greater than 8 weeks of age and/or ~20 g in weight. All animal studies were conducted in a specific pathogen-free environment in accordance with the Internal Institutional Animal Care and Use Committee (IACUC), accredited by the American Association of Laboratory Animal Care under conditions that meet or exceed the standards set by the United States Department of Agriculture Animal Welfare Act, Public Health Service policy on humane care and use of animals, and the NIH guide on laboratory animal welfare.

B16F10 cells (6×10^4) were mixed 1:1 with matrigel (BD Biosciences, Bedford, MA, USA) and injected *s.c.* (0.2 ml) into the shaved flank of female C57BL/6 mice. For early treatment studies (ET), mice were injection-order allocated to treatment groups and therapy was initiated on day 1 or 3 following inoculation. Two bisecting diameters were measured with calipers and tumor volumes were estimated from the formula: (length × width^2)/2. TMZ treatment effect on tumor growth rate was assessed by determining $\%\,T/C_{day}$ X caculated by: [(mean tumor volume of treated group on day X / mean tumor volume of control vehicle group on day X) ×100]. Potentiation of TMZ efficacy ($\%\,T_{TMZ+A}/C_{TMZ}$) was assessed using tumor growth effect in the TMZ monotherapy group (C_{TMZ}) compared to the TMZ combination group (T_{TMZ+A}) calculated by: [(mean tumor volume of TMZ combination group on day X / mean tumor volume of TMZ monotherapy group on day X) ×100]. $\%\,T_{TMZ+A}$ / C_{TMZ} was assessed at a later timepoint than % T/C when the vehicle control groups reached tumor endpoint and could no longer be analyzed.

ABT-888 (0.1-25 mg/kg/d) was orally administered (*q.d.* or *b.i.d.* from 5-8 days) in a vehicle containing 0.9% NaCl adjusted to pH 4.0 using hydrochloric acid. Temozolomide (25-75 mg/kg/d; Schering-Plough, Kenilworth, NJ, USA) was formulated according to the manufacturer's recommendations and administered orally, *q.d.x5*. ABT-888 and TMZ were administered concurrently with various extended dosing of ABT-888 as indicated.

pADPr Western blot. Tumors were excised from humanely euthanized mice treated *in vivo*, flash frozen in liquid nitrogen and protein lysates prepared as described elsewhere (25).

Semi-quantitative pADPr ELISA. Protein lysates were prepared from tumors using sample lysis buffer (Biosource, Camarillo, CA, USA) and protein assay were performed as described elsewhere (25). Specific mouse monoclonal antibody for pADPr (Trevigen, Gaithersburg, MD, USA) was adsorbed on immunoplates (Pierce Endogen, Rockford, IL, USA) with 0.1 M carbonate buffer at pH 9.5 for 2 h at 37˚C. Plates were washed five times in Tris buffered saline-0.05% Tween 20 (TBST) and blocked for 1 h at room temperature with Superblock (Pierce Endogen). Samples and standards (15-1000 pg/ml, purified pADPr polymers; BioMol, Plymouth Meeting, PA, USA) were incubated overnight at 37˚C. Incubation with a rabbit polyclonal detecting antibody (Trevigen) was performed after washing with TBST, then plates were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature and subsequently developed with a chemiluminescent substrate (SuperSignal Femto for ELISA; Pierce Endogen). Plates were read using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Sample concentrations were determined using the four-parameter equation commonly used for immunoassays (34).

Pharmacokinetic studies. Plasma samples were prepared by precipitating proteins with 2 volumes of acidified methanol and tumor samples were prepared by homogenizing with 2 volumes of saline. Subsequent protein precipitation with either 2 volumes of acetonitrile, or liquid-liquid extraction at alkaline pH with ethyl

Table I. *Efficacy of ABT-888 and TMZ monotherapy and combination therapy in the B16F10 murine melanoma flank tumor model. No observable health concerns (e.g. weight loss, dehydration, lethargy) were observed in any of the treatment groups.*

Compound Rx schedule (mg/kg/day)	Mean tumor volume (mm^3±SE)	% T/C_{veh} (% TGI)	Student's *t*-test	Mean tumor volume (mm^3±SE)	% T/C_{TMZ} (% TGI)	Student's *t*-test
Study A		Day 14				
TMZ						
75, *p.o.*	363±49	18 (82)	<0.0001	NA	NA	NA
50, *p.o.*	442±55	22 (78)	<0.0001			
25, *p.o.*	656±64	32 (68)	<0.0001			
Study B		Day 14			Day 19	
TMZ						
50, *p.o.*	442±59	16 (84)	<0.0001	1877±327	NA	
ABT-888						
25, *p.o.*	2252±229	80 (20)	NS	NA	NA	NA
ABT-888/ TMZ						
25/50, *p.o.*	186±7	7 (93)	<0.0001	463±57	25 (75)	
12.5/50, *p.o.*	197±15	7 (93)	<0.0001	945±124	50 (50)	0.0005
5/50, *p.o.*	202±17	7 (93)	<0.0001	1060±119	56 (44)	0.02
1/50, *p.o.*	346±39	12 (88)	<0.0001	1697±202	90 (10)	0.03
b.i.d. (5 days)/*q.d.*						NS
ABT-888/TMZ						
12.5/50, *p.o.*	171±10	6 (94)	<0.0001	741±89	39 (61)	0.004
q.d. (5 days)/*q.d.*						
ABT-888/TMZ						
25/50, *p.o.*	176±12	6 (94)	<0.0001	412±73	22 (78)	0.0004
b.i.d. (8 days)/*q.d.*						
Crossover vehicle group 0/0, *p.o./p.o.*	2799±204	NA	NA	NA	NA	NA

% T/C: drug-treated/appropriate vehicle-treated control tumor volume ×100; % TGI: percentage tumor growth inhibition (100-% T/C). NA: Not applicable, NS: not significant. Student's *t*-test was calculated against appropriate controls (vehicle or TMZ monotherapy groups).

acetate was performed. Samples were analyzed by the method used in the osmotic minipump studies as described elsewhere (25).

Statistical analysis. Differences between groups were analyzed using Student's *t*-test (two- tailed) for comparing groups, with $p<0.05$ considered statistically significant.

Results

Potentiation of TMZ efficacy by ABT-888 in vivo using multiple schedules. To achieve a window for potentiation of TMZ efficacy, a dose titration of TMZ alone was initially determined (Figure 2A) showing a dose response for TMZ administered on a *p.o.*, *q.d.*x5 schedule (TMZ dose used throughout all studies). Significant antitumor efficacy was observed at 75, 50 and 25 mg/kg/d for five days as reflected by % T/C_{day14} (Table I). Since equivalent efficacy was observed with 75 and 50 mg/kg/d, 50 mg/kg/d was chosen for subsequent combination studies because it is the allometric equivalent to the human dose of 200 mg/m^2 (35). Therefore, the five-day TMZ schedule used here models both the frequency of dosing and drug exposure observed in the clinic.

For potentiation of TMZ activity by inhibition of PARP-mediated DNA repair, we anticipated that exposure to ABT-888 *in vivo* should coincide with the occurrence of DNA damage, thereby necessitating the overlay of dosing of ABT-888 with TMZ administration. Different schedules of ABT-888 in conjunction with the clinical schedule of TMZ (*p.o.*, *q.d.*x5) were used to assess the ABT-888 exposure that achieves maximum enhancement of TMZ efficacy. Significant antitumor efficacy of TMZ+ABT-888 was observed in a dose-proportional

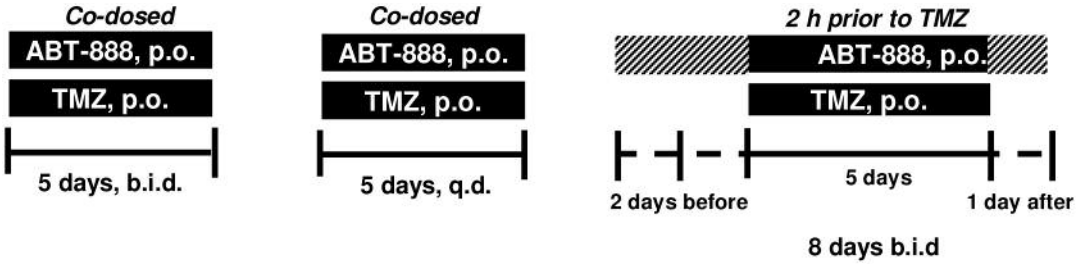

Figure 1. *Schematic diagram of dosing schedules used for potentiation studies.*

manner when ABT-888 (*b.i.d.*x5, mg/kg/day dose is divided into two doses per day) was dosed concurrently with TMZ (Figure 2B, Table I). While ABT-888 did not show significant single agent efficacy, TMZ monotherapy did (16% $T/C_{day\ 14}$). TMZ+ABT-888 at 25 or 5 mg/kg/d demonstrated significant potentiation of TMZ efficacy at day 19 with 25% T_{TMZ+A}/C_{TMZ} and 56% T_{TMZ+A}/C_{TMZ}, respectively compared to TMZ monotherapy. TMZ monotherapy was not significantly different from TMZ+ABT-888 at 1 mg/kg/d (90% T_{TMZ+A}/C_{TMZ}) and this was considered the non-effective dose. No toxicity (*e.g.* overt signs of significant weight loss, dehydration, lack of grooming) was observed in any of the treatment groups in this study, including no exacerbation of TMZ-induced myelosuppression. The exact overlay of TMZ+ABT-888 (*b.i.d.*x5) resulted in significant potentiation of TMZ efficacy and all subsequent studies were compared to these results.

Previous TMZ+ABT-888 combination studies have shown the maximum efficacious dose of ABT 888 to be 25 mg/kg/d (no additional benefit for efficacy was found using higher doses of ABT-888; data not shown). However 12.5 mg/kg/d produced a similar degree of TMZ potentiation (25) and this dose was used for comparison of fractionated dosing of ABT-888 administered either *q.d.*x5 *vs. b.i.d.*x5 and ABT-888 coverage for 5 days *vs.* 8 days (Figure 1). As seen in Figure 2C equivalent potentiation of TMZ efficacy by *b.i.d.* (50% $T_{TMZ+A}/C_{TMZ\ day\ 19}$) and *q.d.* (39% $T_{TMZ+A}/C_{TMZ\ day\ 19}$) dosing of ABT-888 at 12.5 mg/kg/d was observed. Similarly, a longer duration of ABT-888 dosing with 8 days of exposure (22% T_{TMZ+A}/C_{TMZ}, ABT-888 administered 2 h prior to TMZ and 6 h later for the second dose to achieve greater ABT-888 exposure) demonstrated equivalent potentiation of TMZ efficacy compared to TMZ+ABT-888 for 5 days (25% T_{TMZ+A}/C_{TMZ}) as shown in Figure 2D and Table I. These results indicate that neither the fractionation of dosing by *b.i.d. vs. q.d.* nor increased duration (8 d *vs.* 5 d) of ABT-888 exposure provided any advantage and suggests that 5 days of ABT-888 dosed concurrently with TMZ achieves sufficient exposure to ABT-888 to result in significant potentiation of TMZ efficacy. Overall, these *in vivo* efficacy studies are consistent with our previous results (25) that demonstrated significant potentiation of TMZ efficacy by ABT-888 at TMZ exposures similar to those achieved clinically, with no exacerbation of TMZ toxicity. While the preclinical dosing schedule evaluation provided guidance for potential combination dosing strategies for ABT-888 in the clinic for TMZ, it also provided an *in vivo* paradigm for the preclinical development of a biological marker/assay.

pADPr as a biological marker for PARP activity in vivo by immunoassay. Upon DNA damage, PARP activation results in poly(ADP-ribosyl)ation of substrate proteins including itself (1, 2), therefore the level of ribosylated proteins or pADPr polymers may be used as a biological marker for PARP activity. We initially evaluated several assays for detecting the level of pADPr including the biotinylated NAD^+ assay (36), flow cytometry, immunoblot and immunoassay (37). Previously, we have shown correlation in the level of pADPr and ABT-888 activity *in vivo* by Western blot (25). Due to factors such as its quantitative nature, high throughput attributes and proven technology, only the ELISA was considered for further development.

To assess the ability of the pADPr-specific ELISA in demonstrating the reduction in pADPr by ABT-888, we treated mice starting on day 3 after tumor cell injection with ABT-888 (*b.i.d.*x5) ± TMZ in a manner similar to the efficacy studies (Figure 2B). After 5 days, when B16F10 tumors were ≤1,000 mm^3, tumors were collected two hours post final dose for pADPr analysis. This timepoint was chosen because it approximates the plasma C_{max} of ABT-888. As seen in Figure 3A, some variability for baseline pADPr in individual tumors from vehicle-treated mice is observed (mean pADPr 4,800±641 pg/ml), while tumors from ABT 888 treated mice showed greater than 95% reduction in pADPr (mean pADPr 243±21 pg/ml, $p<0.0001$ *vs.* vehicle controls). This experiment provided preliminary evidence that the ELISA was able to detect a reduction in tumor pADPr *in vivo* as a result of the inhibition of PARP activity mediated by ABT-888 treatment.

Next we evaluated the effect of a dose response of ABT-888 (12.5, 5, 1, 0.3, and 0.1 mg/kg/d) on the reduction of pADPr (Figure 3B). The dose-proportional inhibition of tumor pADPr

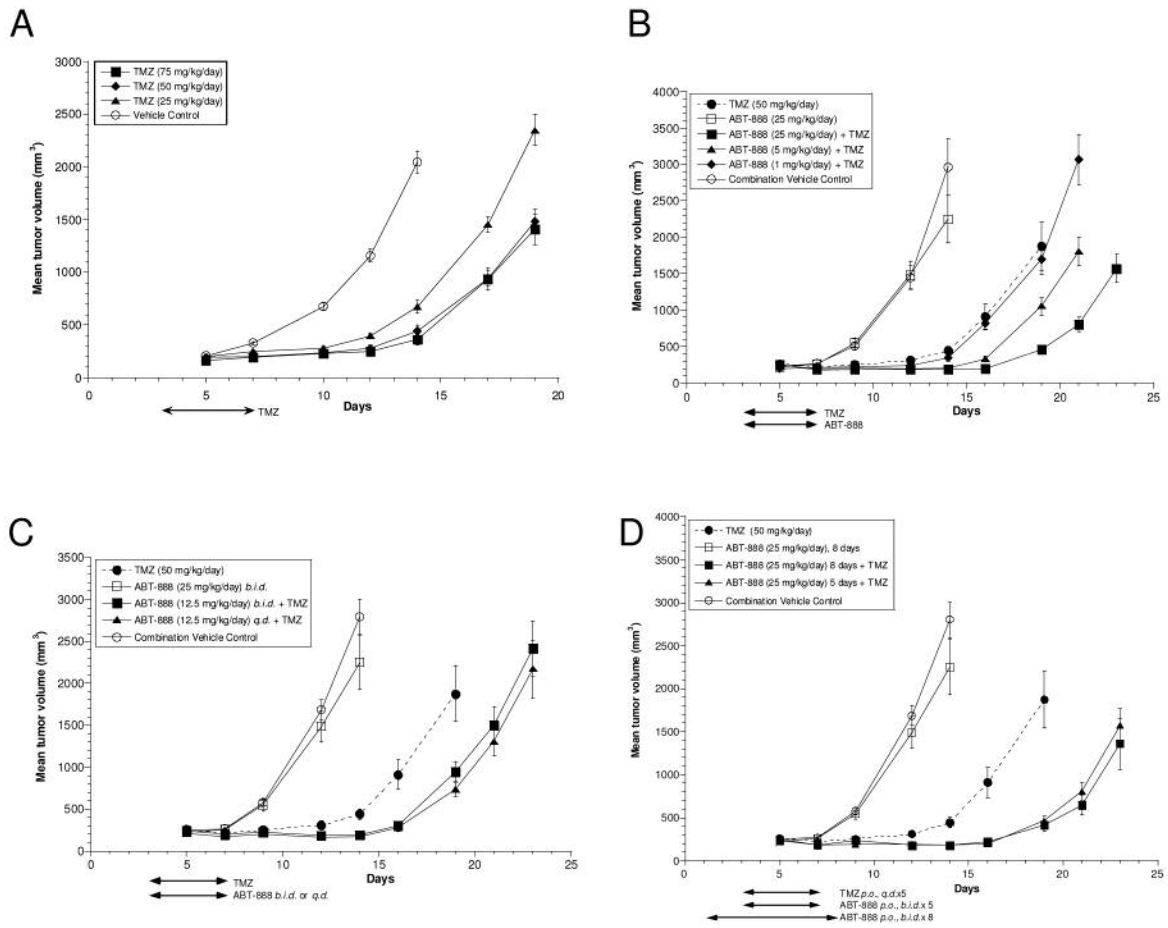

Figure 2. *Potentiation of TMZ efficacy by ABT-888 in vivo using multiple schedules in the B16F10 syngeneic melanoma model. A, TMZ was administered on a p.o., q.d.x5 schedule on days 3-7 at 75, 50 and 25 mg/kg/d. Data consist of 10 mice per treatment group; bars, SE. B, TMZ was administered on a p.o., q.d.x5 schedule at 50 mg/kg/d on days 3-7. ABT-888 was co-administered with TMZ on a p.o., b.i.d.x5 schedule at 25, 5 and 1 mg/kg/d. Data consist of 10 mice per treatment group; bars, SE. C, TMZ was administered on a p.o., q.d.x5 schedule at 50 mg/kg/d on days 3-7. ABT-888 was co-administered with TMZ on either a p.o., b.i.d.x5 schedule or p.o., q.d.x5 schedule at 12.5 mg/kg/d. Data consist of 10 mice per treatment group; bars, SE. D, TMZ was administered on a p.o., q.d.x5 schedule at 50 mg/kg/d on days 3-7. ABT-888 was administered concurrently with TMZ on either a p.o., b.i.d.x5 schedule or 2 h prior to TMZ on a p.o., b.i.d.x8 schedule at 25 mg/kg/d. Data consist of 10 mice per treatment group; bars, SE.*

was observed after ABT-888 treatment alone (inhibition of 89%, 84%, 71%, 62%, 60% for 12.5-0.1 mg/kg/d, respectively; $p<0.02$ *vs.* vehicle control). This reflected a corresponding trend in the potentiation of TMZ+ABT-888 efficacy as seen in Figure 3C. In the efficacy study, 12.5 and 5 mg/kg/d of ABT-888+TMZ provided marked potentiation with 35% and 42% T_{TMZ+A}/C_{TMZ}, respectively ($p<0.0001$ *vs.* TMZ monotherapy), while the lower doses of 1-0.1 mg/kg/d ABT 888+TMZ did not (66-82% T_{TMZ+A}/C_{TMZ}). Altogether, the trends in dose-proportional combination efficacy that correlate with an overall reduction in tumor pADPr reduction indicate that the ELISA can detect the inhibition of PARP activity by ABT-888 *in vivo*. However, when tumors were collected 2 h post final dose, significant pADPr reduction was still observed at the lower doses that did not show *in vivo* efficacy (Figure 3B). Therefore, subsequent studies assessed later timepoints (24 h) to determine if a better correlation of tumor efficacy and pADPr reduction by ELISA could be determined.

Our previous studies have shown a significant reduction in pADPr at 2 h by ABT-888 *in vivo* by Western blot (25). Additionally, various other xenograft models (melanoma, ovarian, lung, prostate, lymphoma) examined have also shown significant reduction in pADPr level *in vivo* after ABT-888 treatment (data not shown). We sought to confirm the detection of pADPr observed with the ELISA at 2 h (Figure 3 A-B) and determine the level of pADPr at 24 h post final dose. Mice were treated with ABT-888 at 25, 12.5 and 1 mg/kg/d (*b.i.d.*x5), and tumors collected at 2 h or 24 h timepoints. As shown in Figure 3D, at the 2 h timepoint, a

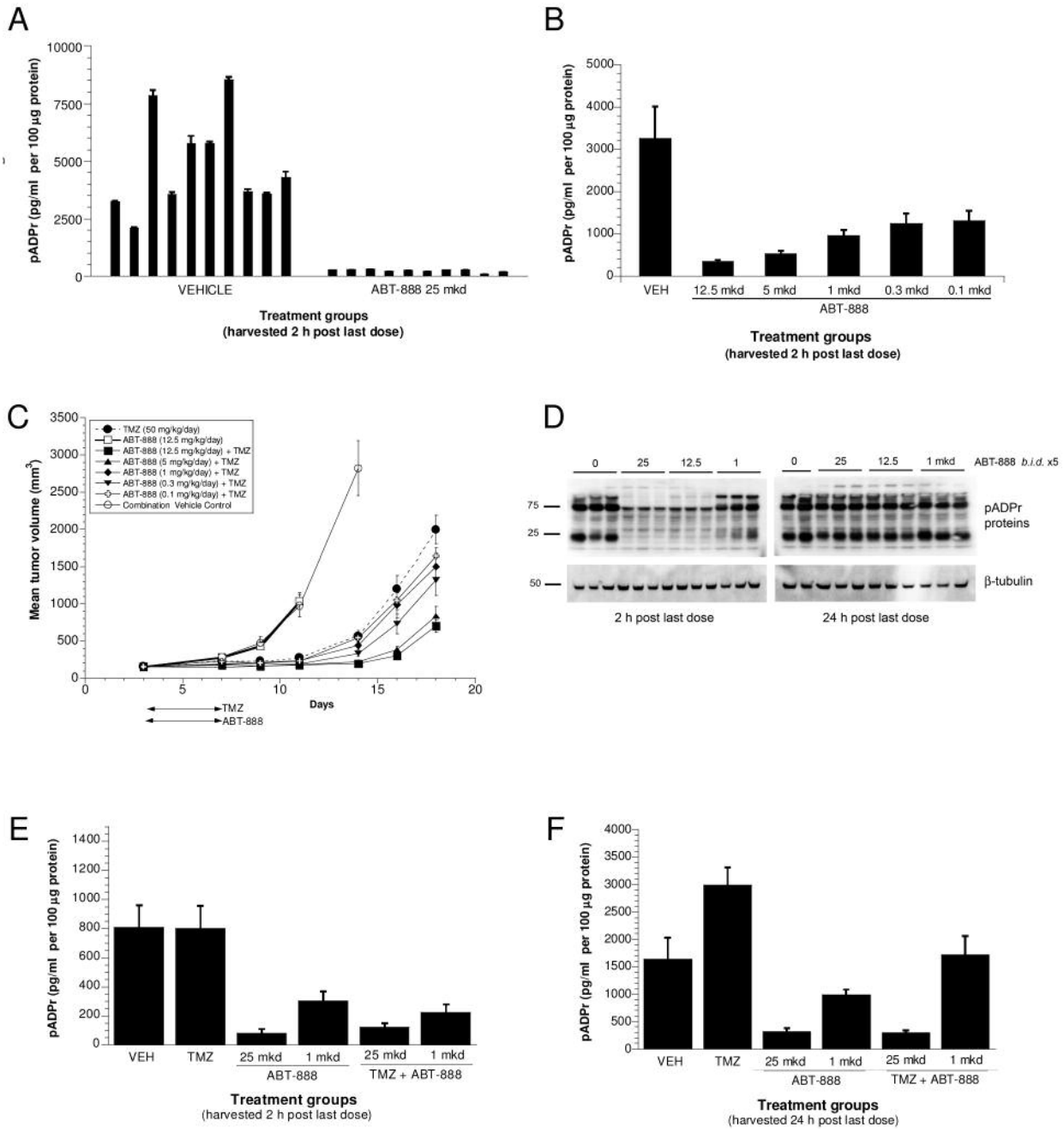

Figure 3. *Reduction in pADPr polymer by ABT-888 in vivo. Animals were treated with TMZ (p.o., q.d.x5) alone or co-administered with ABT-888 (p.o., b.i.d.x5) at different doses [mg/kg/d, (mkd)]. Tumors were then harvested at various timepoints as indicated. A, pADPr graph showing significant reduction in pADPr after ABT-888 treatment as measured by the ELISA. Mice were treated with ABT-888 alone, at the maximum efficacious dose of 25 mg/kg/d and harvested 2 h after the final dose. Data represent individual tumors, 10 mice per treatment group; bars, SE. B, A significant and dose-proportional reduction of pADPr was observed at 12.5, 5, 1, 0.3 and 0.1 mg/kg/d of ABT-888. Tumors were harvested for pADPr analysis by ELISA at 2 h post final dose of ABT-888 alone. Data represent n=6-7 mice per treatment group; bars, SE. C, A separate arm of the study shown in (B) was used for an efficacy study at the same doses of ABT-888 in combination with TMZ. The graph depicts a dose-responsive efficacy for ABT-888 at 12.5, 5, 1, 0.3, and 0.1 mg/kg/d, co-administered with TMZ. Data represent 10 mice per treatment group; bars, SE. D, Western blot analysis of B16F10 tumors from mice treated with ABT-888 at similar doses, 25, 12.5 and 1 mg/kg/d (p.o., b.i.dx5) 2 and 24 h post final dose. A significant reduction in pADPr level was observed after ABT-888 that was both dose- and time-dependent where less significant reduction in pADPr was observed at lower doses and later timepoints. E and F, Tumors from mice treated with ABT-888 at 25 or 1 mg/kg/d either alone or in combination with TMZ was harvested at either 2 h (E) or 24 h (F) post final dose. A significant pADPr reduction was observed with or without cytotoxic treatment. Data represent 5 mice per treatment group; bars, SE.*

marked but similar degree of reduction in tumor pADPr *in vivo* after ABT-888 treatment was observed with the 25 and 12.5 mg/kg/d dose, while a slight reduction was detected at 1 mg/kg/d, corresponding to the 2 h results by ELISA. However by Western blot, at 24 h post final dose there was no significant reduction in tumor pADPr at any of the doses, suggesting the effects of ABT-888 on pADPr level detected by Western blot dissipate by 24 h post last dose. Alternatively, Western blot may not be sufficiently sensitive to detect minimal differences in the degree of pADPr inhibition.

Reduction of tumor pADPr by ABT-888 after cytotoxic treatment in vivo. No consistent trend in pADPr levels was observed after TMZ treatment (no consistent significant increase in pADPr after TMZ/DNA damage though TMZ was expected to increase PARP activity, hence pADPr levels) similar to findings in clinical studies in combination with another PARP inhibitor (38). Since the increased level of pADPr as a result of DNA damage resulting in PARP activation is not yet clear, further study may be necessary to show a clear kinetic profile for pADPr in response to TMZ. However, there was a profound effect on pADPr levels in tumors from ABT-888 monotherapy and TMZ+ABT-888 groups at 2 h and 24 h post final dose by ELISA and only those of 2 h post final dose mirror the Western blot results (Figure 3E). In contrast, at 24 h post final dose (Figure 3F), less reduction in pADPr was observed at the nonefficacious dose for 1 mg/kg/d ABT-888 monotherapy and TMZ+ABT-888 combination. However, significant reduction of pADPr at the efficacious dose of 25 mg/kg/d ABT-888 monotherapy and TMZ+ABT-888 combination was still evident. While the ELISA results demonstrate the ability of ABT-888 to inhibit pADPr after cytotoxic treatment, lending to its utility in demonstrating pADPr inhibition after combination therapy in the clinic, there is somewhat of a discrepancy between the Western blot and ELISA at the 24 h timepoint, possibly reflecting the differential sensitivity of an ELISA format compared to the Western blot, warranting further analysis. Nevertheless, the ability of the ELISA to demonstrate the sustained reduction in pADPr at 24 h for the higher dose of ABT-888 (25 mg/kg/d) seems to indicate a prolonged duration of PARP inhibition that appears to coincide with the observed equivalent efficacy using various schedules examined in Figure 1. Therefore, in order to understand the kinetics *in vivo*, studies further examined the correlation of pADPr reduction with ABT-888 plasma/tumor drug level *in vivo*.

ABT-888 Plasma and tumor levels correspond with pADPr in a dose-/time-dependent manner in vivo. To better examine pharmacokinetic and pharmacodynamic correlation of ABT-888 and pADPr reduction without the potential contribution from multiple dosing, tumors were collected at 2, 6 and 24 h after a single dose of ABT-888 spanning doses used in efficacy studies. Plasma drug levels (Figure 4A), tumor drug levels (Figure 4B) and corresponding pADPr level measured by the ELISA (Figure 4C) were analyzed. High

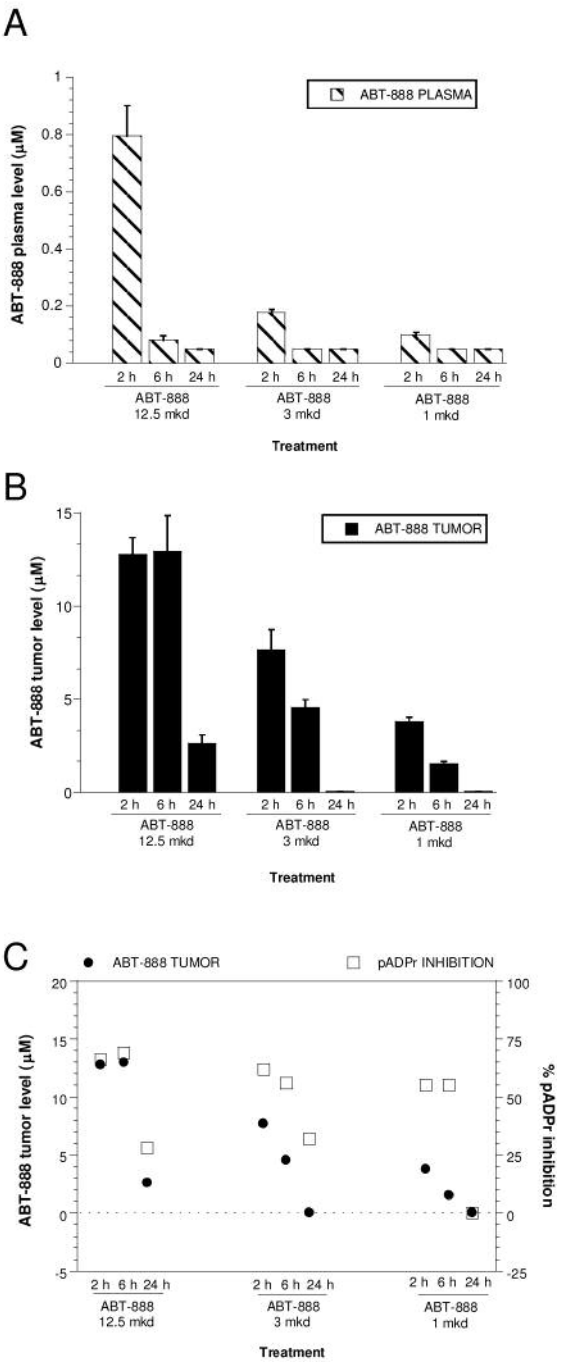

Figure 4. *Drug level and correlation of the reduction in pADPr polymer by ABT-888 in vivo. The level of ABT-888 [12.5, 3 and 1 mg/kg/d (mkd)] in A, plasma and B, tumor were analyzed after a single dose at 2, 6, and 24 h. C, Corresponding pADPr levels were also analyzed by ELISA. Data represent 3-5 mice per treatment group; bars, SE.*

tumor:plasma ratios of ABT-888 were initially observed but by 6 h, plasma drug levels dropped to the limit of detection (<0.01-0.02 μg/ml) at all doses, while significant tumor drug levels were maintained at 6 h (0.38-3.17 μg/ml). By 24 h, only the 12.5 mg/kg/d dose group demonstrated considerable tumor drug level (>0.49 μg/ml) but were significantly lower compared to the 2 h and 6 h timepoint (>2.44 μg/ml). These results clearly show that the level of ABT-888 in plasma dissipated by 6 h and tumor ABT-888 concentration was both dose- and time-dependent. However, while the tumor level of ABT-888 generally paralleled the degree of pADPr inhibition, the correlation was non-linear: i.e. markedly lower tumor drug concentrations were observed at the 3 and 1 mg/kg/d (0.24-1.71 μg/ml) but greater than 50% reduction in pADPr was still observed at 6 h. This may suggest that pADPr levels reflect more than just tumor efficacy and perhaps tumor growth inhibition only occurs if ≥70% pADPr reduction is achieved and maintained (~24 h) similar to findings with AG014699 where >50% pADPr reduction is observed (39). Collectively, these single dose experiments demonstrated an overall correlation of pADPr reduction measured by the ELISA with drug level proving the utility of the ELISA to detect PARP inhibition mediated by ABT-888 *in vivo*.

Discussion

Multiple mechanisms of resistance acquired by tumor cells in response to genotoxic stress have limited the effectiveness of chemotherapy (40) and the resistance to alkylating agents such as TMZ may be mediated by DNA repair enzymes such as OGAT (O^6-alkylguanine-DNA alkyltransferase), BER (repair of N^3 and N^7 methylguanine) and mismatch repair (MMR, tolerance to O^6 methylguanine alkyltransferase or OGAT) (23, 41). In fact, TMZ has shown activity in glioma and melanoma that correlates with OGAT levels and MMR proficiency (30, 31, 41). Thus, the impairment of such DNA repair mechanisms (BER) by PARP inhibition may overcome tumor resistance mechanisms (22, 23). Additionally, PARP inhibitors in combination with appropriate DNA damaging agents may potentially provide improvement of clinical response to existing regimens thereby potentiating cytotoxic agent efficacy, making PARP inhibition by agents such as ABT-888, an attractive therapeutic strategy.

Pharmacological inhibition or genetic ablation of PARP has been associated with an increase as well as a delay in accumulation of phosphorylated H2AX (γ-H2AX, indicating DNA damage) (11, 42), highlighting the important but complex interactions in response to DNA damage. We have previously demonstrated a concomitant increase in TMZ-induced DNA damage measured by γ-H2AX foci in combination with ABT-888 *in vitro*, (26) suggesting that impairment of DNA repair by ABT-888 may be achieved with TMZ *in vivo*. Consistent with our previous studies, TMZ monotherapy was moderately active in the B16F10 melanoma model (25) and thereby allowed for demonstration of an effect by combination therapy. We found that concurrent dosing of TMZ+ABT-888 was sufficient for the enhancement of TMZ *in vivo* efficacy, but whether concurrent dosing is optimal for other DNA-damaging therapies remains to be determined. Since longer duration of PARP inhibition may lead to a significant reduction of PARP-mediated DNA repair, administration of ABT-888 using various schedules was also examined. TMZ co-administered with ABT-888 demonstrated robust, consistent efficacy in the B16F10 melanoma model. Neither fractionated dosing nor longer than 5 days ABT-888 administration in the 5 d TMZ dosing regimen afforded improved activity, perhaps due to the sustained level of intratumor ABT-888 compared to plasma, and further corroborated by the kinetics of pADPr reduction. Combining drugs during therapy can alter the exposure to one or more of the drugs involved, however, neither the alteration of ABT- 888 drug levels by TMZ nor *vice versa* was found (data not shown). Collectively, the potentiation of TMZ efficacy by ABT-888 is consistent with mechanism, that is, the role of PARP in facilitating DNA repair and the ability of ABT-888 to inhibit PARP activity *in vivo*.

Since ABT-888 exhibits no single agent activity, determination of the optimal dose can pose a challenge and highlights the importance of a biological marker/assay that reflects PARP activity *in vivo* to facilitate the assessment of pharmacological and mechanistic effect *in vivo*. pADPr polymers serve multiple functions including autoregulation of PARP itself (automodification), recruitment of ribosylated DNA repair proteins (heteromodification) to the site of DNA damage through pADPr-binding motifs and relaxation of high order chromatin structure (ribosylation of histones) to provide accessibility to the site of damage (43). The level of pADPr appears to be an accurate determinant of PARP activity (44, 45), reflecting inhibition of enzyme activity mediated by PARP inhibitors such as ABT-888. Similar approaches in assessing PARP activity through pADPr analysis has been described with other PARP inhibitors employing a modified dot blot (AG014699) (38) and immunoassay (CEP-8900, GPI 21016 and KU59436) (19, 46, 47). We initially assessed several assays for pADPr (37). The biotinylated NAD^+ assay, while sensitive, entails permeabilization of cells from fresh tissue and flow cytometry or immunoblot displayed reduced sensitivity, therefore the ELISA was chosen for further development. The pADPr ELISA described in this study is characterized by ease of use, standard preparation of protein lysates, robustness, ability to sample at multiple timepoints and use of frozen biological material without need to further process

(permeabilize or assay) immediately. Overall, this is a consistent platform that reflects PARP activity and is amenable for use in the clinic, however, the optimization of a validated clinical ELISA is beyond the scope of this paper.

Overall, the dose and kinetics of pADPr generally paralleled tumor drug levels, providing strong evidence for the ability of ABT-888 to mediate PARP inhibition *in vivo*. In addition, a reduction in pADPr after cytotoxic treatment was demonstrated that mimics a clinical scenario where ABT-888 in combination with DNA damage agents could result in improved efficacy. PK/PD studies revealed significant drug levels in the tumor compared to plasma. While the degree of pADPr reduction at 2 h cannot clearly differentiate efficacious and non-efficacious doses (>50% pADPr inhibition and significant tumor drug levels at all doses), pADPr reduction at 24 h more closely reflects ABT-888 dose and drug level. The degree and duration of pADPr reduction after 5 days of dosing possibly reflect the accumulation of drug after multiple doses demonstrated by high tumor:plasma ratios as well as the residence time of ABT-888 which affect the relationship between pADPr level and *in vivo* efficacy. Although, the pADPr level may be a feasible biological marker for ABT-888 activity *in vivo*, it may be limited to assessment of pharmacological effect and dose rather than a predictive determinant of efficacious dose (the activity of ABT-888 *in vivo* correlates overall with tumor drug level and efficacy, where at least a ≥70% pADPr reduction is observed at effective doses). Nevertheless, a biological marker that correlates with *in vivo* efficacy and drug level can be very helpful in monitoring pharmacological effect in the clinic. Furthermore, our subsequent studies also demonstrated pAPDr reduction after ABT-888 treatment in peripheral blood mononuclear cells (PBMCs, data not shown), thereby supporting pADPr ELISA as a potential tool for the evaluation of pharmacological effect and correlation of pharmacokinetic levels and pADPr reduction in humans. A validated assay for PBMCs is currently providing guidance for determining timepoints for tumor biopsy collection in the clinical trials (48).

The ultimate use of biological markers is not only to monitor pharmacological effect, but also predict clinical outcome (49). While pADPr ELISA is able to demonstrate the level of PARP activity *in vivo*, inhibition thresholds that predict clinical efficacy or outcome are not yet fully understood. Multiple factors such as the type of cytotoxic used in combination therapy, PARP expression level and polymorphisms (associated with enzyme activity and/or progression in certain cancers) (50, 51), genetic factors (11, 12, 27) and epigenetic factors (52) can contribute to the overall response to therapy. Understanding the relationship of pADPr levels and these factors is critical in establishing predictability of therapeutic effect. Comprehensive clinical studies may be necessary to fully establish such efficacy–biomarker relationships as has been done for biological markers that exist for cancer today (*e.g.* CA-125 and *her2* expression for breast and PSA for prostate) (49). In conclusion, we have demonstrated potent antitumor efficacy of ABT-888 in combination with TMZ that correlated with drug level and reduction in tumor PARP activity *in vivo* by ELISA. ABT-888 was the first oncology agent to be evaluated in an exploratory Phase 0 clinical trial that utilized a validated pADPr biomarker ELISA based on the assay described here (28).

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