DISSECTING THE BIOLOGY OF MUTANT P53 WITH THE CELLULAR THERMAL SHIFT ASSAY (CETSA)

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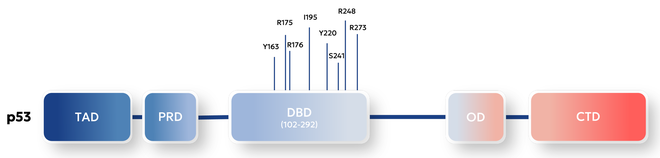
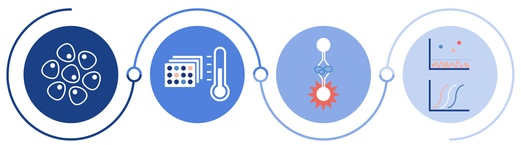
INTRODUCTION

The tumor suppressor protein p53 is one of the most important safeguards against malignant transformation of mammalian cells, but it is also one of the most frequently mutated proteins in cancers. Therefore, p53 constitutes an attractive drug target in oncology. However, there has been little success in finding clinical candidate drugs that directly manipulate the function of p53. The p53 protein offers several challenges for investigators wanting to manipulate its function e.g., the absence of accessible binding pockets, lack of enzymatic activity, and its intracellular location. Moreover, another complication with p53 is the plethora of mutations that alters the protein's characteristics. We have applied the Cellular Thermal Shift Assay (CETSA) to characterize tool compounds that are annotated to bind and/or modulate the function of both WT and mutant p53. Since CETSA exploits the stability of the intracellular protein and identifies compound induced changes in protein melting behavior it is important to know the melting characteristics of the protein under investigation. We decided to investigate three different mutational variants present in three separate breast cancer cell lines: SK-BR-3 (R175H), BT-474 (E285K), and MCF-7 (p53WT) and their ability to bind to well known tool compounds.



CETSA PRINCIPLE

The Cellular Thermal Shift Assay (CETSA) is a patented label-free technology that assesses protein-ligand interactions in the native cellular environment. The thermal stability of proteins is in many cases affected by high affinity interactions with small molecules, which can result in either protein stabilization or destabilization. In this work we have employed CETSA with both antibody based or mass spectrometric readouts. When screening for compound binding using HTS with CETSA we utilize the AlphaLISA® technology for detection. For mass spectrometric readout we use TMT® based or DIA based detection.



EVALUATION OF TOOL COMPOUNDS

Direct binding of tool compound to p53 was investigated in three breast cancer cell lines with a targeted CETSA assay in both intact cells and lysate. In paralell, we investigated the effect on p53 regulated trancription (CDKN1A) and viability (Toxilight and Celltiter Glo). The proteome wide CETSA effects of each tool compound were also investigated in lysate from SK-BR-3 cells using mass spectrometric readout (Figures 1 and 2).

Compound	MCF-7 (p53 wt) CETSA EC_{50} (µM)	SK-BR-3 (p53 mut) CETSA EC_{50} (µM)	BT-474(p53 mut) CETSA EC_{50} (µM)	SK-BR-3 Proteome wide CETSA (hits)
Butein	NA	21.9	NA	19
MIRA-1	35.3	7.7	11.4	2
NSC319726	34.9	27.9	24.6	27
PRIMA-1met	NA	NA	NA	0
PhiKan-083	NA	NA	NA	1
Pifithrin µ	NA	NA	NA	18
RITA	NA	NA	NA	9

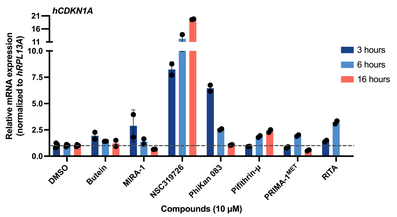
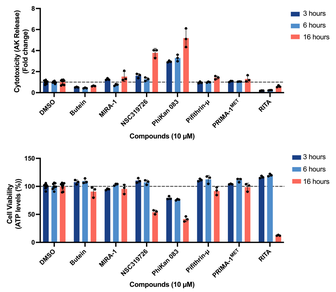


Figure 1. A) Only three of the investigated tool compounds resulted in an EC50 value.
B) NSC319726 showed a persistent effect on CDKN1A mRNA levels at all investigated time points.



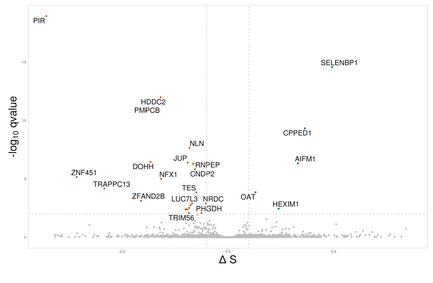
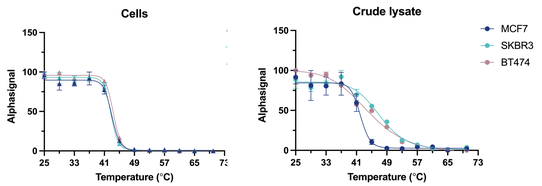


Figure 2. A) Three of the compounds showed effects on cell viability after 16 h.
B) Proteome wide CETSA with NSC319726 in SK-BR-3 lysate show thermal stability change of cell cycle regulator CPPED1 and AIFM1, which are involved in regulation of cell cycle and apoptosis. These two proteins may constitute alternative targets for NSC319726 besides p53.

MUTATED AND WT P53 DISPLAY DIFFERENT MELTING BEHAVIOR

The melting behavior of p53 protein was similar irrespective of genomic status of p53 in intact cells (Figure 3). However, in lysed cells there was a striking difference in p53 protein melting between MCF-7 cells that carry WT p53 and the two other breast cancer cell lines that carries mutations in the gene coding p53. The Tm for WT p53 was similar in both lysate and intact cells, whereas there was a marked difference between intact cells and lysates derived from the two cell lines that expressed mutated p53.



	Cells T_m (°C)	Lysate T_m (°C)
MCF7	43.0	41.1
SK-BR-3	42.7	46.6
BT-474	43.4	45.5

Figure 3. Melting profiles of p53 in intact cells and lysate for WT and p53 with the R175H and E285K substitutions.

As p53 is a transcription factor, we investigated if the thermal stability was an effect of presence of DNA in the lysate, which could yield different stability of the protein. Including nuclease in the lysis procedure showed no effect on the melting behavior of the different p53 proteoforms in lysate (Figure 4). We have previously shown that addition of cognate DNA has a stabilizing effect on WT but not on the R273H substitution (Figure 5).

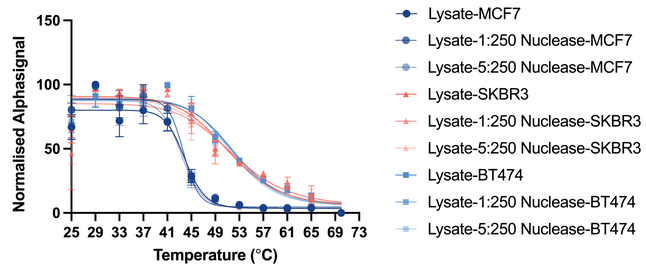


Figure 4. Melting behavior of p53 is unaffected by addition of Nuclease – indicating that the p53 protein is not complexed to DNA upon lysis of the cells.

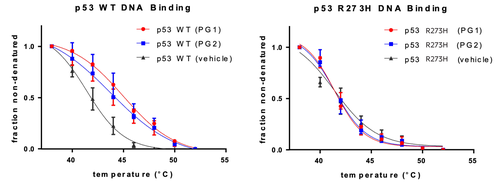


Figure 5. p53 with two cognate duplex-DNA oligonucleotides (PG1; Orange, and PG2; blue) added to A549 cell extract containing wild-type p53 and HT-29 cells containing the p53 R273H mutant. When wild-type p53 is exposed to its cognate effector DNA it is stabilized, while the p53 R273H mutant, known to not bind these effector sequences, shows no stabilization. (Modified from Savitski et al Science 2014)

Moreover, mutated forms of p53 are rescued from proteasomal degradation by association with chaperone proteins like Hsp90. Therefore, cell lysates were treated with the HSP90 inhibitor Geldanamycin. Similar to nuclease treatment, Geldanamycin had no effect on the thermal stability of either WT or mutated p53. However, we could detect destabilization of p53 that was not temperature dependent when treating with Geldanamycin. This effect was much more potent in cells with mutated p53, especially in SK-BR-3 cells (Figure 6)

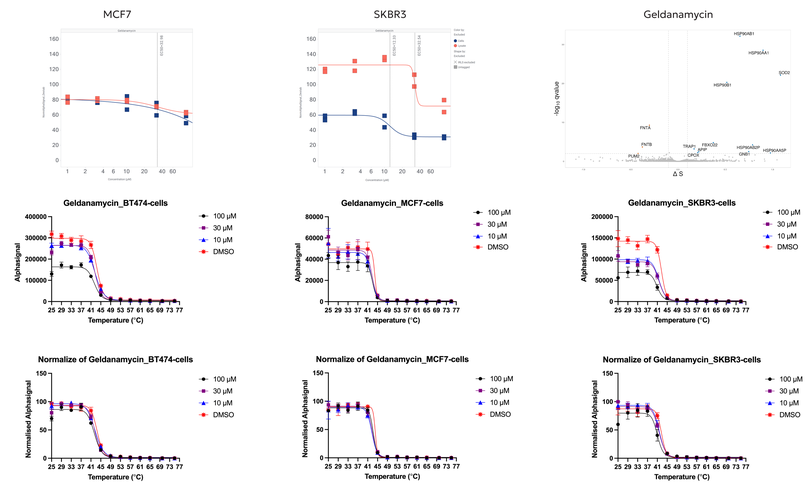


Figure 6. Concentration response curves for p53 in the presence of the HSP90 inhibitor Geldanamycin. Geldanamycin shows selective binding to HSP90 when assayed with mass spectrometry readout. Melt curve analysis shows difference in the response rate for different mutated p53 vs WT

CONCLUSIONS

- CETSA monitors protein thermal stability in intact cells and lysate.
- The method can be used either to identify targets of compound treatment or to monitor differences in melting behaviour of specific proteins.
- Melt curve monitoring gives information on protein activity state and allows for establishing correlation between melting temperature and cellular phenotypic effects.
- Downstream effector assays can further be employed to connect how molecular phenotypes lead to cellular phenotypes.

References

1. Martinez Molina, D. et al. Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. Science 341 (2013)
2. Savitski, M. et al Tracking cancer drugs in living cells by thermal profiling of the proteome. Science 346 (2014)