Supplementary Materials

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Supplement to: Kinders RJ, Hollingshead M, Khin S, et al. Preclinical Modeling of a Phase 0 Clinical Trial: Qualification of a Pharmacodynamic Assay of Poly (ADP-Ribose) Polymerase in Tumor Biopsies of Mouse Xenografts

Supplementary Materials and Methods

PAR Assay Method

Extracts were assayed for total protein concentration using a Bicinchoninic Acid (BCA) Assay Kit (Pierce; Rockford, IL) adapted for use in a 96-well plate format according to the manufacturer's instructions. The standard was bovine serum albumin (BSA), also purchased from Pierce. Biopsy extracts were diluted 1:5, tumor extracts 1:10 for the assay, and further diluted if necessary. PAR Standards are prepared from pure PAR (Cat.#SW-311; BioMol International; Plymouth Meeting, PA) diluted in Superblock (Cat.# 37535; Pierce) to a final concentration of 100 ng/mL. These stocks may be stored frozen at -80ºC for up to 6 months, but should not be refrozen after thawing. Assay standards are prepared by 1:2 serial dilution into Superblock, with the high standard set to 2 ng/mL, and the final dilution at 7.8 pg/mL; assay dynamic range is 31 pg/mL to 2000 pg/mL PAR, with an LLQ of approximately 15 pg/mL PAR. Standards may be stored at 2ºC to 8ºC overnight for re-use. Pierce Reactibind (Cat.# 15042) plates were coated with Trevigen Monoclonal anti-PAR (Cat.# 4335) diluted to a concentration of 4 µg/mL in pH 9.6 carbonate buffer, 100 µL per well, overnight at 2ºC to 8ºC. Plates were aspirated and washed once with PBS/0.1% Tween 20 (Sigma-Aldrich; St. Louis, MO) on a BioTek ELx405 plate washer (BioTek Instruments, Inc.; Winooski, VT). Blocking was performed with 250 µL/well Superblock at 37ºC for one hour. Plates were washed on the plate washer 4 times prior to proceeding with the assay.

Tumor lysate (10 µL) adjusted to an extract protein concentration of 0.05 µg/µL to 1 µg/µL was added to wells containing 65 µL Superblock (Pierce), and the specimen was incubated in the plate for 16 hours ± 1 hour at 2ºC to 8ºC. Prior to adding tumor lysate, clarified extracts were assayed for total protein using a BCA Assay (Pierce) and then diluted in Superblock for immediate assaying, or in 2% BSA/1% SDS (Sigma-Aldrich) for storage at –80ºC. All specimens were assayed in duplicate. The standards were pure PAR polymers (BioMol International) diluted in Superblock to a final concentration of 10 ng/mL. Control samples, Colo829 extracts, were used at Superblock dilutions covering the range of the standards (0, 15.6 pg PAR/mL–2000 pg PAR/mL).

a. Assay Standards were added, 75 µL per well, in duplicate wells first, and then specimen extracts were added to wells according to the templates, and finally controls were added to wells.

b. Specimen extracts were diluted to a protein concentration of 0.1 µg to 1 µg per µL. Using an assay template, 65 µL of Superblock was added to all wells to be used for specimen assay. Wells along the bottom and top rows of the plate were filled with 75 µL Superblock and served as assay zeros. Extract (10 ng/mL) was then added to each specimen well. Final protein concentrations per well were held in the range of 1 µg to 5 µg, using the appropriate dilution of the extract.

c. Assay controls, composed of extracts of Colo829 cells, were prepared as described above and diluted in Superblock + 2% BSA (W/V, ELISA-grade; Sigma-Aldrich). To obtain readings at critical points in the standard curve, approximately 50, 100, 300, and 1000 pg/mL PAR, 10 µL per well were added to the first and twelfth rows of the plate and run with every assay.

d. Plates were covered with acetate sealers (Cat.# 3501; Thermo Labsystems; Franklin, MA), and incubated overnight (16 ± 1 hour) at 2ºC to 8ºC.

e. The next morning, probe antibody reagent was prepared as follows: rabbit anti-PAR (Cat.#4336; Trevigen; Helgerman, CT) was diluted 1:500 into PBS/2% BSA supplemented with 1% mouse serum (V/V; Sigma-Aldrich); this solution was allowed to stand at ambient temperature for 1 hour prior to use. Plates were washed 4 times on the platewasher then 75 µL of the probe solution was added to each well. Plates were covered with fresh acetate sheets, and placed into an incubator at 25ºC for 2 hours.

f. Conjugate solution was prepared at least one hour in advance in the same diluent as the probe. HRP-conjugated affinity-purified goat anti-rabbit (Cat.#074-15-061; KPL, Inc.; Gaithersburg, MD) was added to a final concentration of 1 µg/mL. Plates were washed 4 times, then 75 µL of the conjugate solution was added per well. Plates were covered with fresh acetate sheets, and placed into an incubator at 25ºC for 1 hour.

g. At the end of the conjugate incubation, plates were washed 4 times, and 75 µL of Supersignal Pico Chemiluminescent Substrate (Cat.# 37070; Pierce) was added to each well in a room without direct sunlight and with fluorescent lights dimmed. Plates were read on a Tecan Infinite or Tecan Genios Pro Luminometer (Tecan; Mannedorf, Switzerland), programmed to shake the plates for 5 seconds followed by a 30-second wait period, before commencing

reads. Reads were routinely performed from 1 minute to 10 minutes after addition of substrate without loss of assay performance.

PAR Immunoassay Validation

Quantitative validation was carried out to establish assay accuracy and precision; accuracy was evaluated by spike/recovery of pure PAR standards in tumor biopsy extracts and by analysis of dilution linearity of specimens spiked with pure PAR. Assay precision measured inter-operator, inter-site, and inter-day variability of results. Precision experiments were repeated on three separate days.

Assay specificity was checked by Western blotting cell extracts to confirm specific reactivity with PAR. Cross-reactivity of the rabbit polyclonal anti-PAR with serum albumin was noted on the Western blots (data not shown), hence the use of BSA in the probe and conjugate diluents to absorb out this cross-reactivity. Recovery experiments were performed to assess accuracy and confirm the absorption of the serum albumin cross-reactivity of the rabbit anti-PAR antibody; success of absorption was determined by spike recovery. Antibodies tested negative for cross-reactivity with nucleic acids by the manufacturer.

Assay interference by double-stranded DNA was assessed by adding DNA or DNase I to specimens and PAR-spiked specimens. DNA interference was passive, primarily due to increases in specimen viscosity. DNase I treatment improved specimen viscosity and overall assay precision, but the effect was not additive to the boiling step described above for tumor extract preparation. No interference by nicotinamide adenine dinucleotide (NAD+), ABT-888, or adenosine diphosphate (hydroxymethyl) pyrrolidinediol (ADP-HPD) was detected.

Recovery experiments were performed as follows: pure PAR was prepared in Superblock as for a standard curve determination and was then spiked into extracts from two A375 xenografts from vehicle-treated mice (XG125 and XG17) at a final concentration of 31.25, 62.5, 125, 250 and 500 pg/mL. Extracts were pre-diluted in Superblock to 2, 4, 8, and 10 µg protein per 37.5 µL. Extracts were added to wells containing either 37.5 µL of the assay diluent or 37.5 µL of pure PAR standards in duplicate wells, and then assayed as described in the methods section. Assay

controls and standards were run on each plate. Each recovery experiment was performed twice. The overall recovery of pure PAR spiked into xenografts was 94% ± 5.7% (mean ± SD; Supplementary Table S1 and Supplementary Fig. S1). The high correlation coefficient for XG17 was due to non-linear dilution below 2 µg extract per well.

Xenograft extracts were also tested by diluting the extracts to a specific protein concentration from 0.5 µg to 25 µg protein per well (as determined by the BCA assay), and then back-calculating the concentration of PAR and normalizing per 100 µg protein load. Strong signal suppression was observed in all tumor extracts tested at protein loads above 10 µg per assay well. Specimen dilution non-linearity was observed and controlled by limiting the amount of specimen loaded per assay well to 0.5 µg to 5 µg protein, as determined by the microtitre protein assay (BCA). Total assay imprecision was estimated to be <9%, and accuracy was 100% ± 15% (mean ± SD, as assessed by recovery. Intra-assay coefficient of variation (CV) was typically around 2%. Linearity of dilution was observed only in the range of 2 µg to 5 µg protein per well for all tumor extracts tested. Non-linearity was attributable to three factors: 1) the use of pure PAR standard as the basis of concentration calculation combined with the use of an antiserum raised to PAR conjugated to protein; 2) the cross-reactivity of the probe antibody to albumin; as tumors were not perfused, the amount of albumin present in an extract is not controlled, but will dilute as extract protein dilutes; 3) the dilution of detergents in the extraction buffer, which did not interfere with antibody binding to pure PAR, but probably interfered with binding to PAR conjugated to proteins in the xenograft extracts. Therefore at higher extract dilutions PAR readouts will fluctuate unpredictably based on the three competing factors. Thus, specimen dilution non-linearity was observed and controlled for by limiting the amount of specimen loaded per assay well to 2 µg to 5 µg protein, as determined by the BCA protein assay.

Dilution linearity was tested by diluting Colo829 cell extract assay controls into Superblock and back-calculating the PAR concentration in the starting material at each dilution tested. Dilutions covered the range of the standards (0 pg PAR/mL and 15.6 – 2000 pg PAR/mL), resulting in low, medium, and high controls. Dilution linearity results for the high and low controls are provided in Supplementary Table S2.

Assay precision was also validated to establish inter-operator, inter-site, and inter-day variability of results. Four control A375 cell line extracts and three pure PAR solutions (31.25, 62.5 and 125 pg/mL) plus the assay zero were assayed by two technicians on the two Tecan instruments for 3 days. On the second day, the technicians switched instruments. Percent CV of apparent specimen concentrations based on reading the standard curve are reported except for the assay zero, which is reported as the percent CV of the instrument. Inter-plate duplicates assayed using Colo829 cells were within 2% CV (Supplementary Fig. S2).

Results

No significant differences were observed in PAR levels between the first and second quadrants from the large or small tumors (Supplementary Table S3). PAR levels were similar in these four groups, with comparable ranges, as indicated by the 95% CIs. The first and second piece of the large tumors contained mean PAR levels of 5258 units and 5910 units, respectively, while the mean PAR levels in the first and second piece of the small tumors were 4176 units and 4115 units, respectively. An "average" tumor PAR level was also calculated from bilateral xenograft tumors of each individual animal by pooling all measurements (two quadrants from each of the large and small tumors). The CI for the PAR levels in the first and second piece of the large and small tumors included the lower limit of the CI for the PAR levels in this "average" tumor specimen from all four groups. The upper limit of the mean CI from the "average" tumor specimen was not included within the CI range for the small tumor group, suggesting slightly lower mean PAR levels in the small tumors.

The PAR content of the individual biopsy specimens ranged from 3363 units to 16,430 units, and PAR levels were randomly distributed between the bilateral tumor sites and across individual animals (Supplementary Table S4). PAR levels were not related to the mass of the biopsy specimen, and the wide range in biopsy yields was due to the needle completely passing through the small xenograft tumors. PAR levels in extracts from all biopsies were sufficiently high to extend the dynamic range of the assay to at least 5% of the control PAR levels, proving that drug-induced reductions in PAR levels of 40% to 95% could be quantified (the lower limit of quantitation [LLQ] was 14 units, defined here as three standard deviations above the mean zero divided by the slope of the standard curve). Two specimens (Supplementary Table S4, 1a and 2b) had a substantially higher protein content (>45%) than the other samples (15%–23%), which were also higher than the values expected for animal soft tissue from classical biochemistry, suggesting the presence of excessive plasma proteins or connective tissue in the samples.

SUPPLEMENTARY TABLES

Supplementary Table S1. Recovery of pure PAR spiked into A375 xenografts.

	Xenograft 125		Xenograft 17	
Protein Load	**Intrinsic pg/mL PAR**	**% Recovery**	**Intrinsic pg/mL PAR**	**% Recovery**
1 µg	192.2	87	179	87.9
2 µg	336.1	98.4	358	101.7
4 µg	617.3	90.7	716	98.9
5 µg	833.2	98.5	895	91.5
Overall Recovery		91		95

Supplementary Table S2. Dilution linearity for Colo829 cell extract high and low controls.

Tumor Extract Dilution	**Readout**		**Dilution Corrected**
	pg PAR/mL	**%CV**	
C829 High 1:2	937.4	7.2	1875
C829 High 1:4	524.7	13.7	2099
C829 High 1:5	463.3	8.9	2316
C829 High 1:8	322	2.8	1932
C829 Low 1:2	354	7.2	708
C829 Low 1:4	175	2.8	700
C829 Low 1:8	101	3.9	808
C829 Low 1:10	75	6.2	750
C829 Low 1:12	58	4.1	696

Supplementary Table S3. PAR levels in two quadrants ("first piece" and "second piece") from resected large and small tumors of bilateral untreated Colo829 xenografts.

Sample type	Large tumor PAR levels		Small tumor PAR levels		Average tumor* PAR levels	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
First piece†	5258	2663–7853	4176	2311–6041	–	–
Second piece†	5910	3425–8395	4115	2310–5920	–	–
Combined pieces of large and small tumors‡	–	–	–	–	5057	3899–6215

*Total extracts from all the samples assayed; †n=6 animals per group (untreated); two quadrants ("first piece" or "second piece") were dissected from large or small xenografts, which occurred randomly to either the right or left flank of each mouse; ‡n=6 animals per group x 2 tumors per animal x one half of each tumor. All units are pg PAR/mL per 100 μg protein. CI=confidence interval.

Supplementary Table S4. Mass and yield of total protein and PAR levels from 18-gauge Temno needle biopsies of Colo829 xenografts.*

Specimen number	Weight (mg)	Protein lysate (µg)	Readout (pg PAR/mL)	PAR Levels
1a†	4	1811	616	5101
1b	7	1568	1200	11480
2a	6	1332	417	4694
2b†	3	1350	1479	16430
3b‡	7	1496	508	5094
4a	7	1503	431	4305
4b	7	1840	412	3363
5a	8	1643	559	5104
5b	12	1883	1891	15060
6a	6	1592	1478	13930
6b	12	1842	1012	8237

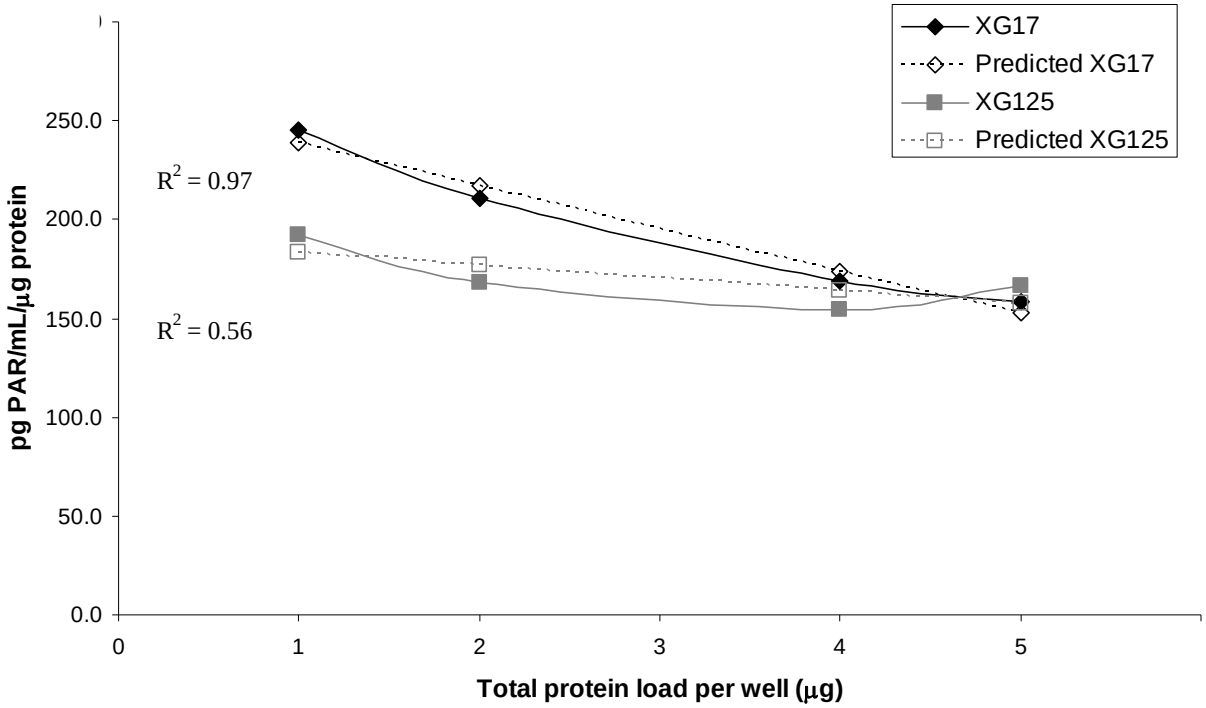
*Biopsies (2/animal) were collected from available xenografts in tared snap-cap microfuge tubes and processed in 200 µL lysis buffer; †specimens with high-protein levels (>45%), suggestive of excessive plasma or connective tissue in the sample; ‡only one biopsy was obtained. All units are pg PAR/mL per 100 µg protein.

Supplementary Table S5. PAR levels from needle biopsy samples of subcutaneous tumors in Colo829 and A375 xenografts.*

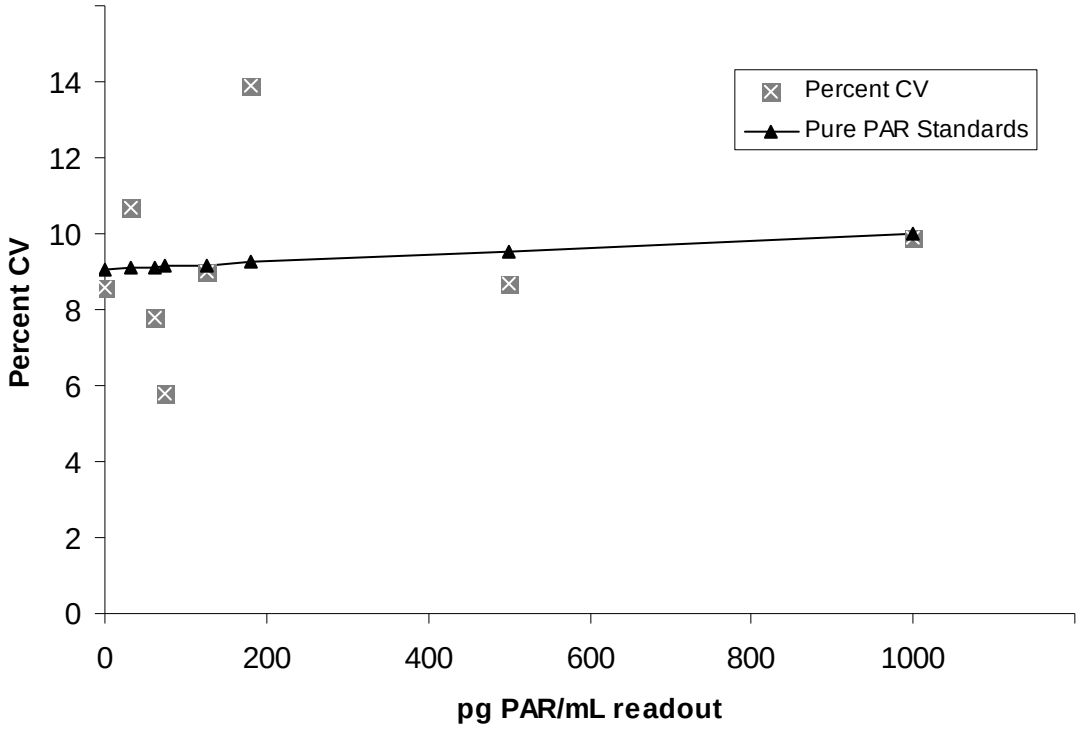
	Colo829		A375	
	Vehicle	**ABT-888 12.5 mg/kg**	**Vehicle**	**ABT-888 12.5 mg/kg**
Pre-treatment baseline				
Average (%CV)	5289 (20%)	6532 (27%)	32,811 (13%)	34,790 (17%)
95% CI	(4333–6245)	(5421–7643)	(28,766–36,856)	(30,790–38,790)
4-hours post dose				
Average (%CV)	4261 (30%)	491 (22%)	31,635 (7%)	327 (79%)
95% CI	(2699–5823)	(356–626)	(25,985–37,285)	(6–648)

*n=5 animals/group; biopsy samples were collected at baseline and 4-hours post single dose of ABT-888 (12.5 mg/kg) or vehicle. CI=confidence interval; CV=coefficient of variation. All units are pg PAR/mL per 100 μg protein.

SUPPLEMENTARY FIGURES



Supplementary Fig. S1. Dilution linearity of A375 xenograft extracts versus protein load. Extracts were assayed in triplicate and the results normalized to a 1 μg total protein load. The predicted dilution curve (open symbols) for PAR concentration per μg protein would be flat with a low R^2 value. Thus, XG17 yielded an unexpected inverse correlation between protein load and PAR concentration at loads below 2 μg per well (solid diamond). Variation between replicates was less than 2%; error bars are concealed by the symbols.



Supplementary Fig. S2. Inter-plate precision, assay standards, and controls. Standards and controls were run as unknowns and read against the standard curve (to yield a PAR concentration for precision analysis) on 3 consecutive days. Solid triangle, pure PAR standards (0, 31, 62, 75, 125, 180, 500, and 1000 pg/mL); X in square, Colo829 cell extract assay controls. Error bars for within-run replicates were less than 2%.