

Development of a Validated Immunofluorescence Assay for γ H2AX as a Pharmacodynamic Marker of Topoisomerase I Inhibitor Activity

Robert J. Kinders¹, Melinda Hollingshead⁴, Scott Lawrence², Jiuping Ji¹, Brian Tabb², William M. Bonner⁵, Yves Pommier⁵, Larry Rubinstein⁶, Yvonne A. Evrard³, Ralph E. Parchment¹, Joseph Tomaszewski⁶, and James H. Doroshow^{5,6}; for the National Cancer Institute Phase 0 Clinical Trials Team

Abstract

Purpose: Phosphorylated histone H2AX (γ H2AX) serves as a biomarker for formation of DNA double-strand break repair complexes. A quantitative pharmacodynamic immunofluorescence assay for γ H2AX was developed, validated, and tested in human tumor xenograft models with the use of clinically relevant procedures.

Experimental Design: The γ H2AX immunofluorescence assay uses a novel data quantitation and image processing algorithm to determine the extent of nuclear-specific γ H2AX staining in tumor needle biopsies and hair follicles collected from mice bearing topotecan-responsive A375 xenografts. After method validation with the topoisomerase I (Top1) inhibitor topotecan, the assay was used to compare pharmacodynamic properties of three structurally related indenoisoquinoline Top1 inhibitors.

Results: γ H2AX response to topotecan was quantified over a 60-fold dose range (0.016-1.0 times the murine single-dose maximum tolerated dose), and significant pharmacodynamic response was measured at the mouse equivalent of the 1.5 mg/m² clinical dose as well as the lowest dose tested. Responses were within a time window amenable for biopsy collection in clinical trials. These studies enabled characterization of dose and time responses for three indenoisoquinolines, resulting in selection of two for clinical evaluation. γ H2AX response to Top1 inhibitors in hair follicles was also observable above a minimal dose threshold.

Conclusions: Our γ H2AX assay is sufficiently accurate and sensitive to quantify γ H2AX in tumor samples and will be used in correlative studies of two indenoisoquinolines in a phase I clinical trial at the National Cancer Institute. Data suggest that hair follicles may potentially serve as a surrogate tissue to evaluate tumor γ H2AX response to Top1 inhibitors. *Clin Cancer Res*; 16(22); 5447-57. ©2010 AACR.

A validated pharmacodynamic assay is a fundamental component of early-phase proof-of-concept clinical trials of molecularly targeted agents in which a pharmacodynamic end point serves as a study objective (1-3). Assay results can inform and expedite drug development decisions if the assay is sufficiently accurate, sensitive, and precise to result in statistical significance for changes in the pharmacodynamic target that are biologically significant (1, 4). An initiative of the National Cancer Institute (NCI) Division of Cancer Treatment and Diagnosis is to develop and validate "clinically ready" pharmacodynamic assays well in advance of first-

in-human clinical studies to select the most promising new drugs for further evaluation (<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>).

Validation of clinical biomarker assays in preclinical models replicates the sample collection, handling, and analytic procedures that will be used in the clinical setting to ensure that reproducible results can be obtained by different laboratories. Variability attributable to the inherent molecular and histologic heterogeneity of each sample can be determined in preclinical tumor models to establish both whether target modulation is measurable and

Authors' Affiliations: ¹Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Support Directorate, ²Pathology/Histotechnology Laboratory, Laboratory Animal Sciences Program, and ³Applied/Developmental Research Support Directorate, Science Applications International Corporation-Frederick, Inc., National Cancer Institute-Frederick; ⁴Biological Testing Branch, Developmental Therapeutics Program, National Cancer Institute, Frederick, Maryland; and ⁵Laboratory of Molecular Pharmacology, Center for Cancer Research, and ⁶Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland

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Corresponding Author: Robert J. Kinders, Laboratory of Human Toxicology and Pharmacology, Applied/Developmental Research Support Directorate, Science Applications International Corporation-Frederick Inc., National Cancer Institute-Frederick, Post Office Box B, Frederick, MD 21702. Phone: 301-846-6410; Fax: 301-846-6536; E-mail: kindersr@mail.nih.gov.

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Translational Relevance

Optimal clinical development of a molecularly targeted agent requires the incorporation of validated pharmacodynamic assays to establish drug activity on target. This article details the development and validation of a quantitative immunofluorescence assay for phosphorylated histone H2AX (γ H2AX) as a biomarker for topoisomerase I (Top1) inhibitor activity. The assay was qualified in human tumor xenograft models with the use of clinically relevant procedures and will be used in a phase I clinical trial conducted at the National Cancer Institute to evaluate the extent to which structurally similar indenoisoquinoline Top1 inhibitors activate γ H2AX. In conjunction with toxicity and pharmacokinetic data, the pharmacodynamic results obtained will inform subsequent clinical development decisions. The γ H2AX assay was also optimized to explore use of minimally invasive skin biopsies as surrogates for tumor biopsies; these studies will also be evaluated clinically and could have important implications for monitoring patients' pharmacodynamic responses to chemotherapy with agents that damage DNA.

the extent to which a drug must modulate that target to show significant activity (5, 6). Method validation also ensures that the biopsy samples provided by patients for research purposes are handled according to the highest standards to allow meaningful analysis; this is especially important in phase 0 trials in which pharmacologically active but nontherapeutic doses of an agent are given to small numbers of patients (6). Our first validated pharmacodynamic assay, an immunoassay for poly(ADP-ribose), successfully showed proof-of-concept in a phase 0 clinical trial measuring pharmacodynamic response to the poly(ADP-ribose) polymerase inhibitor ABT-888 in patients with cancer (4, 7). Our second validated method, measuring nuclear phosphorylated histone H2AX at serine 139 (γ H2AX) levels in patient circulating tumor cells after chemotherapeutic treatment, was recently published and is being used in correlative studies in phase I trials at the NCI (8, 9). This study provided evidence of the specificity of the γ H2AX response after treatment with traditional DNA-damaging chemotherapeutic agents, such as topotecan and temozolomide, versus antiproliferation drugs that do not induce DNA damage (e.g., rapamycin).

γ H2AX is one of the earliest markers for DNA double-strand breaks, and signal levels directly correlate with the number of breaks formed (10, 11). Thus, γ H2AX has been suggested as a potential pharmacodynamic biomarker for assessing the activity of chemotherapeutic agents that inhibit double-strand break repair (12, 13). Treating cells with inhibitors of topoisomerase I (Top1), an enzyme involved in the relaxation of supercoiled DNA during replication and transcription, stabilizes the normally transient

DNA-Top1 cleavage complex, resulting in the formation of double-strand breaks at replication forks and transcription sites (11, 14, 15). The generation of γ H2AX foci after drug treatment has been shown, *in vitro*, to begin within 1 hour of double-strand break formation at levels proportional to the number of breaks formed (16–19).

Although the literature on γ H2AX as a metric for double-strand breaks induced by ionizing radiation is extensive (20, 21), less is known about the utility of γ H2AX as a marker for monitoring chemotherapy-induced DNA damage. Banuelos and colleagues did a feasibility study to monitor changes in γ H2AX foci formation with the use of an immunofluorescence-based assay on paraffin-embedded biopsies from patients with cervical cancer treated with cisplatin and ionizing radiation (22). Their study provided evidence that assaying γ H2AX within the first few days after treatment is important to eliminate loss of signal once DNA repair begins and tumor-infiltrating normal cells begin to populate the tumor. Recent immunofluorescence-based γ H2AX studies report fluorescent signal intensity in tissue sections; however, this approach can be problematic due to variable levels of background fluorescence (13, 23). Variability of intensity measurements can also arise during sample preparation, in which section thickness and overlapping nuclei within tissue sections can affect fluorescence intensity. The γ H2AX immunofluorescence assay described here uses mean intensity background autofluorescence to set signal intensity as a yes/no proposition, reducing overall background interference and measurement variance. Total positive nuclei are then quantified to yield a numeric estimate of the biomarker response.

Method validation for this study was done with the use of both positive and negative control tissues, and a calibrator panel of topotecan-responsive xenograft biopsies to standardize assay performance across different clinical research sites while providing a measure of assay performance over time for quality control. In addition, tumor needle biopsies and tissue samples were collected and handled following standard operating procedures established for the clinical poly(ADP-ribose) immunoassay (7). The assay was then used to compare three non-camptothecin indenoisoquinoline Top1 inhibitors; previous *in vitro* studies indicate that these agents produce more persistent DNA-Top1 cleavage complexes than camptothecin or topotecan, and have activity in camptothecin-resistant cell lines and the A375 human melanoma xenograft model (15, 18, 24, 25). Finally, skin biopsies in mice were collected after topotecan and indenoisoquinoline treatment to evaluate the potential use of hair follicles as surrogate tissues for measuring γ H2AX response.

Materials and Methods

Cell lines and animal models

Cell lines and animal models used for method development, optimization, and validation are detailed in the Supplementary Data.

Drug administration

Topotecan (NSC 609699) was obtained through the NCI Developmental Therapeutics Program, and the indenoisoquinolines NSCs 724998, 725776, and 706744 were initially synthesized by Dr. Mark Cushman (Purdue University, West Lafayette, IN; ref. 26), and provided by the Developmental Therapeutics Program Repository, NCI. Detailed drug administration information can be found in the Supplementary Data.

Method optimization and validation

Sample collection and handling conditions were optimized to match the specimen handling conditions that could be met in the clinical setting at both the NCI Clinical Center and associated extramural NCI sites. The biopsy method used was optimized to preserve protein phosphorylation status and tissue morphology, and has been successfully used in several NCI clinical trials (24, 27). Methods from biopsy processing through paraffin embedding were developed to balance tissue morphology and fluorescence-staining specificity. Details on method optimization and validation are detailed in the Supplementary Data.

Tumor biopsy and skin snip collection, and slide preparation

Biopsies were done as previously described (7). Details for biopsy and skin snip collection, fixation, and slide preparation are described in the Supplementary Data.

Antibody detection of γ H2AX

The certificate of analysis for the biotinylated γ H2AX monoclonal antibody JBW301 (Millipore Corp.) included western blot data showing its specificity. Before acceptance for use, JBW301 specificity was validated, and performance of specific lots of antibody was screened by western blot and on calibrator slides as detailed in the Supplementary Data. Biotinylated γ H2AX antibody binding was detected with the use of Alexa Fluor 488-labeled streptavidin (Invitrogen) and processed in a Bond-max Autostainer (Leica Microsystems). Detailed information on Bond-max Autostainer staining protocols, including antibody dilutions, and antigen retrieval steps can be found in the Supplementary Data. Prolong Gold containing 4',6-diamidino-2-phenylindole (Molecular Probes) was then placed on the sections, and slides were cover-slipped and allowed to dry for at least 1 hour in the dark before viewing. The adapted methodology for use on clinical samples is set forth in a standard operating procedure to be posted on the Division of Cancer Treatment and Diagnosis Web site (<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>).

 γ H2AX assay instrumentation and image selection, capture, and quantitation

Assay instrumentation, image capture, quantitation, and quality control for both xenograft tumor biopsies and hair follicles are detailed in the Supplementary Data.

Calibrators and controls

Selection and preparation of calibrator and control slide panels are detailed in the Supplementary Data.

Statistical analysis

Regression analysis, Student's *t*-test, ANOVA, and descriptive statistics were done with Microsoft Excel. ANOVA was calculated with the significance level (α) set at 0.05 (95% confidence level) for a two-tailed test. *P*-values up to 0.10, for these small group sizes, are reported in the tables. *P*-values ≥ 0.10 are reported as not significant.

Results**Assay instrumentation and image selection**

The image acquisition software and procedure were initiated after the field selection process. Fields for acquisition were selected in phase contrast or in the 4',6-diamidino-2-phenylindole (blue) channel. Criteria were evidence of good tissue morphology and nuclear integrity, and specifically excluded necrotic areas. Background fluorescence variation effects on assay measurements were controlled by the design of a macro that allowed dynamic scripting of the image capture criteria and were incorporated into the Image-Pro Plus control program (see Supplementary Data). The dynamic range of intensity was established with the use of γ H2AX-negative areas on tissue section slides to calibrate the camera settings and match a predetermined signal level on the light-response curve (background autofluorescence). Background was independently determined for each slide; this method allowed all slides to be examined against the same background fluorescence intensity. Selection of the γ H2AX-positive response threshold was based on multiple computer-based analyses of vehicle-treated xenograft biopsies. This approach converts imaged nuclei (location of the specific γ H2AX signal) into positive or negative events. Computer software was then used to score the biopsy images for percent nuclear area positive (%NAP). Quantitative characterization and estimated imprecision with the use of this image analysis method are described in the Supplementary Data and Supplementary Table S2.

Controls and calibrator panel

Specificity of the biotinylated γ H2AX monoclonal antibody, JBW301, was confirmed in each specimen run with the use of murine testis and small intestine as positive and negative controls, respectively (28, 29). Murine testis serves as a positive control for the assay because of the presence of γ H2AX at DNA double-strand breaks generated during meiotic recombination in the spermatocytes (30). Control and calibrator tissues were run on the same slides, and all tissues were subjected to the same probe antibody and reporter dye concentrations and incubation conditions. Few or no γ H2AX-positive cells were detected in untreated small intestine (positive cells, when present, were restricted

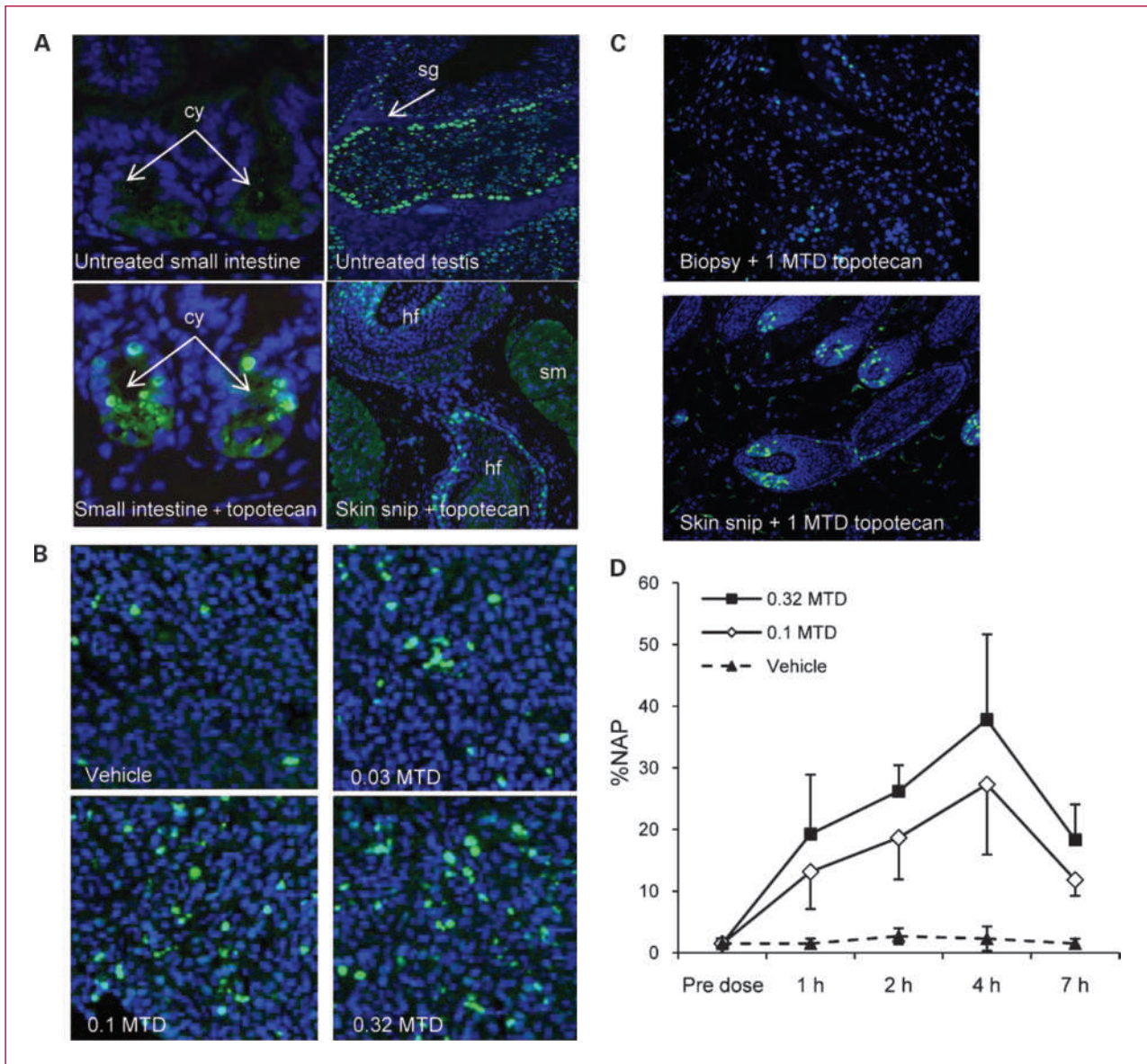


Fig. 1. Induction of γ H2AX in topotecan-responsive and nonresponsive xenograft tumor biopsies and hair follicles from mice treated with topotecan. A, assay specificity for γ H2AX immunostaining of untreated mouse small intestine (negative control), untreated mouse testis (positive control), and mouse small intestine 2 hours after treatment with 0.32 MTD topotecan. Bottom right, a representative section of γ H2AX staining in hair follicles isolated from skin snip samples taken 2 hours after treatment with 0.32 MTD topotecan. Magnification, $\times 400$. B, γ H2AX-stained topotecan-responsive A375 xenograft biopsy samples 2 hours post-treatment with vehicle, or 0.03, 0.1, or 0.32 MTD topotecan. Magnification, $\times 400$. C, γ H2AX staining in nonresponsive SK-MEL-28 xenograft biopsy (top) and hair follicle (bottom) samples from the same animal 5 hours post-treatment with 1 MTD topotecan. Magnification, $\times 200$. D, mean %NAP for γ H2AX in A375 xenograft biopsies from mice treated with vehicle control, or 0.1 or 0.32 MTD topotecan collected 1, 2, 4, and 7 hours post-dose; four mice per cohort. The mean %NAP for all topotecan-treated groups was statistically different from vehicle, with a significance level of $P \leq 0.001$, as determined by Student's *t*-test. cy, small intestine crypts; sg, spermatogonia; hf, hair follicle; sm, smooth muscle.

to the base of the crypts), whereas high numbers of γ H2AX-positive cells were detected within the testis (Fig. 1A, top). As expected, γ H2AX-positive cells were observed within the intestinal crypts of mice 2 hours after a single dose of 0.32 maximum tolerated dose (MTD) topotecan (Fig. 1A, bottom left). The γ H2AX level in the negative control tissue was set as the lower limit of detection for the assay.

Biomarker performance modeling

Fitness for the intended purpose of γ H2AX as a biomarker to measure DNA double-strand break damage induced by chemotherapy agents was established by determining the minimum change in γ H2AX levels that could be measured after treatment with a known effective drug. Topotecan was selected to model biomarker performance because the drug produces DNA double-strand

breaks by targeting the same Top1 molecule as the indenoisoquinolines. An additional consideration was that the NCI Biological Testing Branch has had extensive experience with topotecan and topotecan-responsive and non-responsive tumor models. Inspection of slides from topotecan-responsive A375 xenograft biopsies collected 1, 2, 4, and 7 hours after single-dose treatment with 0.03, 0.1, and 0.32 MTD topotecan revealed a dose-dependent γ H2AX response (Fig. 1B; 2-h time point shown) and served as the foundation for development of the quantitative method. In contrast, biopsies from nonresponsive SK-MEL-28 xenografts displayed a minimal γ H2AX response at 2 and 5 hours after single-dose treatment with 1 MTD topotecan (15 mg/kg), whereas hair follicles from the same mouse showed a strong γ H2AX response (Fig. 1C; 5-h time point shown); lower doses had no effect in SK-MEL-28 xenografts (data not shown).

Optimal sample collection time for peak γ H2AX response was determined in mice with A375 xenografts treated with a single dose of 0.1 or 0.32 MTD topotecan. Biopsy samples were collected 1, 2, 4, and 7 hours after treatment (Fig. 1D). Maximal γ H2AX response was evident at the 4-hour sampling time, after which the γ H2AX response decreased, likely due to cell death or resolution of double-strand breaks. At all post-dose time points, the mean %NAP in A375 xenografts of topotecan-

treated mice was significantly different statistically from that of vehicle control.

Interlaboratory reproducibility

Two laboratories, the National Clinical Target Validation Laboratory (NCI, NIH) and the Pathology/Histotechnology Laboratory (NCI-Frederick), did the γ H2AX immunofluorescence staining procedure on 75 matched xenograft slides (three to four specimens per slide) to determine interlaboratory slide processing, image acquisition, and analysis precision. The matched slides included A375 xenografts from mice treated with vehicle, 0.1 MTD topotecan, 0.32 MTD topotecan, and a range of indenoisoquinoline drugs and doses. Each laboratory stained and imaged a set of slides; slides were then exchanged and imaged at the alternate site. Image analyses and quantitation to determine the γ H2AX %NAP from both sets of slides were done by both laboratories; regression analysis of the data showed a high degree of correlation between the sites ($R^2 = 0.939$; Fig. 2).

Efficacy of indenoisoquinolines in suppressing A375 xenograft growth

Tumor growth inhibition efficacy of NSC 724998 compared with topotecan was tested in dose escalation studies. Both a log cell kill and a delay in tumor growth, as determined by changes in median tumor volume, were observed with increasing dose (Table 1 and Fig. 3A). When given on a schedule of once daily for 5 days, a 12-mg/kg dose of NSC 724998 (estimated to be half the MTD in mice at this schedule) was effective by both log cell kill and delayed growth metrics. The 16-mg/kg cohort of mice, treated for two cycles of daily for 5 days (17-d rest between cycles), resulted in a 259% growth delay; however, the overall log cell kill was lower than that obtained on a single cycle at the next lower dose. The apparent discrepancy was likely due to the method of calculation of the two variables. In addition, the second cycle of drug administration likely altered the calculated results compared with the other treatment groups. These results were indicative of xenograft cell escape from drug-induced growth inhibition between treatment cycles; nonetheless, tumor regression was observed. Additional experiments with topotecan-resistant HT-29 xenografts resulted in no significant growth inhibition with either 24 mg/kg NSC 724998 or topotecan at the equivalent 4.7 mg/kg dose level given daily for 5 days (data not shown). Regression analysis of γ H2AX %NAP response 4 hours after drug administration on day 5 of cycle 1 compared with tumor volume 23 days after first drug administration yielded an R^2 value of 0.867 (Fig. 3B). The group median tumor volume 23 days after treatment daily for 5 days with NSC 724998 at the 16 mg/kg dose level and for the 4.7 mg/kg topotecan dose level was lower than the tumor volume at staging, indicating tumor shrinkage. Experimental logistics resulted in biomarker and efficacy experiments being run in parallel on matching lots of test compounds and vehicles (i.e., biomarker response and growth inhibition were tested independently).

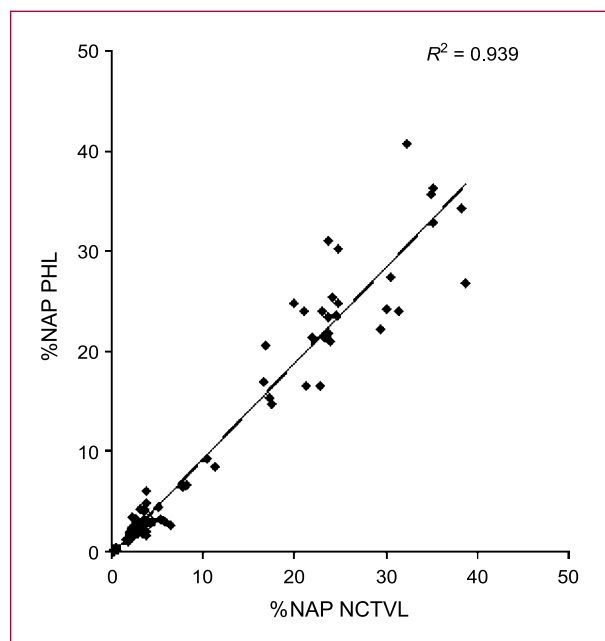


Fig. 2. Interlaboratory method validation. Comparison of 75 duplicate slides processed for γ H2AX immunofluorescence at two independent laboratories, the National Clinical Target Validation Laboratory (NCTVL) and the Pathology/Histotechnology Laboratory (PHL). Image quantitation and analysis were run by both laboratories independently, and %NAP for γ H2AX values were compared across sites.

Table 1. Antitumor activity of NSC 724998 and topotecan in A375 xenografts

	No. of mice*	Drug-related deaths [†]	Maximum % mean body weight loss (d)	Tumor growth inhibition		
				Optimal % T/C (d)	Growth delay % (T-C)/C	Net log cell kill
Vehicle	16	0	0.9 (12)			
NSC 724998						
16 mg/kg QD×5 [‡]	8	0	7.2 (12)	1 (19)	259	0.8
12 mg/kg QD×5	8	0	7.1 (12)	3 (19)	116	1.4
8 mg/kg QD×5	8	0	3.6 (12)	12 (15)	68	0.6
4 mg/kg QD×5	8	0	3.2 (12)	36 (15)	28	-0.1
Topotecan						
4.7 mg/kg QD×5	8	2	28.4 (15)	3 (15)	143	1.8
1.5 mg/kg QD×5	8	0	6.4 (12)	13 (15)	54	0.3

Abbreviations: QD×5, treated for 5 sequential days at designated dose; T, treated group; C, control group; d, day.

*No mice were tumor-free by study day 70.

[†]A death is considered treatment-related if the animal dies within 15 days of the last treatment and either the tumor weight is less than the lethal burden in the control mice or its net body weight loss at death is 20% greater than the mean net weight change in the control animals at death or sacrifice.

[‡]The NSC 724998 16-mg/kg treatment group was treated daily for 5 days for two cycles with a 17-day rest between treatments; all other groups were treated for one cycle.

γH2AX immunofluorescence assay application: indenoisoquinolines

Induction of γH2AX foci formation by the three indenoisoquinolines (NSCs 724998, 725776, and 706744) and topotecan was compared over a range of doses in the A375 xenograft model to assist in selection of which indenoisoquinoline(s) to move forward to clinical studies. The previously described controls and calibrator panels were used to verify γH2AX response. ANOVA of γH2AX response to NSC 724998 showed a significant response in xenograft biopsies collected 2 hours post-dose over a dose range of 0.05 to 1 MTD compared with vehicle ($P \leq 0.05$; Table 2). At 2 hours post-dose, γH2AX response after NSC 725776 treatment was statistically significant for three of the four dose levels tested (0.05, 0.33, and 1 MTD) compared with vehicle ($P \leq 0.05$; Table 2). NSC 706744 was the only indenoisoquinoline not to induce a measurable γH2AX dose-response curve and lacked a growth inhibitory response in A375 xenografts (data not shown). NSC 706744 was not pursued in further studies. Although the data for NSCs 724998 and 725776 showed a positive correlation between γH2AX response and indenoisoquinoline dose at 2 hours post-dose (data not shown), in general, γH2AX response was not statistically different between dose levels. However, all dosing groups were statistically significantly different from vehicle controls (Table 2). Intragroup variability of response resulted in overlapping statistical ranges in adjacent dosage levels. For both drugs, within-treatment-group heterogeneity in γH2AX response was thought to affect the significance level.

To clarify the dose and time effect of NSC 724998 on γH2AX biomarker induction, xenograft-bearing mice

were treated with doses ranging from 0.16 to 0.67 MTD NSC 724998, and biopsies were collected at 1, 2, 4, and 7 hours post-dose (Fig. 4). Although a small γH2AX signal was observed at the 2-hour time point, the high degree of intragroup heterogeneity precluded significance. However, a significant drug response compared with vehicle was seen for all doses of NSC 724998 at 4 and 7 hours post-dose. γH2AX levels between dose levels and sampling times overlapped at the 4-hour and 7-hour time points, and were not significantly different from each other, mirroring results obtained in growth inhibition experiments (Fig. 3A).

Hair follicle surrogate tissue analysis

Hair follicles in skin samples collected from mice bearing A375 xenografts had γH2AX-positive nuclei 2 hours after single-dose topotecan treatment at doses ranging from 0.016 to 1 MTD (Fig. 1A, bottom right; 0.32 MTD shown). Vehicle-treated animals had an average of three γH2AX-positive nuclei per follicle (Supplementary Fig. S1A). The minimal clinical cutoff for γH2AX response to drug treatment was set at 4 SDs above the average vehicle response, or 10 γH2AX-positive nuclei per follicle. When topotecan was given at 0.32 MTD, a significant γH2AX response compared with vehicle was seen in hair follicles at 1, 2, and 4 hours post-treatment, with the peak response at 2 hours (Supplementary Fig. S1A). Decreasing the dose of topotecan to 0.1 MTD resulted in a peak γH2AX response at 4 hours (Supplementary Fig. S1B). At 4 hours post-treatment, 0.016 to 1 MTD topotecan yielded γH2AX-positive follicles, with all dose levels statistically distinguishable from vehicle (data not shown).

γ H2AX response in hair follicles after indenoisoquinoline treatment was determined for skin snip samples collected 1, 2, 4, and 7 hours after single-dose administration of NSC 724998 (0.16, 0.33, 0.50, and 0.67 MTD), NSC 725776 (0.67 and 1 MTD), or vehicle. When 0.33 MTD and higher doses of NSC 724998 were used, mean peak γ H2AX response was observed at 4 hours post-dose and was statistically different from vehicle (Supplementary Fig. S1B; Supplementary Table S1). As with the xenograft biopsy analysis, no statistically significant differences were seen between doses at 4 and 7 hours due to individual mouse response heterogeneity, although the signal strength trended downward at the later time points. γ H2AX response was also significantly different statistically from vehicle at 4 hours post-dose for both of the NSC 725776 doses tested (Supplementary Fig. S1C).

Validated assay performance

Performance of the validated assay includes a calibrator/control slide, which includes positive and negative control samples, as well as vehicle, low, and high calibrator samples for quality control. Based on the %NAP observed in xenograft samples treated with topotecan and the indenoisoqui-

nolines, a 4-hour sampling time was chosen for the calibrator panel to best match potential clinical sampling times. The lower limit of quantification for the assay was set as the mean %NAP in vehicle-treated A375 xenograft samples, and the negative control specimen was used as an assay zero. A calibrator panel was established that comprised biopsies collected 4 hours post-dose from mice bearing A375 xenografts treated with vehicle and single-dose 0.1 and 0.32 MTD topotecan (labeled Calibrators 1, 2, and 3, respectively; Supplementary Fig. S2). The acceptable ranges for the γ H2AX signal on the calibrator sections were established as follows: Calibrator 1, <2.5% NAP with staining restricted to the nucleus; Calibrator 2, between 7.5% NAP and 15% NAP; and Calibrator 3, between 15% NAP and 25% NAP.

Further assay validation for quality control included that the %NAP of the negative control sample be less than the vehicle calibrator and that the positive control sample be intensely stained (>15%NAP). Two sets of calibrator/control slides are run with each set of clinical samples; assay quality control requires that there be at least three analyzable fields, in aggregate, across the two calibrator/control slides at each calibrator level. For

Fig. 3. Tumor growth delay is observed after treatment with increasing doses of NSC 724988 or topotecan. Mice with A375 tumor xenografts were treated with topotecan (1.5 or 4.7 mg/kg) or NSC 724998 (4, 8, or 12 mg/kg) for one cycle given once daily for 5 days or with NSC 724998 (16 mg/kg) for two cycles given once daily for 5 days (17-day rest between cycles). A, xenograft tumor volume was measured throughout the study period. Data points, median \pm SE; 8 mice per treatment group and 16 mice per control group. B, regression analysis of γ H2AX %NAP measurements 4 hours post-dose on day 5 of cycle 1 compared with tumor volumes on day 23 of cycle 1. Dashed vertical line, average tumor volume at staging was 200 mm³. Data points, median \pm SE; 4 mice per treatment group and 8 mice per control group.

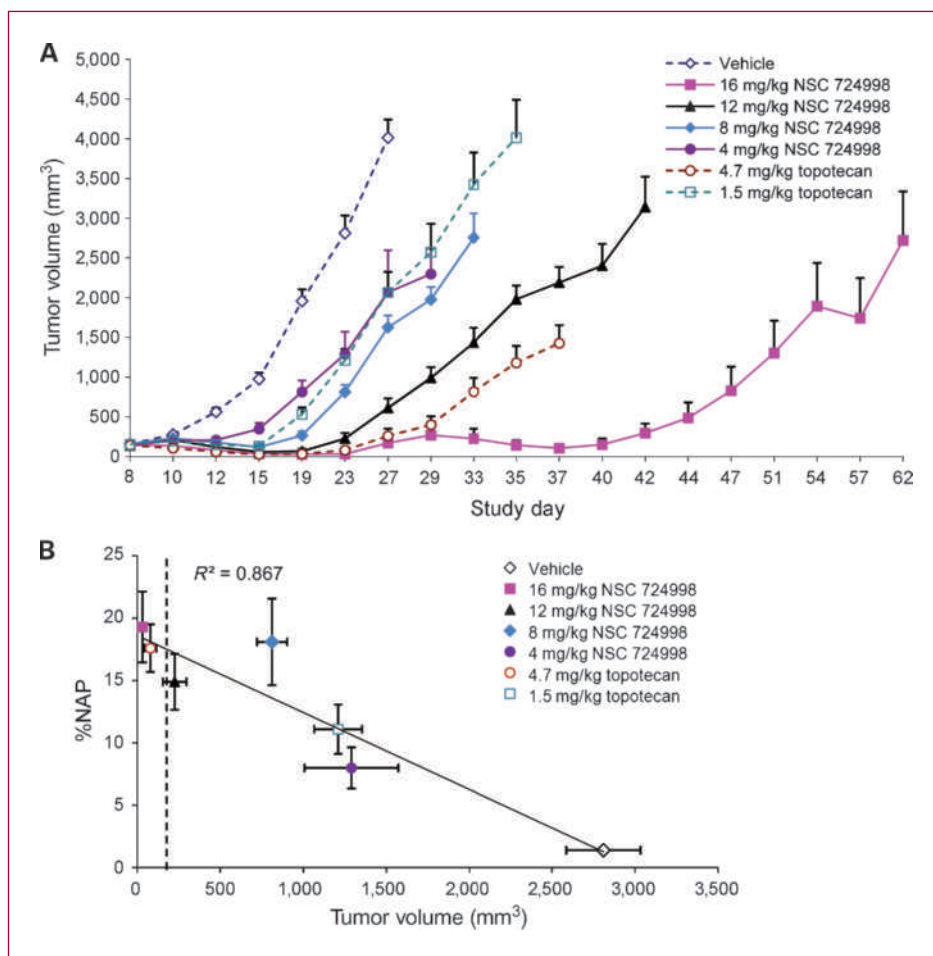


Table 2. Intergroup statistical differences in γ H2AX response in A375 xenograft biopsies collected 2 hours post-dose

NSC 724998*	Vehicle	0.05 MTD	0.1 MTD	0.33 MTD	1 MTD
Vehicle	—	—	—	—	—
0.05 MTD	0.026	—	—	—	—
0.1 MTD	0.017	NS	—	—	—
0.33 MTD	0.001	NS	NS	—	—
1 MTD	0.034	0.0381	NS	0.090	—

NSC 725776*	Vehicle	0.05 MTD	0.1 MTD	0.33 MTD	1 MTD
Vehicle	—	—	—	—	—
0.05 MTD	0.010	—	—	—	—
0.1 MTD	0.103	NS	—	—	—
0.33 MTD	0.002	NS	NS	—	—
1 MTD	0.034	NS	0.038	NS	—

NOTE: One-way ANOVA, with the significance level (α) set at 0.05, two-sided, was used to determine *P*-values for statistical differences across treatment groups. *P*-values were calculated with the use of pairwise comparisons of γ H2AX response to identify specific intergroup response differences. *P*-values ≤ 0.10 are reported due to the small group sizes. *P*-values > 0.10 are reported as NS.

Abbreviation: NS, not significant.

*Four mice per group.

clinical specimens, all three images from each section must be analyzable by immunofluorescence, or a second set of tissue sections from the specimens will be stained and analyzed. The %NAP for each sample will be established as the average of the separate quantified images per section.

Discussion

We developed an immunofluorescence-based assay to measure γ H2AX response after Top1 inhibitor treatment. The assay uses control and calibrator tissues, and was tested with the use of clinically relevant needle biopsy collection and handling procedures (4, 7). The background fluorescence-induced variability and nonuniformity of nuclear volume within tissue sections, common problems in many assays measuring immunofluorescence, were mitigated with a novel data quantitation and image processing algorithm. Evaluation variables included specificity of response, amplitude of response measured as percentage of positive tumor cell nuclei or hair follicle cells timing of response after treatment, and correlation of biomarker response to the biologically effective dose of the Top1 inhibitor topotecan and NSC 724998. After establishing that all of these marker characteristics were

consistent with the expected effects of topotecan, we validated the assay for detection of double-strand breaks caused by topotecan given over a 60-fold dose range (0.016-1.0 MTD) in both topotecan-sensitive and topotecan-insensitive human cancer cell line xenografts, and in normal murine testes and small intestine. The assay was then used to measure γ H2AX response to three structurally related indenoisoquinoline inhibitors of Top1, NSCs 724998, 725776, and 706774.

It has previously been shown that indenoisoquinolines have activity in camptothecin-resistant cell lines, are chemically more stable than camptothecin, overcome drug efflux-related multidrug resistance, and produce DNA breaks resistant to reversal of the trapped DNA-Top1 cleavage complex (24, 25, 31-33). Despite considerable intragroup heterogeneity in γ H2AX response in A375 xenografts after indenoisoquinoline treatment, a response that was significantly different statistically from vehicle was detected for NSCs 724998 and 725776. Notably, γ H2AX response was significant compared with vehicle at all doses of NSCs 724998 tested at 4 hours post-dose. This response persisted at 7 hours post-dose. These findings, in conjunction with additional preclinical data, supported the selection of NSCs 724998 and 725776 for clinical evaluation (25). The observation that a significant γ H2AX response compared with vehicle could readily be measured in xenografts by 4 hours after drug administration also informed the design of the phase I indenoisoquinolines trial being conducted at the NCI.

It is well established that γ H2AX formation occurs in response to DNA double-strand breaks, and apoptosis generates double-strand breaks; the γ H2AX assay outlined in this article cannot separate these two processes. The literature on γ H2AX as a marker for DNA double-strand breaks induced by ionizing radiation is extensive; however, less is known about the utility of γ H2AX as a marker for monitoring chemotherapy-induced DNA damage. When Rogakou and colleagues used ionizing radiation as a source of double-strand break damage and apoptosis, maximum γ H2AX response was estimated to be 9 to 30 minutes post-exposure (10), which is significantly less than the time course observed in our studies. In contrast, the same group reported that γ H2AX started to accumulate 1.5 hours after anti-Fas and tumor necrosis factor-related apoptosis-inducing ligand were used to induce apoptosis *in vitro*, corresponding to the appearance of DNA double-strand breaks (34, 35). Our interpretation of the difference in γ H2AX response between our observed drug response and previously published radiation-treated animal models is the difference in the timing of DNA damage. Ionizing radiation results in immediate double-strand break damage. For chemotherapeutic agents, injected as well as oral, there is a time lag between their administration and arrival at the tissue being sampled. In addition, for those agents that act by interfering with DNA metabolism, time is needed for the inhibition and subsequent repair to occur, with most of the double-strand breaks forming during the attempted

repair of the breaks themselves. Our ability to determine drug dose response within groups with statistical significance required collecting and testing tumor biopsies no sooner than 4 hours post-dose, which would seem to be a very early time point for detecting apoptosis, considering the biodistribution of the compound that must take place in the test animal.

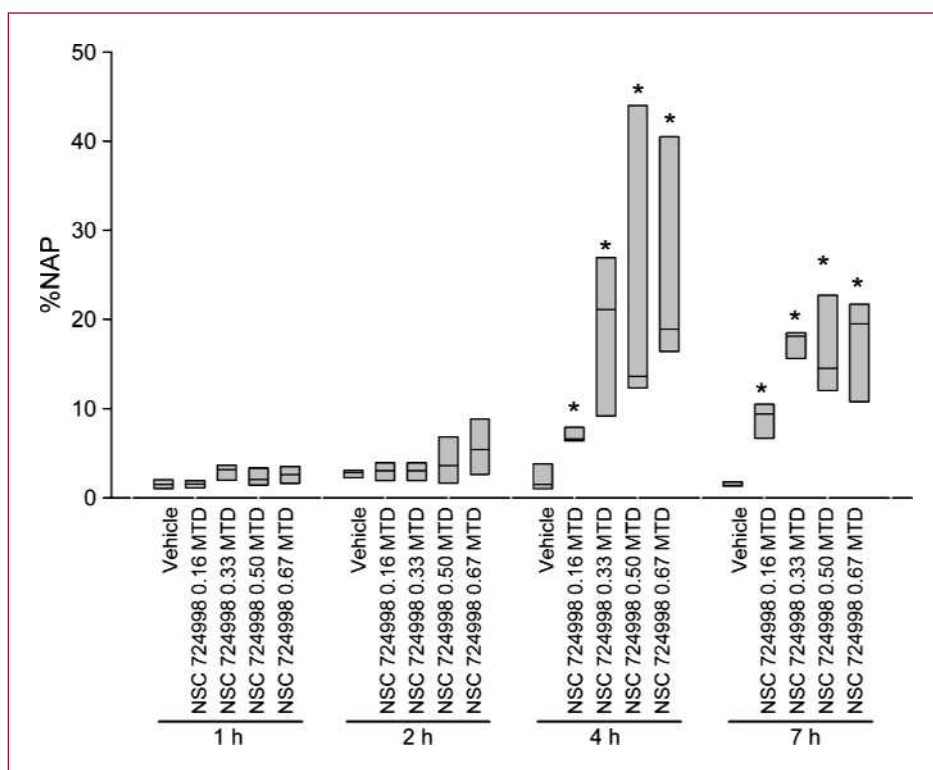
Our use of γ H2AX as a pharmacodynamic biomarker also raises the question of quantifying the number of DNA double-strand break repair complexes and using that information to predict cell death in response to drug treatment. It has been reported that cancer cell lines have a wide variation in the number of γ H2AX foci per nucleus in the absence of radiation or drug treatment, and these cells continue to grow and divide (36, 37). The question of whether an increase in the fraction of γ H2AX-positive cells reflects an increase in cell death remains to be further explored (35), although data presented here point in that direction (Fig. 3B). We are currently validating an apoptosis-specific assay and plan to use it to develop a multiplex immunofluorescence assay for simultaneous measurement of DNA damage and apoptotic markers on the same specimens.

There was significant within-treatment group variability for both biomarker levels (tumor and hair follicle) and tumor growth inhibition. We suspect that the within-group variability in response to exposure to topotecan and indenisoquinolines in xenografts may, in part, be related to variable intratumoral levels of Top1 (38–41). A number

of tumor cell lines respond to Top1 inhibitors by selective S-phase arrest during DNA replication, and γ H2AX formation in response to these treatments is restricted to replicating cells (19, 42, 43). In addition, it has been reported in the Fluorouracil, Oxaliplatin, CPT-11: Use and Sequencing (FOCUS) trial that response to irinotecan is a function of Top1 levels in the tumor at the start of treatment (40). It is reasonable to suggest that the reduction in Top1 levels during and after Top1 inhibitor treatment can result in decreased drug response in the next treatment cycle. We have recently analytically validated an ELISA-based total Top1 assay in tissues (41), and are interested in confirming the FOCUS trial as well as documenting the effect of repeat Top1 inhibitor treatment on biomarker and xenograft growth response. The Top1 assay is being used as a correlative study in clinical trials at the NCI. We have also reported, with the use of that assay, that there is significant variability in the amount of Top1 in xenografts from different mice and that topotecan-sensitive xenografts express higher levels of Top1 protein than toptecan-resistant xenografts (38).

The within-treatment group heterogeneity of γ H2AX response measured in our preclinical model also suggests there might be similar variability in patient samples. To address this, the assay was designed to score γ H2AX response based on the γ H2AX background level above the patient's pretreatment biopsy sample background, with normalization to sample autofluorescence. Although it was not feasible to do repeat sampling in the mouse

Fig. 4. A significant γ H2AX response was observed compared with vehicle control at all doses of NSC 724998 in A375 xenograft tumor biopsies collected 4 and 7 hours post-treatment. Box plots, interquartile range and median %NAP for γ H2AX for three to five mice per dose group per time point. Biopsies were collected 1, 2, 4, and 7 hours after a single dose of 0.16 to 0.67 MTD NSC 724998 or vehicle control. *, treatment cohort mean was statistically different from vehicle mean at same time point, with a significance level of $P < 0.05$, as determined by Student's *t*-test.



model, the assay quantitation described here should allow each patient's predose sample to serve as a baseline read-out, and each sample to serve as its own control to offset intersample and intrasample molecular and histologic heterogeneity. In addition, a calibrator panel, and positive and negative control samples will be run during each clinical assay to ensure that quality control standards are met. It is expected that these quality control procedures will allow more accurate detection of a γ H2AX response when applied to patient samples.

Use of hair follicles as a surrogate tissue for drug response was also examined, and data presented here suggest that γ H2AX can be successfully measured in hair follicles after treatment in mouse models, albeit at higher doses of indenoisoquinolines and topotecan than required for response in xenografts. Hair follicle cells have long been recognized as a possible surrogate tissue for testing drug efficacy in patients undergoing chemotherapy; epithelial in origin, as are 80% of cancers, they can be obtained by noninvasive or minimally invasive procedures (44, 45). Hair follicle cells are among the most rapidly proliferating cells in the body and are therefore sensitive to agents that damage DNA. Other studies have shown that antibody-based protein detection methods are effective in hair follicle cells (46, 47). One obvious limitation of our model is that decreased concentrations of the drugs across the surface area of the skin may result in higher doses being required to observe an effect in hair follicles. In addition, hair follicles are not abundant in athymic nude mice; additional studies are underway in rats and dogs to evaluate hair follicles as surrogate tissues. Because it was challenging to process individual hairs with intact follicles and sheaths on our automated tissue processing system, skin snips were collected to preserve the entire follicle. We identified the optimal clinical sampling time by determining that the mean number of nuclei per hair follicle positive for γ H2AX in the A375 xenograft mice peaked 4 hours after treatment with topotecan and NSCs 724998 and 725776. Vehicle-treated animals generally had up to 5 positive nuclei per follicle (mean, 3 positive nuclei per follicle), so the minimal cutoff for "positive" drug effect for clinical samples was set at 10 γ H2AX-positive nuclei per follicle, or 4 SDs above the average vehicle response. A higher cutoff was selected due to the lack of published data on the amount of variability to be expected in specimens from patients with cancer and may be adjusted after clinical trials with the indenoisoquinolines. Correlating γ H2AX dose-response to Top1 inhibitors in hair follicles and tumor biopsies in clinical trials will establish the value of hair follicles as minimally inva-

sive surrogates for drug effect in future trials of this class of drug.

Finally, a correlation between the γ H2AX biomarker and effects on tumor growth was established at comparable dose levels in parallel experiments. However, the correlation of biomarker response to compound effect on tumor growth is fundamentally limited by the requirement for multiple administrations of any of the Top1 inhibitors we investigated to achieve xenograft growth suppression. In contrast, elevated γ H2AX levels were observed within a few hours of a single administration of the compound. This rapid response to a single dose is a critical consideration in selecting a biomarker for use in the clinical population, in which, often, only a single post-treatment biopsy is allowed.

In conclusion, γ H2AX is a sensitive biomarker for monitoring the pharmacodynamics of anticancer therapeutics that damage DNA, and its measurement could lead to enhanced monitoring of chemotherapeutic effects in the clinic. Evaluation of γ H2AX response in tumor biopsies or hair follicles allows for monitoring of pharmacodynamic effects in patients over the course of drug treatment. We anticipate that the use of this pharmacodynamic assay will inform clinical drug development decisions and promote the examination of surrogate tissues to evaluate the biochemical effects of Top1 inhibitors.

Disclosure of Potential Conflicts of Interest

R.J. Kinders: consultant, KPL, Inc. (unpaid) and Trevigen, Inc.

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Development of a Validated Immunofluorescence Assay for γ H2AX as a Pharmacodynamic Marker of Topoisomerase I Inhibitor Activity

Robert J. Kinders, Melinda Hollingshead, Scott Lawrence, et al.

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