APPLICATION NOTE

Cyclin-dependent kinase signaling in oncology: evaluation of phosphorylation events triggered by drug-induced DNA damage.

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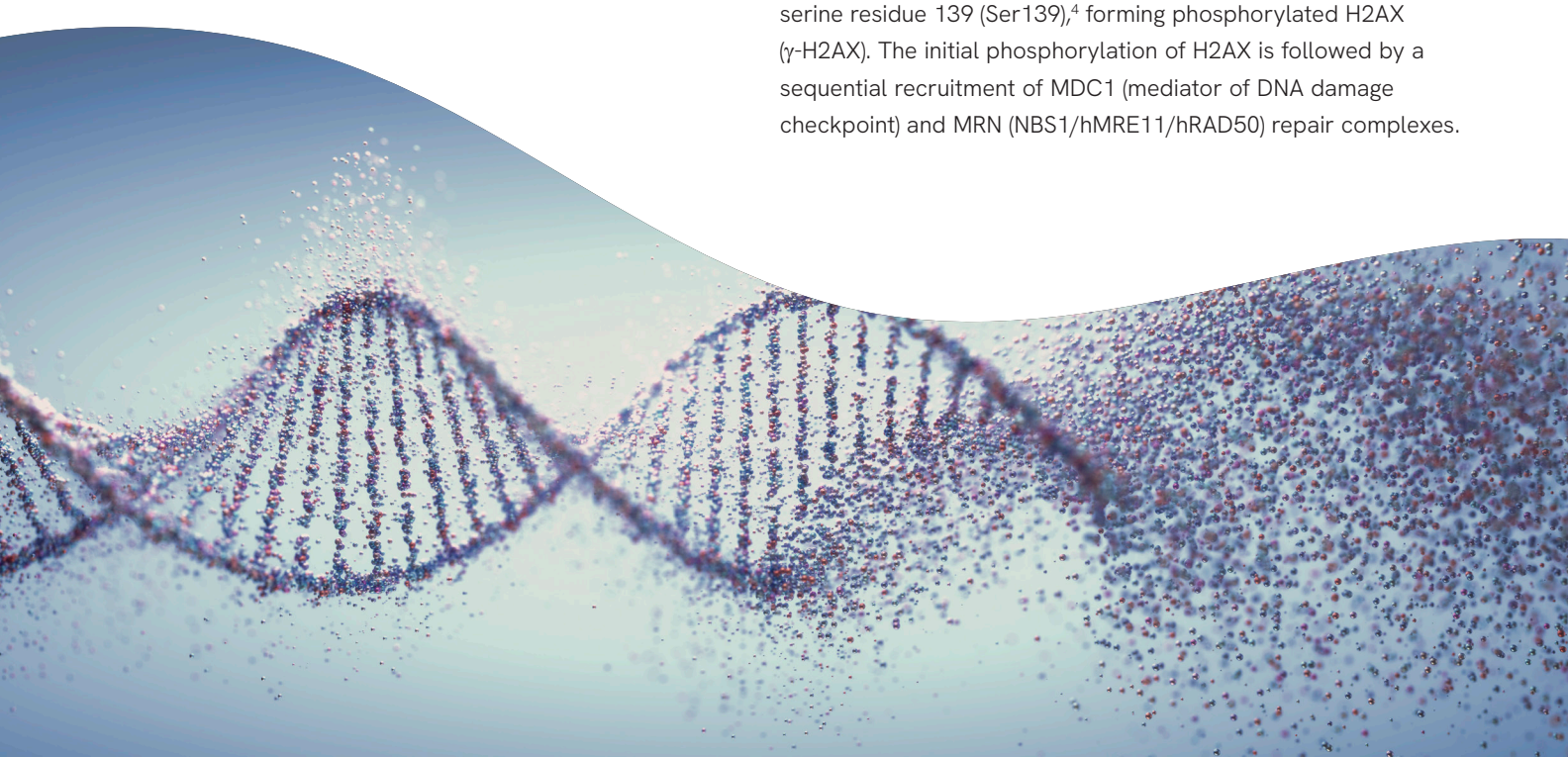
Introduction

Etoposide is a widely used chemotherapy drug for the treatment of multiple types of human malignancy, including leukemia, lymphoma, and solid tumors. It acts as an inhibitor of topoisomerase II, resulting in cytotoxic accumulation of DNA double-strand breaks.[1] Topoisomerase II is critical for the survival of proliferating eukaryotic cells, playing essential roles in cell proliferation, replication, transcription, chromosome structure, and segregation. Rapidly proliferating cells, such as those found in aggressive malignant tumors, contain a high concentration of topoisomerase II and are therefore most susceptible to drugs like etoposide, which generate high levels of covalent enzyme-DNA cleavage complexes.[2]

DNA damage can directly affect cell cycle progression by modulating levels of an important cell cycle regulator – cyclin-dependent kinase 1 (CDK1). CDK1 drives the progression of the cell cycle from G2 to M phase and is sensitive to drugs like etoposide because it is a target for DNA damage response proteins that can trigger G2/M arrest. For example, the CDK inhibitor $p21^{CIP1/WAF1}$ has been shown to be upregulated in response to etoposide treatment, with an increase in expression level at both G1/S and G2/M checkpoints.[3]

DNA double-strand breaks, like those induced with etoposide treatment, trigger a DNA damage response that activates a variety of DNA repair mechanisms, including the phosphorylation of the histone protein component H2AX at serine residue 139 (Ser139),[4] forming phosphorylated H2AX (γ-H2AX). The initial phosphorylation of H2AX is followed by a sequential recruitment of MDC1 (mediator of DNA damage checkpoint) and MRN (NBS1/hMRE11/hRAD50) repair complexes.



This results in further activation of ataxia telangiectasia mutated kinase (ATM) and subsequent phosphorylation of hundreds to thousands of H2AX histone proteins in large chromosomal domains surrounding the double-strand breaks. γ-H2AX interacts with hundreds of other proteins to form heterogeneous foci in the region of DNA double-strand breaks. Therefore, foci containing γ-H2AX have been established as markers for identifying cells containing DNA double-strand breaks. Many different assay methods exist for measuring and quantifying γ-H2AX foci, including fluorescence-based microscopy, flow cytometry, Western blot, and a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®).[5]

In this application note we evaluate DNA damage induced by etoposide treatment in two distinct cell lines using phosphorylated (Ser139) histone H2AX as a biomarker for the detection of DNA double-strand breaks. AlphaLISA™ *SureFire® Ultra™* technology was highlighted to demonstrate utility and functionality in monitoring and characterizing changes in crucial cell cycle events by measuring both phosphorylated and total target protein from the same cell lysate sample.

Materials and methods

AlphaLISA *SureFire Ultra* Assays: Alpha technology is a fast, highly sensitive, homogeneous, no-wash assay platform that can be performed in a microplate format. Alpha assays require two bead types: Donor beads and Acceptor beads. The Donor beads generate singlet oxygen upon illumination at 680 nm. The singlet oxygen can diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead, resulting in light production. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. AlphaLISA *SureFire Ultra* assays are utilized to measure total protein as well as key phosphorylation events that are critical to cell signaling. Total Histone H2AX (Revvity, ALSU-TH2X-A500) and Phospho-Histone H2AX (Ser139) (Revvity, ALSU-PH2X-A500) kits were used following the procedure outlined in the AlphaLISA *SureFire Ultra* manual. Figure 1 demonstrates the AlphaLISA *SureFire Ultra* Assay Principle for a phosphorylated target protein. The same concept is used for total protein, with each target antibody recognizing a distinct epitope on the analyte of interest.

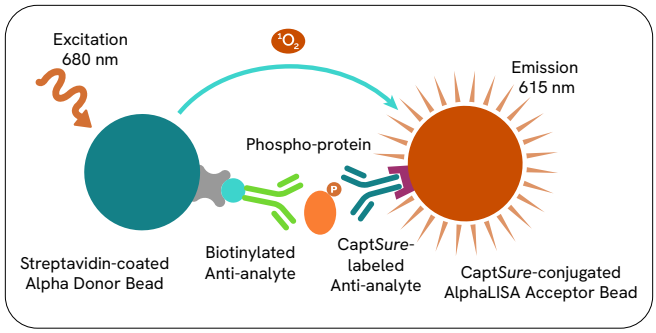


Figure 1. AlphaLISA *SureFire Ultra* Assay Schematic.

The schematic shows the detection of a phosphorylated target protein whereby the biotinylated antibody recognizes the target and a second CaptSure-labeled antibody recognizes the phosphorylated epitope. Each antibody is recognized by the appropriate Donor (streptavidin-coated Alpha Donor Bead), or Acceptor (CaptSure-conjugated AlphaLISA Acceptor Bead) and the Alpha signal is directly proportional to the amount of phosphorylated analyte present in the sample.

Cell-based Materials and Compounds: Jurkat cells (ATCC, TIB-152), A549 cells (ATCC, CCL-185), RPMI (ATCC, 30-2001), F-12K (ATCC, 30-2004), Fetal Bovine Serum (FBS) heat inactivated (Thermo Fisher, 10082147), and Etoposide (MedChemExpress, HY-13629).

Cell-based Experiments: 10,000 cells/well were plated in 75 µL volume in a ViewPlate-96 well black, clear bottom assay plate (Revvity, 6005182) overnight with no FBS present in the media. Removing the serum from the media starves the cells and reduces the baseline level of phosphorylation present in cells. This step is recommended in the AlphaLISA *SureFire Ultra* Assay Optimization Startup Guide with starvation times ranging from 1 hour to overnight. A549 cells were prepared in F-12K Media and the Jurkat suspension cell line was prepared in Hanks' Balanced Salt Solution (HBSS). Etoposide drug treatments were prepared at 4X final concentration in HBSS and 25 µL of drug or vehicle (DMSO) was added to each assay plate for 1 hr, 2 hr, or 4 hr as noted. Cells were lysed in 50 µL final volume of AlphaLISA *SureFire Ultra* Lysis buffer following the recommended procedure in the AlphaLISA *SureFire Ultra* manual. Media was aspirated from adherent cells and 50 µL of 1X lysis buffer was added to each well. For suspension cells, 75 µL of media was removed from the assay plate and 25 µL of 2X lysis buffer was added to each well. Assay plates were placed on an orbital shaker at 350 rpm for 10 minutes. After visual inspection to confirm lysis was complete, assay plates were sealed and placed at -80°C until processing of each sample was completed. Next, 10 µL of lysate was used from each sample and biological replicates were tested for total and phosphorylated H2AX following the AlphaLISA *SureFire Ultra* manual. The assay plates (Alphaplate-384, Revvity, 6005350) were read on an EnVision 2105 Multimode Plate

Reader with the stock AlphaLISA settings.

Results and Discussion

Phospho-H2AX (Ser139) is a biomarker for DNA double-strand breaks

Etoposide is a widely used cancer drug that inhibits topoisomerase II and leads to an accumulation of DNA double-strand breaks in cells. Phosphorylation of histone H2AX at Ser139 is known to be a biomarker for DNA double-strand breaks.[4] Increasing doses of etoposide were used to treat A549 and Jurkat cells in a time course experiment. Histone H2AX phosphorylation in response to DNA damage caused by the drug was measured by AlphaLISA *SureFire Ultra* assay and Figure 2 shows the result in A549 cells. A mild increase in phosphorylation was seen with the 10 µM dose; however, a more marked increase was observed at the 100 µM dose. There was no apparent increase in signal over time in the A549 cells. This suggests that the response to etoposide-induced damage is rapid in this cell type, taking place within one hour, as measured by phosphorylation of H2AX at Ser139.

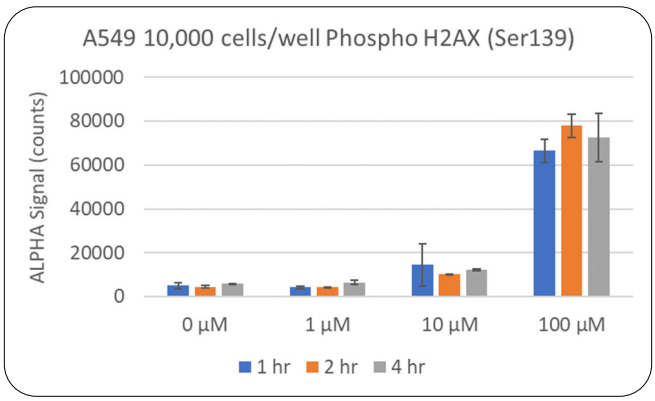


Figure 2. Phospho-H2AX (Ser139) AlphaLISA *SureFire Ultra* detection in A549 cells.

Etoposide-induced DNA damage was measured by phosphorylation of the histone H2AX at Ser139. Drug doses and timing of stimulation are shown. Phosphorylation increased notably with 100 µM etoposide and was observed as early as 1 hr of treatment.

Figure 3 shows the AlphaLISA *SureFire Ultra* results measuring the phosphorylation of H2AX in response to etoposide-induced DNA damage in Jurkat cells. As seen with A549 cells, there was a slight increase in phosphorylation of H2AX over baseline with the 10 µM dose and a more pronounced effect with the 100 µM dose of the drug. We observed a strong time-dependent phosphorylation of H2AX in Jurkat cells indicating the accumulation of DNA damage takes longer in this cell type compared to A549 cells, with the highest phosphorylation response measured at 4 hours. This tracks well with the literature where an etoposide treatment time course from 0.5 hours up to 12 hours in Jurkat cells showed a maximal response of phosphorylated H2AX (Ser139) at the 4-hour timepoint measured by Western blot.[6]

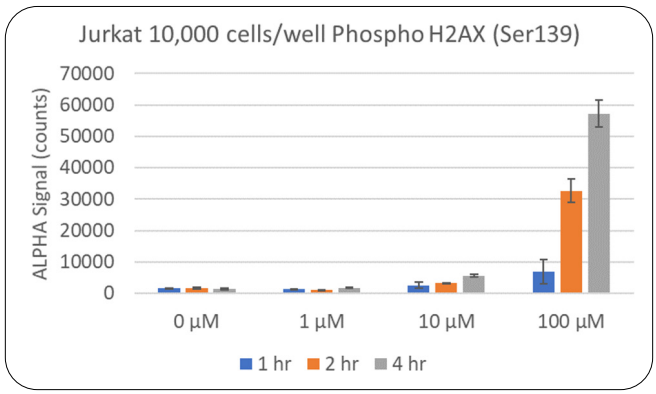


Figure 3. Phospho-H2AX (Ser139) AlphaLISA *SureFire Ultra* detection in Jurkat cells.

Etoposide-induced DNA damage is measured by phosphorylation of the histone H2AX at Ser139. Drug doses and timing of stimulation are noted. Phosphorylation increases markedly with 100 µM etoposide and displays a time-dependent response with the highest stimulation detected at the 4 hr incubation.

Total H2AX protein not affected by drug-induced DNA damage

When measuring phosphorylation in response to DNA damage it is important to monitor changes in the total target protein pool to ensure that an observed increase in detected phosphorylation is not simply due to an increase in baseline signal corresponding to elevated levels of the target protein in the cells. Figure 4 demonstrates no observable increase in total H2AX levels in A549 cells upon treatment with etoposide. There may be a slight decrease in total H2AX in cells treated with 100 µM etoposide, but this effect appears to be negligible.

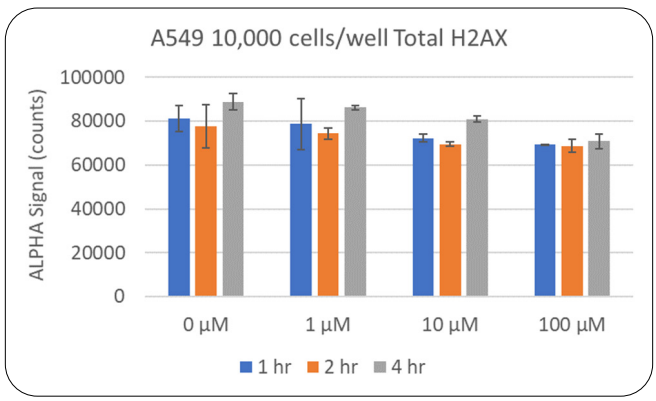


Figure 4. Total H2AX AlphaLISA *SureFire Ultra* detection in A549 cells.

Etoposide doses and stimulation timing is as noted. No major change of the average Alpha Signal count was seen with treatment of etoposide on the A549 cells up to 4 hr of stimulation, suggesting the total H2AX pool remains unchanged.

Similarly, in the Jurkat cell line, total H2AX was measured by AlphaLISA *SureFire Ultra* and again showed no increase in total H2AX levels upon treatment with etoposide at any dose tested (Figure 5). The overall Alpha signal was approximately four times lower, suggesting a reduced level of total H2AX in Jurkat cells compared to A549 cells.

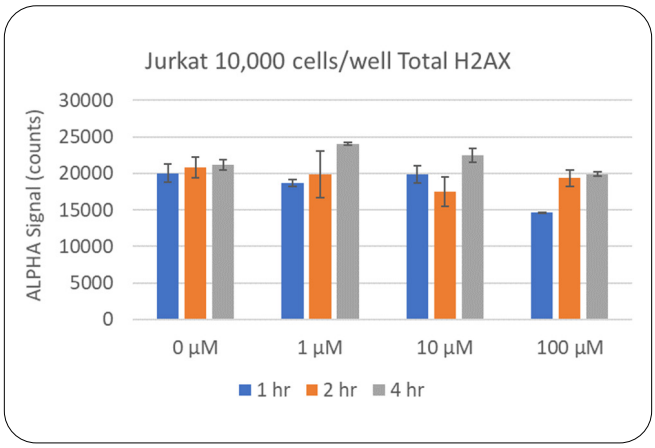


Figure 5. Total H2AX AlphaLISA *SureFire Ultra* detection in Jurkat cells.

Etoposide doses and stimulation timing is as noted. No major change of the average Alpha signal count detected with treatment of etoposide on the Jurkat cells up to 4 hr of stimulation, indicating no change in the total H2AX pool.

Summary

The ability to accurately monitor drug-induced DNA damage is an important part of drug discovery when screening efficacious doses used for the treatment of various malignancies. Phosphorylation of histone H2AX has been shown to be a biomarker of DNA double-strand breaks in cells.4 We were able to measure the phosphorylation at Ser139 of H2AX in two separate cell lines (A549 and Jurkat) with differing temporal responses to etoposide treatment. The A549 cell response and accumulation of DNA double-strand breaks were rapid, reaching a maximal signal within 1 hour. Comparatively, the response in Jurkat cells was slower, reaching a maximal signal at the longest time point of 4 hours, steadily increasing from 1 hour through 2 hours.

Overall, monitoring DNA damage induced by drug treatment can be easily accomplished using AlphaLISA technology demonstrated using Total and Phosphorylated (Ser139) Histone H2AX AlphaLISA *SureFire Ultra* kits. AlphaLISA technology offers a rapid and reliable method for tracking and characterizing changes to cell cycle and cellular homeostasis with AlphaLISA *SureFire Ultra* providing the additional benefit of tracking potential changes to total target protein, in addition to monitoring target-specific phosphorylation events in cells.

References

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