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A Novel Immunoassay (ELISA) for Quantitative γH2AX Detection and Pharmacodynamic Monitoring of DNA Damage Induced by Chemotherapeutic Agents and PARP Inhibitors

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Introduction

A quantitative immunofluorescence assay (IFA) for γH2AX, a marker for DNA damage response (DDR) (1) and apoptosis (2, 3), was previously developed by our group (4). To accommodate situations where high-throughput quantitation is needed, we developed a 96-well plate- based enzyme-linked immunosorbent assay (ELISA) for quantifying γH2AX. The assay uses a pair of high-affinity γH2AX antibodies for protein capture and detection and a synthetic peptide calibrator. We compared the ELISA with γH2AX detection by both Western blot and IFA and assessed assay utility in a mouse xenograft model. Treatments evaluated preclinically using the γH2AX ELISA included the topoisomerase I inhibitors topotecan and irinotecan (4), the apoptosis-inducing biomolecule TRAIL (TNF-related apoptosis-inducing ligand) (3), ionizing irradiation, and poly(ADP-ribose) polymerase (PARP) inhibitors ABT-888, AZD2281, and MK-4827 (5).

Methods

The human colon cancer cell line HCT 116 was used for ELISA development and comparisons to Western blot and IFA; the human melanoma cell line A375 was used for xenograft mouse studies. Both cell lines were acquired from ATCC and cultured with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. In vitro treatments were topotecan, TRAIL, and ionizing radiation. Cells were extracted in 100 µL (per million cells) of Cell Extraction Buffer (Invitrogen) with protease inhibitors (Roche) and 1% PMSF (Sigma-Aldrich) on ice for 30 min for ELISA and Western blot analysis. In parallel, cell aliquots were spotted on slides by cytospin and γH2AX detection was performed as previously described (4). γH2AX levels were determined using Adobe Photoshop (Adobe Systems) by measuring the mean green intensity in at least 50 nuclei.

To show that the γH2AX ELISA was useful in drug screening assays, a xenograft model was established in female athymic *nu/nu* (NCr) mice with A375 by s.c. injection (1.0×10^7 cells per 0.1 mL/mouse) on the lateral body wall, just caudal to the axilla. Treatment agents included 3 PARP inhibitors, ABT-888 (NSC 752840), AZD2281 (NSC 753686), and MK-4827 (NSC 754355), and irinotecan (NSC 616348). The vehicle treatment was sorbitol and lactic acid in sterile water at a dose of 0.1 mL/10 g. Doses and administration routes are described in the figures. Mice were anesthetized by isoflurane gas inhalation before biopsy.

For the γH2AX ELISA, 96-well Pierce reacti-bind plates were coated with phospho-H2AX (S139) mouse monoclonal antibody (Millipore). The antibody were diluted to a concentration of 4 µg/mL in 0.1 M pH 9.6 carbonate buffer (Sigma-Aldrich) at 100 µL/well and incubated for 2 h at 37°C. A γH2AX synthetic peptide standard or whole-cell extract was added to each well and incubated for antigen capture overnight at 4°C. Plates were then incubated with 100 µL/well of 2 µg/mL γH2AX rabbit polyclonal antibody (Abcam) diluted in 2% BSA/PBS supplemented with 1 µL/mL mouse serum for 2 h at 24°C. Subsequently, 100 µL/well HRP-conjugated affinity-purified goat anti-rabbit (KPL) was added at a final concentration of 1 µg/mL diluted in 2% BSA/PBS, supplemented with 1 µL/mL mouse serum, and incubated for 1 h at 24°C. Finally, 100 µL/well of freshly prepared SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific) was added to the plate and immediately read on a Tecan Infinite M200 plate reader. The RLU (relative light unit) values were exported as an Excel file from the plate reader to generate the γH2AX standard curve. The average γH2AX level for each cell extract was then determined using the standard curve.

For the xenograft data, γH2AX levels were compared by Student's t-test (unpaired, 2-sided, assuming unequal variances) for irinotecan with or without PARP inhibitor for each of the 3 inhibitors. For each PARP inhibitor, the comparisons were made for both dose levels and at 6 h and 24 h.

Results

Comparison of γH2AX quantitation using ELISA, immunofluorescence assay, and Western blot in HCT 116 cells

γH2AX Readouts

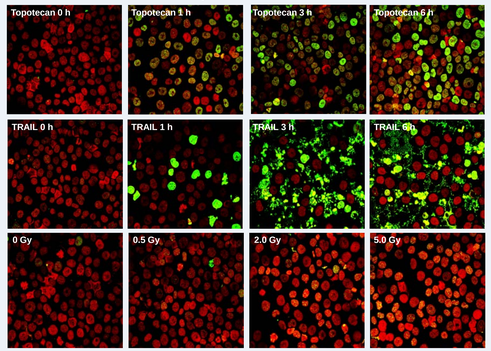
Treatment		ELISA (pg/mL)	IFA (RLU)	Western blot (RLU)
Topotecan (1 µM)	Control	418	2.5	2.5
	0 hr	384	0.7	4.7
	1 hr	7850	23.0	8.1
	3 hr	9700	43.9	6.9
	6 hr	14834	51.2	14.3
TRAIL (0.1 µg/mL)	0 hr	414	2.0	2.7
	1 hr	8555	34.5	17.3
	3 hr	28708	72.2	45.8
	6 hr	23840	49.6	43.5
Ionizing radiation	Control	383	1.3	2.5
	0.1 Gy	378	2.2	2.8
	0.5 Gy	482	2.4	4.2
	1.0 Gy	481	4.3	4.1
	2.0 Gy	497	7.9	5.2
	5.0 Gy	877	13.2	4.3

Assay Correlation

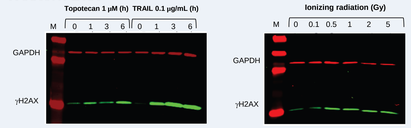
Comparators	Pearson's correlation (*r*) ELISA vs IFA	ELISA vs Western blot	IFA vs Western blot
1 µM topotecan*	0.96	0.89	0.77
1 µg/mL TRAIL*	0.96	0.99	0.94
Ionizing radiation*	0.92	0.32	0.49

*Pearson's correlation determined for the pooled dose or time response to the indicated treatment types.

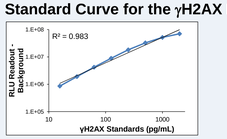
Immunofluorescence Assay



Western Blot

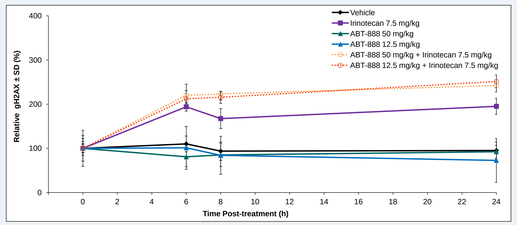


Standard Curve for the γH2AX ELISA

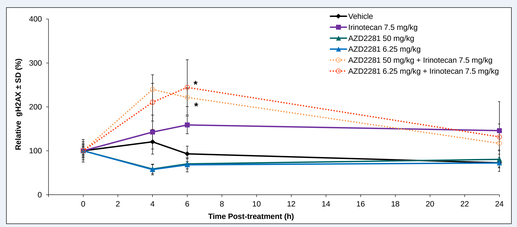


γH2AX quantitation after irinotecan and PARP inhibitor treatment in the A375 xenograft model

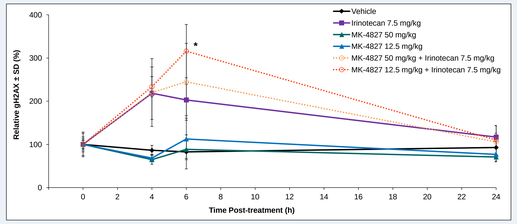
ABT-888



AZD2281



MK-4827



Mice were treated with irinotecan or PARP inhibitor alone or in combination at the doses indicated (n=6 mice/treatment group). Data presented as γH2AX levels relative to vehicle (%). The γH2AX ELISA was used for γH2AX quantitation.
*Statistically significant difference in γH2AX levels compared to irinotecan treatment alone ($P<0.05$, unpaired t-test).

Summary

- The γH2AX ELISA will be useful in situations when higher throughput and accurate quantitation are needed and γH2AX analysis does not need to be restricted to nuclear foci.
- The ELISA can quantify γH2AX concentrations as low as 16 pg/mL in crude extracts from cancer cells and solid tumors.
- The assay has a lower limit of detection of < 4 pg/mL, upper limit of quantitation of 2000 pg/mL, and a coefficient of variation of 20%.
- The ELISA detected changes in γH2AX induced by a variety of treatments in vitro and was overall highly correlated to both IFA and Western blot following treatment with irinotecan or TRAIL. With the less active treatment of ionizing radiation, only a modest correlation between ELISA and Western blot was observed, but the high correlation with IFA remained.
- In a mouse xenograft model, the assay detected apparent potentiation of DNA damage with combined irinotecan and PARP inhibitor treatment, an effect that reached statistical significance versus irinotecan alone for the lower dose of MK-4827 with irinotecan; and both doses of AZD2281 with irinotecan at the 6-h post-dose time point.
- The assay shows utility for drug discovery screening, molecular pharmacology studies, and pharmacodynamic monitoring.

References

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Acknowledgments

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