Supplementary Appendix

γH2AX in PBMCs

Blood Collection and Preparation

Whole blood was collected in 8-mL Becton Dickinson Vacutainer CPT tubes then centrifuged at 1500 x *g* for 30 minutes at 25°C. The plasma layer was carefully removed and the mononuclear cells collected into a sterile tube. Cells were washed twice with Plasma-Lyte A USP (Baxter) and pelleted at 430 x *g* for 10 minutes at room temperature. PBMCs were counted, resuspended to 2 x 10^6 cells/mL, and fixed in 2.2% (w/v) paraformaldehyde in Plasma-Lyte A USP for 20 minutes at room temperature. Cells were washed twice with Plasma-Lyte A USP and pelleted at 300 x *g* for 10 minutes at 4°C. Finally, cells were permeabilized in cold 70% ethanol for 5 minutes and stored at 4°C until processed for γH2AX immunofluorescence.

γH2AX Immunofluorescence Staining of PBMC Cytospin Slides

For each PBMC sample, cytospin spots of 1.5 x 10^6 fixed PBMCs were prepared on Superfrost plus slides using the Shandon Cytospin 4 (Fisher Scientific). Slides were air-dried for 2 minutes and then stained using the automated Bond-Max System (Leica Microsystems). In brief, slides were rinsed once with 5% hydrogen peroxide and then 3 times with Bond Wash Solution (Leica Microsystems) at room temperature. Slides were incubated for 1 hour at room temperature with 150 µL of 10 µg/mL biotin-conjugated anti-phospho-histone H2AX (Ser139), clone JBW301 (Millipore Corp) in Bond Antibody Diluent (Leica Microsystems) and then washed 3 times for 5 minutes

each at room temperature with Bond Wash Solution. Slides were incubated for 30 minutes with 150 µL of 5 µg/mL Alexa Fluor 555 goat anti-mouse IgG (H+L; Invitrogen) in Bond Antibody Diluent, washed twice for 5 minutes each at room temperature with Bond Wash Solution, and washed 3 times for 5 minutes each at room temperature with deionized water. Once slides were removed from the Bond-Max System, cover slips were mounted using ProLong gold anti-fade reagent with DAPI (Invitrogen).

Slides were examined using Zeiss LSM 510 meta (inverted) confocal microscope with plan-apochromat 63X/1.40 oil DIC M27 objective and Carl Zeiss AIM software (Carl Zeiss Microscopy). For each cytospin spot, 3 to 6 images were captured so that a total of 200 to 300 nuclei could be analyzed. The percentage of nuclear area positive (%NAP) for γH2AX was calculated using Image-Pro software (Media Cybernetics).

Pharmacokinetics

Samples were centrifuged, and plasma split for topotecan and ABT-888 analysis. Plasma for ABT-888 analysis was stored at –70°C. For analysis of topotecan lactone and carboxylate, an aliquot of plasma was immediately deproteinized by 4-fold dilution with dry ice–cold methanol (–20°C), vortexed for 30 seconds, and centrifuged at 4000 x *g* for 10 minutes. The supernatant was collected, transferred to a cryotube, and stored at –80°C until analysis.

All urine collected during each 8-hour time period was mixed, and then a sample of 10 mL reserved and stored at –80°C until analysis. Extraction of topotecan from patient

plasma was achieved by first acidifying topotecan with 10 µL H_3PO_4 (85%) to change all topotecan in patient plasma (200 µL) to its lactone form and then precipitating the plasma proteins with MeOH (1 mL) followed by 30-second vortexing. The mixture was spun at 10,000 rpm for 3 minutes at 4°C to obtain supernatant. Fifty microliters of the supernatant was injected into the HPLC system for quantitative analysis. The topotecan HPLC system was composed of an analytical column LiChrospher 60 select B (125 x 4 mm, 5 µm) preceded by a pre-column LiChrospher 100 RP-18 (4 x 4 mm, 5 µm). An isocratic mobile phase (pH 6.0) containing MeOH (65%), 250 mM docusate sodium salt (4%), 1 mol/L Na_3PO_4 (2.3%, pH 6.0), triethyl amine (0.3%), and H_2O (28.4%) was used to elute topotecan from the columns at a flow rate of 1 mL/min.

ABT-888 was extracted from plasma using a separation column. After adding 500 µL plasma sample to the separation column, the column was pre-washed with 1 mL H_2O, 1 mL 0.1% formic acid, and 1 mL MeOH, separately. ABT-888 and its internal standard were eluted by 2 mL of 0.4 mol/L ammonium formate in MeOH. The eluents were collected in 5-mL conical bottom glass tubes and dried at room temperature under a stream of nitrogen. Dried residues were reconstituted in 100 µL of 10% ACN, vortexed, and then transferred into glass micro-tube inserts for HPLC analysis. The HPLC system was composed of an Aquasil C18 (250 x 4.6 mm, 5 µm) preceded by a Brownlee NewGuard RP-18 precolumn (15 x 3.2 mm, 7 µm). A gradient elution profile composed of mobile phase A [H_2O:ACN (32:68, v/v) and 0.1% formic acid] and B [H_2O:ACN (89:11, v/v) and 0.1% formic acid] was used to elute ABT-888 and its internal standard from the columns at a flow rate of 1 mL/min.

Human Tumor Xenograft Models

NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (1).

Athymic nude (nu/nu NCr) mice obtained from the animal production facility at NCI-Frederick were given autoclaved feed and hyper-chlorinated water ad libitum. Female athymic nude mice were implanted subcutaneously with A375 human melanoma (topotecan-sensitive) tumors when the mice were 6 to 8 weeks of age as described (2). Cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and grown in humidified incubators supplemented with 5% CO_2; the cell-culture media were maintained according to ATCC recommendations. A375 cells were cultured for less than 6 months, and were not reauthenticated. Tumor implantation was considered study day 1.

Tumor Measurements and Statistical Analysis

Data for the left and right bilateral tumors were pooled for each group. Statistical comparisons between the groups were performed by Student's t-test. The median tumor weights for each treatment group were used to calculate the following parameter: $\%T/C = (T_x - T_0/C_x - C_0) \times 100$, where T_x and C_x are the median tumor weights of the treated and control groups at any measurement day beyond the tumor staging day and T_0 and C_0 are the median tumor weights on the tumor staging day. The optimal %T/C is

defined as the minimum %T/C occurring in a particular treatment group for all of the time points measured; an optimal %T/C of 40% or less meets our minimum criteria for activity. Tumor growth was monitored by bi-dimensional caliper measurements. Tumor measurements (mm) were converted to tumor weights (mg) using the formula for a prolate ellipsoid: tumor weight (mg) = [tumor length x (tumor width^2)]/2. Serial body weights were also measured to evaluate the toxicity associated with repeated dosing. Toxicity was assessed as doses resulting in euthanasia or body weight loss in excess of 20%.

Drug Administration

ABT-888 was prepared in a vehicle containing 105 mg sorbitol and 5.17 mg citric acid monohydrate per mL of distilled water at a final concentration of 0.313 mg/mL and was administered at 0.1 mL/10 g body weight given by oral gavage (PO). Topotecan was prepared at a concentration of 0.15 mg per mL of water for dosing at 0.1 mL/10 g body weight by intraperitoneal (IP) injection.

Mice were randomized to receive vehicle (0.1 mL/10 g body weight once daily for 5 days [QDx5] PO); ABT-888 (3.13 mg/kg once daily for 9 days PO); topotecan (1.5 mg/kg QDx5 IP); or the combination of ABT-888 with topotecan. The number of doses and relative time of administration of ABT-888 varied (Fig. 2), but the doses were identical. Drug administration commenced on day 9 post–tumor implantation, when tumors were approximately 250 mg. Each cohort contained 8 mice (16 in vehicle control group) randomized into groups using the StudyLog randomizer (StudyDirector).

The ABT-888 dose of 3.13 mg/kg is associated with a reduction in tumor PAR levels in previous single-dose experiments in mice (2, 3). The topotecan dose of 1.5 mg/kg per day for a total of 5 doses is less than the murine maximum tolerated dose for topotecan of 4.7 mg/kg but was considered more likely to result in a clinically achievable plasma concentration of topotecan (4, 5).

References

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Supplementary Table S1.

Schedule	Optimal %T/C[a] (Day 17)	%T/C[a] (Day 31)
Topotecan QDx5	7	30
Topotecan QDx5 + ABT-888 QDx1	-6	13
Topotecan QDx5 + ABT-888 QDx2	-8	6
Topotecan QDx5 + ABT-888 QDx3	-8	8
Topotecan QDx5 + ABT-888 QDx5	1	15
ABT-888 QDx9	77	72

[a]%T/C = $(T_x - T_0/C_x - C_0) \times 100$, where T_x and C_x are the median tumor weights of the treated and control groups and T_0 and C_0 are the median tumor weights on the tumor staging day.

Supplementary Table S2. Pharmacodynamic assay results by patient and dose level after ABT-888 and topotecan

Dose level	Patient No.	PAR response		γH2AX response		
		Tumor biopsy[a]	PBMCs[b]	Tumor biopsy[c]	PBMCs[d]	CTCs[e]
1	1	ND	–	ND	ND	ND
	2	ND	+*	ND	ND	ND
	3	ND	–	ND	ND	ND
	4	+	ND	ND	ND	ND
	5	ND	+	ND	ND	ND
	6	ND	+	ND	ND	ND
–1	7	ND	+	ND	ND	ND
	8	+	+	–	ND	ND
	9	ND	–	ND	ND	ND
–2	10	ND	+	ND	+	ND
	11	ND	+	ND	+	ND
	12	ND	+	ND	+	ND
–3	13	ND	+	ND	+	ND
	14	ND	+	ND	–	ND
	15	+	+	–	–	ND
	16	ND	+	ND	–	ND
	22	ND	+	ND	+	ND
	23	ND	+	ND	–	–
	24	ND	–	ND	–	+
1A	17	ND	+	ND	ND	ND
	18	ND	+	ND	ND	ND
	19	ND	+	ND	ND	+
	20	ND	+	ND	ND	ND
	21	ND	+	ND	ND	+

[a]Greater than 75% decrease in PAR levels in tumor biopsies collected 3 to 7 hours after combination treatment, compared to baseline.

[b]Greater than 50% decrease in PAR levels in peripheral blood mononuclear cells (PBMCs) at any time point (2, 4, 7, or 24 hours) after combination treatment, compared to baseline.

*Patient 2 had a decrease in PAR only after administration of ABT-888 only and not the combination treatment.

[c]Increase in percent nuclear area positive for γH2AX in tumor biopsies collected 3 to 7 hours after combination treatment, compared to baseline.

[d]Increase in percent nuclear area positive for γH2AX in PBMCs at any time point (2, 4, 7, or 24 hours) after combination treatment, compared to baseline.

[e]Increase in the fraction of circulating tumor cells (CTCs) positive for γH2AX on day 2 or 5 of treatment, compared to baseline

Abbreviation: ND, not determined.

Figure Legends

Supplementary Fig. S1. PAR levels relative to baseline (100%) in PBMCs from patients on Schedule A (A), B, (B), and C (C). Samples were collected pre-dose (0 hours) and at 2, 4, 7, and 24 hours after drug administration.