Homogeneous and High Throughput Characterization of DNA Damage Based on Cell-Based Detection of p53 Phosphorylation.

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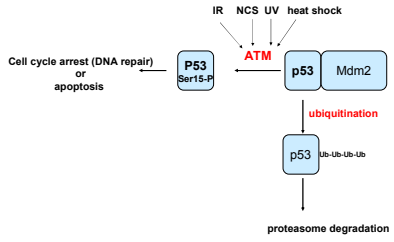
1 Introduction

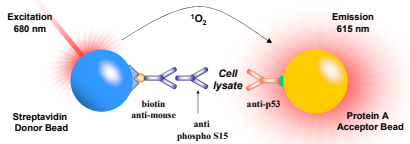
p53 is a transcription factor that possesses a sequence specific DNA-binding activity and acts as a tumor suppressor. In response to different degrees of DNA damage, intracellular levels of p53 increase, inducing cell cycle arrest, leading to either repair of DNA damage or a commitment to cell death by apoptosis. The stability of p53 appears to be associated with a diverse array of posttranslational modifications including phosphorylation at over 18 phosphosites. ATM and ATR are some of the kinases that have been directly implicated in p53 phosphorylation induced by ionizing radiation (IR) or radiomimetic chemicals. Upon DNA damage, ATM mediates rapid phosphorylation of p53 on Ser 15 whereas ATR seems to be involved in maintenance of the phosphorylation state.

Regulation of p53 activity is mainly regulated by its association with the cellular proto-oncogene Mdm2. The later protein controls p53 activity by blocking its transcriptional activity, exporting p53 into the cytoplasm and/or by promoting its proteasome based degradation. Phosphorylation of p53 on serine 15 has been shown to play an important role in decreasing the ability of p53 to associate with Mdm2, which stabilizes p53 by inhibiting its ubiquitination and subsequent degradation.

Enediynes, such as neocarzinostatin (NCS), are a class of DNA strand-breaking agents that show the promise of becoming anticancer drugs. They are extremely potent cytotoxic agents and some have demonstrated selectivity for neoplastic cells relative to normal human bone marrow. In order to screen for such compounds, we have developed an AlphaScreen® assay that measures induction of p53 phosphorylation upon treatment with NCS in human osteosarcoma derived cell line U2OS.



2 Cell - Based Detection of p53 Serine 15 Phosphorylation Using AlphaScreen



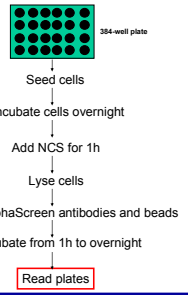
In this AlphaScreen assay, following NCS treatment, endogenous phospho p53 is captured in cell lysate of U2OS cells using a monoclonal antibody against phospho ser15 and a polyclonal antibody against p53. A biotinylated anti-mouse antibody is then used to capture the mouse anti phospho ser15 antibody. An AlphaScreen signal is generated when beads are brought into close proximity via capture of the biotinylated antibody with the Streptavidin Donor beads simultaneously with the capture of the rabbit anti-p53 by protein A Acceptor beads.

3 Materials

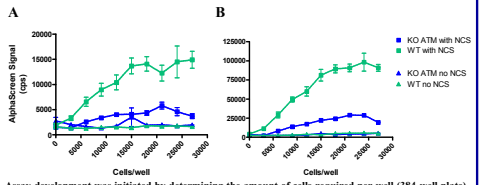
- U2OS wild type
- U2OS delta ATM cells

  ATM was knocked down by expression of shRNA containing sequences that correspond to positions 1266-1284 of the ATM ORF (Ziv et al., 2006)
- Neocarzinostatin (NCS): Sigma Cat. No. N9162
- Lysis buffer : Developed by TGR BioSciences
- Anti-p53 antibody: developed by CST Cat. No. 2353B
- Anti-phospho p53ser15: developed by CST Cat. No. 9286
- Biotinylated goat anti-mouse antibody: Jackson Immunoresearch Cat. No. 71904
- AlphaScreen protein A detection kit: PerkinElmer Cat. No. 6760617

4 Methods



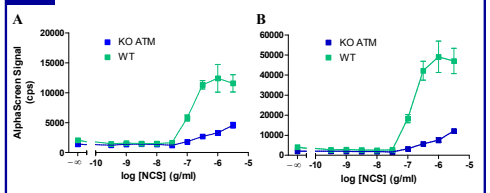
5 Assay Optimization (Cell Number)



Assay development was initiated by determining the amount of cells required per well (384-well plate) to obtain a maximal response using 400 ng/mL of NCS. As a negative control, cells depleted of ATM kinase using RNA interference were tested in parallel (KO ATM).

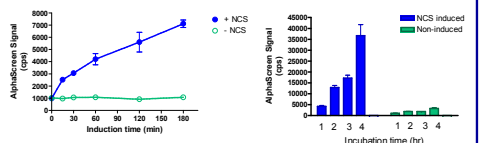
Maximum signal was observed using 24 000 cells/well with signal to background of (A) 10 and (B) 25 following 4 and 17 h of detection reaction, respectively (+/- NCS). Signal was 4-times lower in the ATM knock out cell lines.

6 NCS Titration



The specificity of the reaction was also evaluated in response to NCS in both wild type and ATM knock out cell lines (KO ATM). Dose-dependent increase of endogenous p53 phosphorylation on serine 15 was detected as function of the concentration of NCS present with an EC_{50} around 200 nM for the wild type U2OS cells. Effect of NCS on KO ATM cells was drastically shifted. Maximum signal was detected at 1 µM of NCS in this cell-based assay model.

7 Time Course



Phosphorylation of p53 was followed by looking at (A) the time course of induction by NCS, as well as (B) time requirement for the detection reaction. (A) Cells were treated for different time with NCS (1µg/mL) before the addition of detection reagents. (B) Cells were treated with NCS 1h before detection reagents were added and incubated at different time points before read out.

We observed that NCS induction increased up to 3h incubation with NCS. As for the detection time, signal increased up to 17 hrs (data not shown).

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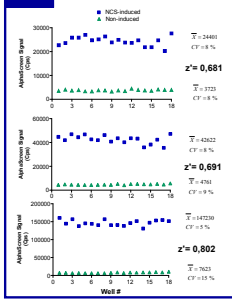
8 Preliminary Z'-Factor Determination



Preliminary Z'-factor evaluation was performed using the following protocol:

(final concentration when indicated):

1. **Seed cells at 24 000 cells/well**
2. **Incubate overnight**
3. **Remove medium**
4. **Add medium supplemented with NCS at 1 µg/ml**
5. **Incubate 1h**
6. **Remove medium**
7. **Add detection reagents:**
   - **10 uL of lysis buffer**
   - **5 uL of p53 antibody (1 nM), AlphaLISA beads (20 mg/mL) mix, pre-incubated for 30 minutes.**
   - **Incubate 1h**
   - **10 uL of anti-phospho-p53 (1 nM), biotin anti-mouse (3 nM), AlphaScreen Donor beads (20 mg/mL) mix, pre-incubated for 30 minutes.**
   - **Incubate 2h, 4h and overnight**
8. **Read on an AlphaScreen capable reader.**

9 Summary

- A cell-based assay that allows for simple and sensitive detection of DNA damage was successfully developed using the well recognized phosphorylation of p53 on serine 15 by ATM following DNA strand breaks.
- Two control experiments have demonstrated the specificity of the reaction:

  - In the U2OS cells (human osteosarcoma), cell lines depleted of ATM by RNA interference showed a marked decrease in response to NCS treatment.

  - In this system, phosphorylation of p53 was undetcted in the absence of NCS with maximal detection using 1 µg/mL of NCS with an EC_{50} around 200 nM.
- p53 phosphorylation was observed within minutes following treatment with NCS. These results correlate with the reported time of activation of ATM using different cell line, such as B-lymphoma cells, measured using non-HTS detection systems such as western blot (Banin et al, 1998, and Fingrut et al., 2005).
- Assay optimization for signal intensity can be performed by varying the incubation time of the detection reaction while still maintaining Z'-values around 0.7, as demonstrated by preliminary Z'-factor evaluation.
- This novel cell-based AlphaScreen assay represents a major improvement over Western Blot aimed at measuring endogenous p53 phosphorylation and thus should ease the study and discovery of new compounds that can modulate this important intra cellular pathway.

References:

Banin et al. 1998. Science. 281:1674-1677.

Fingrut et al. 2005. British J Pharmacol. 146:800-808.

Ziv et al. 2006. Nature Cell Biol. 8(8):870-876.