APPLICATION NOTE

Accelerating Data Throughput with the SemaCyte® Multiplex Platform: Unravelling Nutlin-3a Cytotoxicity and p53 Induction from a Cell Panel to Identify Promising Biomarkers

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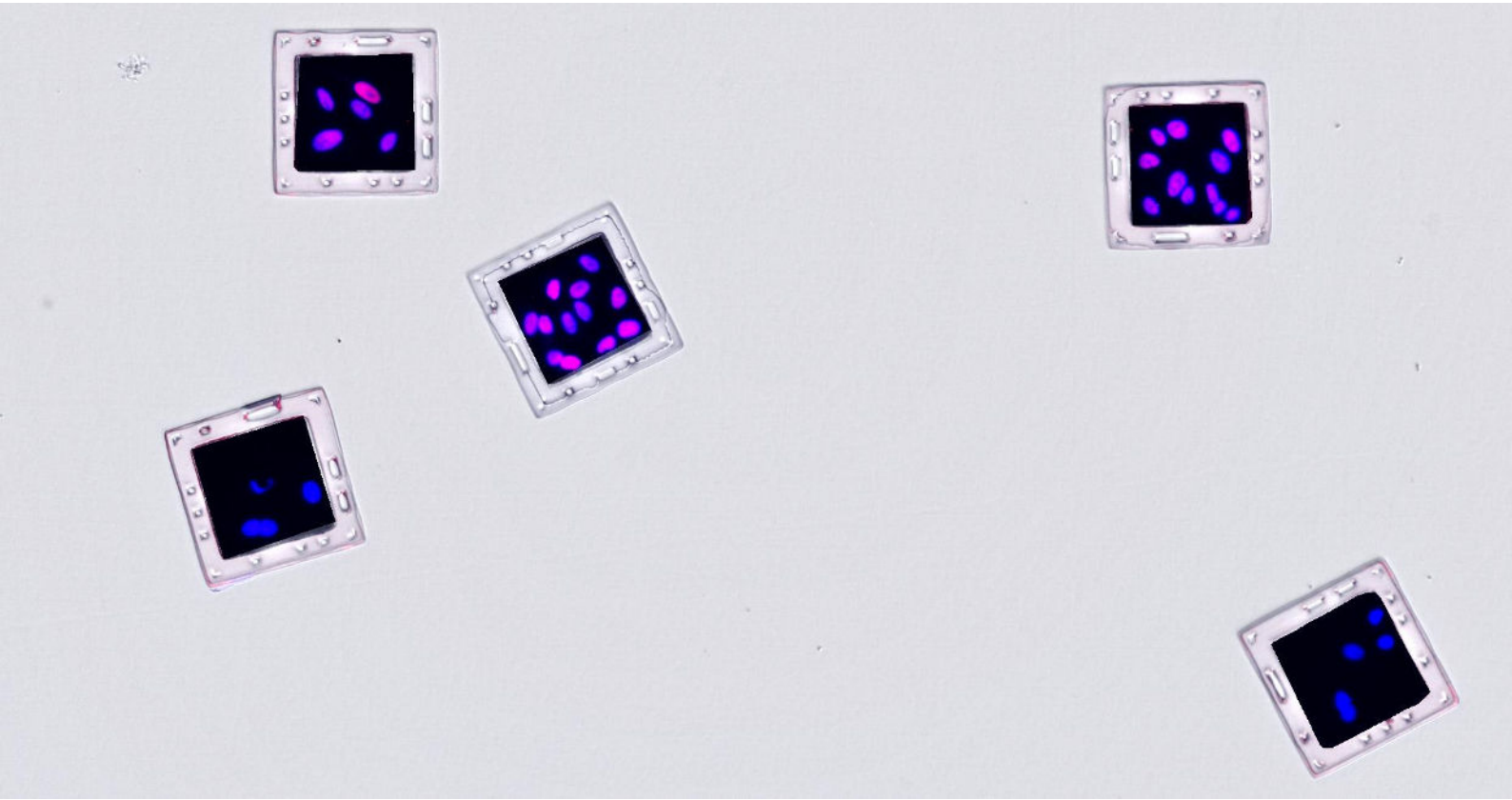
Abstract

In the pursuit of novel therapeutics, identifying the right patient populations is paramount. Correlating *in vitro* drug efficacy with phenotypic or -omics markers from cell line panel screens can significantly enhance this process. However, the challenge lies in assaying numerous cell lines, which can be both costly and time-consuming. The SemaCyte® Multiplex platform directly addresses this challenge, promising a 6-fold cost reduction and 10-fold time savings for cell panel assays. By enabling the multiplexing of 10 distinct cell types within microplate wells, this innovative platform drastically accelerates data generation. In this study, we employ a 4-plex cell panel as a proof of concept. Our results demonstrate that the SemaCyte® Multiplex platform establishes meaningful and biologically plausible associations between Nutlin-3a cytotoxicity and p53 induction. Specifically, we investigate A549, PC3, T98G, and HepG2 cells, which were pooled and exposed to serial dilutions of Nutlin-3a. Notably, while A549, PC3, and HepG2 cells exhibit a robust response to Nutlin-3a, T98G cells display a significantly higher IC50 value — correlating with high basal yet mutant, and thus unresponsive, p53. In summary, our optimized assay setup using the SemaCyte Multiplex® platform achieves results 2 times faster and with 4 times fewer reagents, making it an indispensable tool for accelerating drug discovery and biomarker identification.



Introduction

Cell panel screening, the analysis of the phenotypic effects of drug compounds across tens, hundreds or even thousands of cell lines, can inform which predictive biomarkers or patient cohorts should be tested in subsequent preclinical or clinical studies. Due to the need to assess the response of multiple cell lines in the same assay, cell panel screens can often be costly and time consuming and suffer from inter-assay variability. The SemaCyte® Multiplex platform addresses these issue by allowing for the multiplexing of up to 10 different cell types in a single well of a multiwell plate delivering a potential 6-fold cost and 10-fold time saving benefit.

p53 is a key cell cycle regulator that is frequently inactivated in cancer[1]. This inactivation is achieved by mutation of the TP53 gene or downregulation of p53 protein expression. p53 protein levels are negatively regulated by the E3 ubiquitin-protein ligase MDM2 and the compound Nutlin-3a blocks the interaction between p53 and MDM2 leading to stabilisation of the p53 protein[2]. *In vitro* studies have shown that Nutlin-3a may have efficacy in p53 wild-type tumours through the induction of p53 expression and subsequent cell death[3].

Using the SemaCyte® Multiplex platform we performed a proof-of concept study to assess the association between p53 protein levels and the cytotoxicity of Nutlin-3a using a single 4-plex cell panel (**Figure 1**).

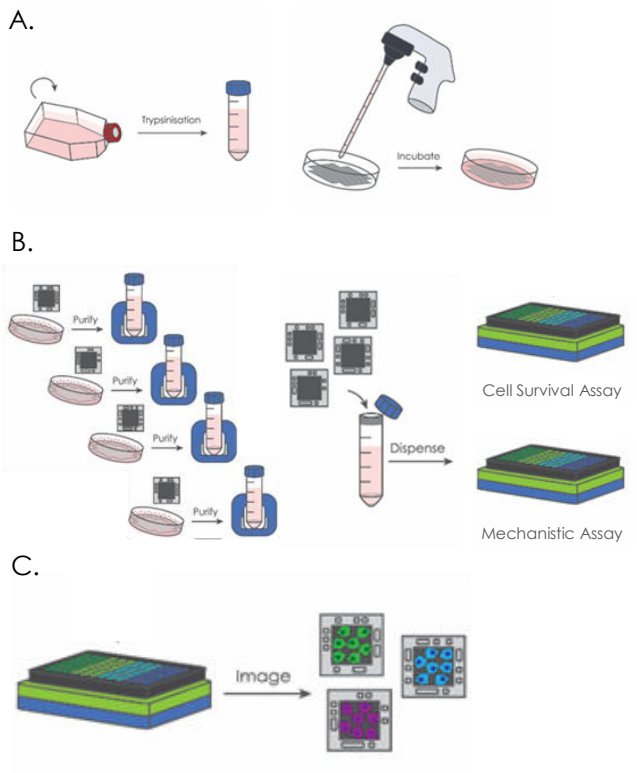


Figure 1. Overview of the use of the SemaCyte® Multiplex platform to perform cell survival and mechanistic endpoint assays on a multiplexed cell panel. A. Each cell line is trypsinized and seeded onto a differently barcoded SD20. **B.** 16-24 hours after seeding, SemaCyte® microcarriers are collected, purified, and pooled before plating into assay plates. **C.** At the end of the assay, plates are imaged and barcodes digitally deconvoluted to allow for analysis of each cell line independently.

Key Advantages

Accelerate Assays: Multiplex cell types to achieve a potential 6-fold cost and 10-fold time-saving benefit.

Workflow Integration: Seamlessly integrate SemaCyte® microcarriers with various data analytics tools, assay types, read-out equipment, and lab automation systems.

Freeze Adhered Cells: Cells adhered to SemaCyte® microcarriers can be frozen in cryovials. Assay these cells within just 1 hour after thawing, enhancing throughput and minimizing batch variability.

Move Adhered Cells: Enable adherent cells to move without affecting their natural morphology.

Ultra-Miniaturize Assays: Reduce the number of cells per well without compromising local confluency.

Materials and Methods

A549, PC3, T98G, and HepG2 cells were seeded onto differently barcoded 20 sq. cm SemaCyte® Seeding Dishes (SD20) at a density of 40,000 cells/cm^2. Cells were incubated for 16 h in EMEM culture medium supplemented with fetal calf serum, glutamine and MEM Non-Essential Amino Acids. After 16 hours of cell attachment, SemaCyte® Seeding Dishes were agitated to release cell-loaded SemaCytes. After magnetic separation, the microcarriers were mixed and approximately 200 microcarriers were plated into each well of a 96 well plate. Appropriate concentrations of Nutlin-3a were then added to each well of the plate.

For cell survival endpoints, SemaCyte® microcarriers were stained with HCS Nuclear Mask Blue (ThermoFisher Scientific) 72 hours post drug addition. Microcarriers were then imaged using the EVOS microscope (10x objective) and nuclear counts performed.

For p53 immunofluorescence SemaCyte® microcarriers were fixed with Paraformaldehyde (PFA) 16 hours post drug addition. For fixation, wells were washed once with PBS and then fixed for 10 minutes with 4% PFA. After fixation, SemaCyte® microcarriers were washed once with PBS and either stored in PBS at 4°C or directly used for antibody staining. Prior to staining, cells were permeabilised with 0.5% Triton X-100 in PBS for 10 minutes. SemaCyte® microcarriers were then incubated with p53 primary antibody (DO-1) for two hours at room temperature. After washing three times with PBS, SemaCyte® microcarriers were incubated with Alexa fluor® conjugated anti-mouse secondary antibody for two hours at room temperature, washed, and then stained with HCS Nuclear Mask Blue (ThermoFisher Scientific). Cells were imaged using an EVOS microscope (10x objective).

Analysis of nuclear counts and nuclear p53 was performed using Semalyse and CellProfiler software. Briefly, images were loaded into Semalyse which performed SemaCyte® microcarrier recognition and barcode deconvolution to produce images containing SemaCyte® microcarriers tiled by barcode (more info). These images were then analysed using cell profiler. Nuclear counts and p53 intensity analysis was performed on triplicate wells with between 20 and 30 SemaCyte® microcarriers analysed per cell line per well.

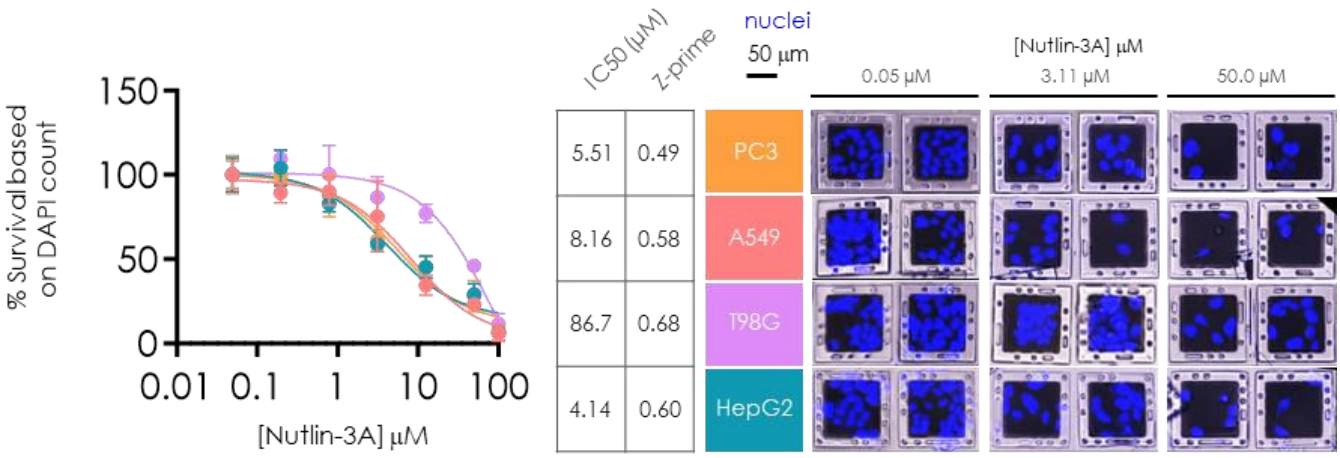


Figure 2. Assessment of the effect of Nutlin-3a on cell survival in a 4-plex cell panel. Barcoded SemaCyte® microcarriers each loaded with a different cell line were mixed and plated into a 96 well plate. After plating, different concentrations of Nutlin-3a were added to each well. After 72 hours of drug incubation, nuclei were stained and a nuclear count performed. Triplicate wells were analysed for each condition, with a minimum of 20 SemaCyte® microcarriers analysed per cell line in each well. Individual dose response curves for the four different cell lines within the multiplex panel are shown. Data shown is the mean and standard deviation of triplicate wells. Example images of SemaCyte® microcarriers at 3 different concentrations of Nultin-3a are shown.

Assessment of the effect of Nutlin-3a on cell survival in a 4-plex cell panel.

In vitro, Nutlin-3a has been shown to cause a dose dependent reduction in cell survival of cancer cell lines[3]. To identify correlates between Nutlin-3a sensitivity and p53 marker expression, we used the SemaCyte® Multiplexing platform to perform multiplexed cell survival assays within a 4-plex cell panel (**Figure 2**). Our cell panel consisted of PC3 (prostate adenocarcinoma) , A549 (lung adenocarcinoma), T98G (glioblastoma), and HepG2 (hepatocarcinoma). As well as being a cancer derived line, HepG2 show a high degree of morphological and functional differentiation so are commonly used as a live toxicity model in drug discovery[4]. In our multiplexed cell survival assay we observed that PC3, A549 and HepG2 had IC50 values in the range of 4-8 µM, whereas, T98G had an IC50 value more than 10-fold greater than the three other lines (**Figure 2**). The differential IC50 values for these cell lines allows for further analysis to investigate associations between Nutlin-3a sensitivity and phenotype.

Assessment of basal and Nutlin-3a induced nuclear p53 levels in a 4-plex cell panel.

In parallel to the cell survival assay, the 4-plex cell panel was plated out to perform Immunofluorescent assessment of p53 nuclear levels with and without Nutlin-3a treatment (**Figure 3**). Of the four lines tested, three of them (A549, PC3 and HepG2) showed significant induction of nuclear p53 in the presence of 12.5 mM Nutlin-3a. No induction of nuclear p53 was observed for T98G, which also had a higher basal level of nuclear p53.

Association between nuclear p53 levels and cell survival effects of Nutlin-3a.

We next performed analysis to identify associations between Nutlin-3a sensitivity and nuclear p53 levels (**Figure 4**).

We observed that both high basal levels of p53 (**Figure 4A**) and a lack of Nutlin-3a induced p53 induction (**Figure 4B**) were associated with high IC50 values in the Nultin-3a cell survival assay. Given the panel size, we were not able to perform any statistical analysis for these associations. However, our observations present hypotheses that could be tested in larger multiplex cell panels to provide statistical robustness. Importantly, based on current literature, these hypotheses are biologically plausible. Analysis of p53 protein levels in a range of tumours has demonstrated that p53 mutation is associated with its high nuclear expression[5] and *in vitro* cell line studies have demonstrated that p53 wild-type but not mutant cancer cell lines show a high degree of sensitivity to Nutlin-3a[6]. Indeed, the Nutlin-3a insensitive, high nuclear p53 cell line T98G has been reported to have a pathogenic p53 mutation[7].

Conclusion

By using the SemaCyte Multiplexing platform we were able to perform parallel analysis of cell survival and p53 nuclear levels in response to Nutlin-3a. Performing the assay in this way led to a 4-fold reduction in cost and time compared to performing the assays on a single cell line at once. Importantly, these assays led to biologically plausible hypotheses that could ultimately be used to predict the response of tumour cells to Nutlin-3a.

References

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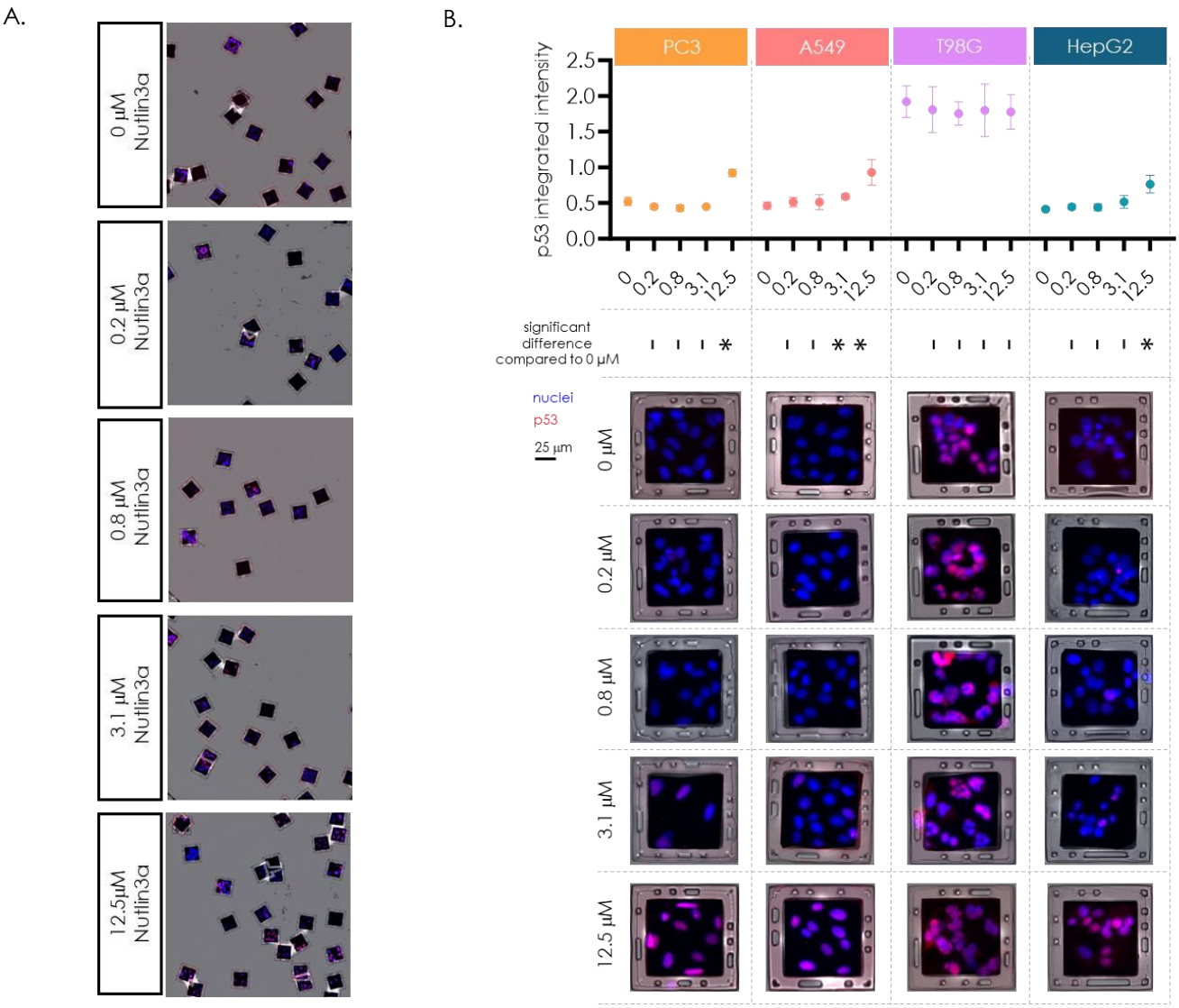

Figure 3. Assessment of the basal and Nutlin-3a induced nuclear p53 levels in a 4-plex cell panel. Barcoded SemaCyte® microcarriers each loaded with a different cell line were mixed and plated into a 96 well plate. After plating, different concentrations of Nutlin-3a were added to each well. After 16 hours of drug incubation, microcarriers were fixed, p53 immunocytochemistry was performed, and nuclear p53 intensity assessed. Triplicate wells were analysed for each condition with a minimum of 20 SemaCyte® microcarriers analysed per cell line in each well. **A.** Example images of SemaCyte® microcarriers from a single field of view. **B.** Individual p53 intensity curves for the four different cell lines. Data shown is the mean and standard deviation of triplicate wells. Example microcarriers from each line and each Nutlin-3a concentration are shown.

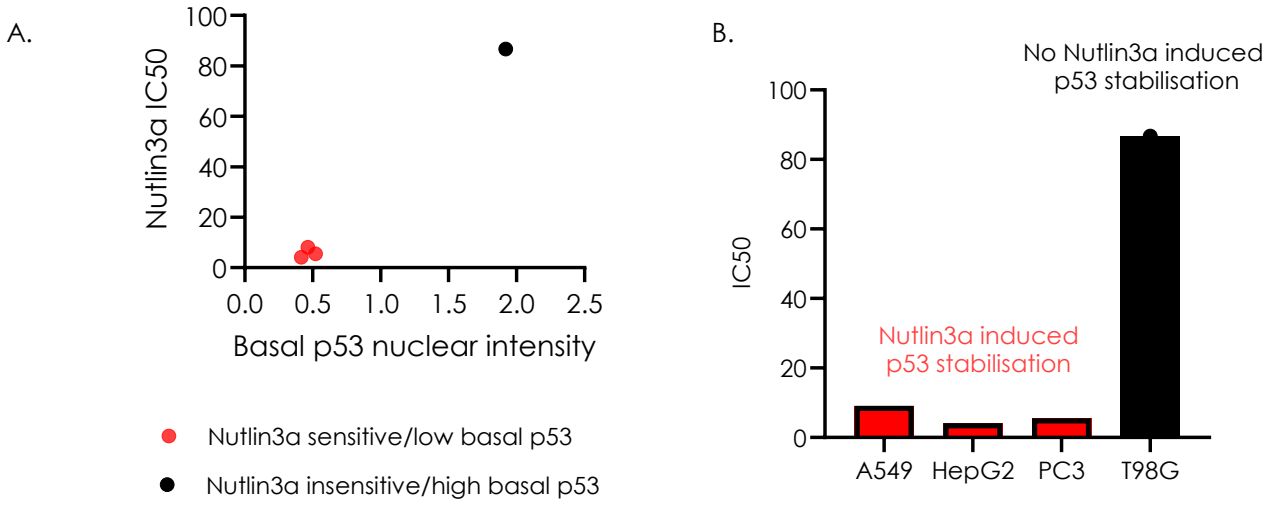

Figure 4. Association between nuclear p53 levels and cell survival effects of Nutlin-3a. **A.** Correlation between Basal p53 nuclear intensity without any Nutlin-3a present and Nutlin-3a IC50. **B.** Plot of Nutlin-3a IC50 values across the panel with lines that show Nutlin-3a induced p53 stabilisation highlighted in red and those with no observable Nutlin-3a induced p53 stabilisation in black.