Development of a Robust Screening Cascade to Identify Small Molecule Inhibitors of a DNA Damage Response Protein

Adam Peall[1], Rob Workman[1], Kathy Dodgson[1], Gary Allenby[1]
[1]Aurelia Bioscience Ltd, Biocity, Pennyfoot Street, Nottingham, UK

Introduction

Developing small molecule inhibitors requires a robust screening cascade which can efficiently identify compounds which engage the target protein, determine selectivity over undesirable related proteins and demonstrate activity in downstream functional assays.

To ensure active hits represent on-target activity, assays in the screening cascade should be biologically relevant and specific to the mechanism of action of the target protein and signalling pathway. All assays in the cascade should be reproducible and robust to ensure that results can reliably inform decisions on structure activity relationships (SAR). In addition, all assays must have a suitable throughput capacity to handle the expected number of compounds at each stage of the screening cascade.

Here we present data on the screening cascade we have successfully developed for a client project to identify small molecule inhibitors of a DNA damage response protein.

Screening Cascade

NanoBRET Target Engagement

Compound binding to target protein? | Selectivity over off-target protein(s)?

Cell Titre Glo Cytotoxicity Screen

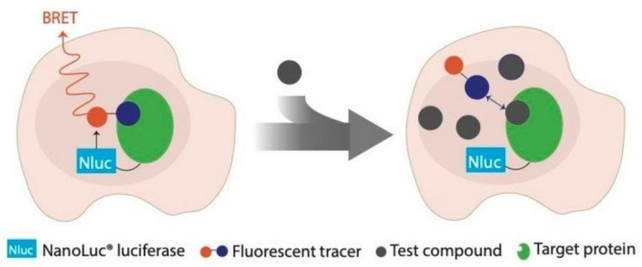
Compound toxicity observed? | Sufficient window of activity below toxic concentration?

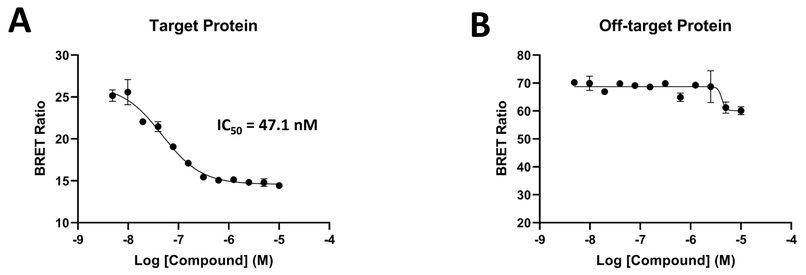
TUNEL Assay Functional Response

Does compound binding translate to functional response? | Can functional activity be repeated in an orthogonal assay?

Cell Cycle Assay Functional Response (Orthogonal)

NanoBRET™ – Target Engagement

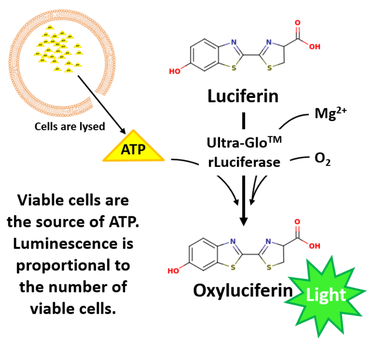




The Promega NanoBRET™ Target Engagement Assay enables compound affinity for the target protein, or off-target binding to related proteins, to be quantified in a cell-based system using full-length target proteins which can also provide information on compound cell permeability and residence time. Cells are transiently transfected with a fusion protein composed of the target of interest and NanoLuc® Luciferase. Addition of a cell-permeable fluorescent NanoBRET™ tracer, a fluorescently tagged compound which reversibly binds the target protein, enables bioluminescence resonance energy transfer (BRET) to occur once the tracer binds and is in close proximity to the NanoLuc® Luciferase. The apparent affinity of test compounds is determined by competitive displacement of the NanoBRET™ tracer and the resultant decrease in BRET signal. We have successfully deployed the NanoBRET™ Target Engagement Assay as an initial screen for inhibitors of a DNA damage response protein whilst simultaneously screening out compounds which bind to a related but undesirable off-target protein. An example of the results for one of the active compounds identified using this assay is shown in **(A)** where potent binding and successful displacement of the NanoBRET™ tracer is observed for the target protein but only minimal binding is identified in the off-target protein **(B)** counter-screen.

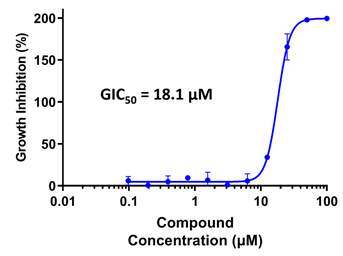
CellTiter-Glo®– Cytotoxicity

Toxic compounds can cause unexpected results in cell based assays and thus it is good practice to include a cytotoxicity assay in any drug discovery screening cascade. This is especially relevant in this project as our downstream cellular assays involve inhibiting the effect of a DNA damage inducing agent. Any compound-induced cytotoxicity would cause deleterious effects on the assay readouts and hence we aim to screen compounds at sub-toxic concentrations.

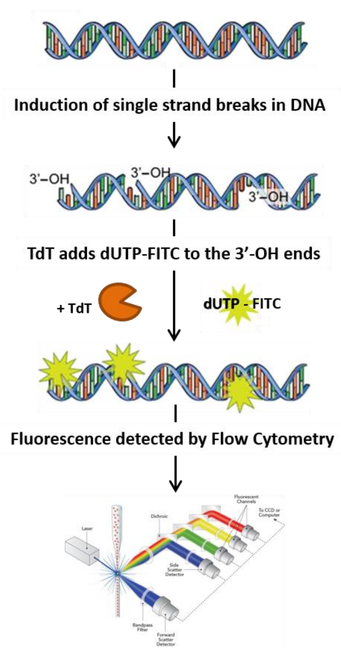


Viable cells are the source of ATP. Luminescence is proportional to the number of viable cells.

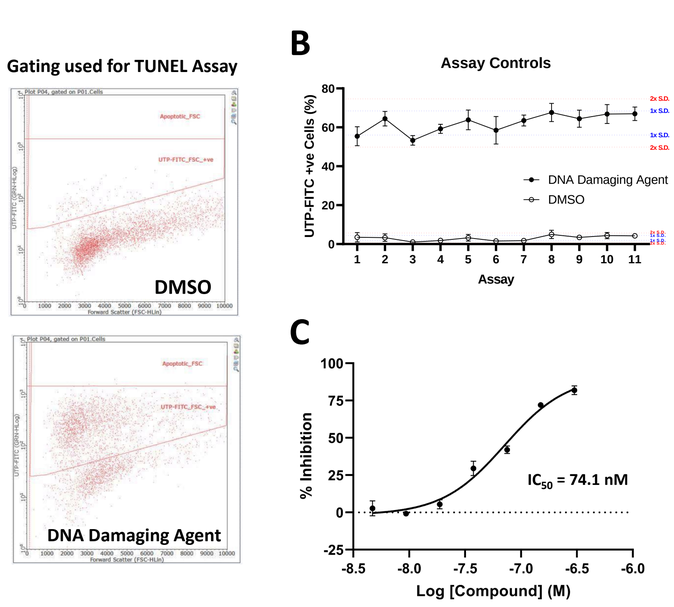
CellTiter-Glo® utilises the ATP dependent conversion of luciferin to oxyluciferin and the resulting luminescence. Cells are used as the source of ATP; the amount of ATP and therefore luminescence signal is proportional to the number of viable cells. The impact of compounds on the number of viable cells can be due to inhibition of cell growth, induction of cell death or perturbation of cell metabolism. Luminescence readouts are normalised to 0h controls and plotted as % growth inhibition, with GIC_{50} being the concentration at which 50% growth inhibition is achieved and growth inhibition >100% indicating cytotoxicity as the number of viable cells has decreased to lower than the seeding density.



TUNEL Assay – On-Target Functional Response



DNA strand breaks reveal free 3'OH groups to which terminal deoxynucleotide transferase (TdT) is able to catalyse the addition of deoxynucleotides. In our assay, cells are treated with compounds and a DNA damaging agent for 48h, fixed, permeabilised and then are incubated with the TdT enzyme and dUTP-FITC. Incorporation of dUTP-FITC is determined by analysing the cells by flow cytometry. Treatment with the DNA damaging agent **(A: Bottom panel)** induces single strand breaks in ~60% of cells (UTP-FITC_FSC_+ve gate), compared to minimal induction in DMSO treated cells. Induction of UTP-FITC +ve cells by the DNA damaging agent is very consistent **(B)** between experiments and enables a robust screening assay. Additional cell stress, such as treatment with toxic concentrations of a compound, induce higher levels of UTP-FITC incorporation **(A)** due to apoptosis-induced double stranded DNA break formation (Apoptotic_FSC gate). Gating of apoptotic cells enables outliers caused by cell death to be identified and unbiasedly excluded from further analysis. Compounds are assessed for their ability to inhibit the DNA damaging agent-induced increase in UTP-FITC cells with data normalised to the controls to generate % inhibition values **(C)**. The use of flow cytometry to analyse the cells has enabled very tight replicates for compound concentration curves. We also observe good correlation between the potency of compounds in the Target Engagement assay and the TUNEL assay.

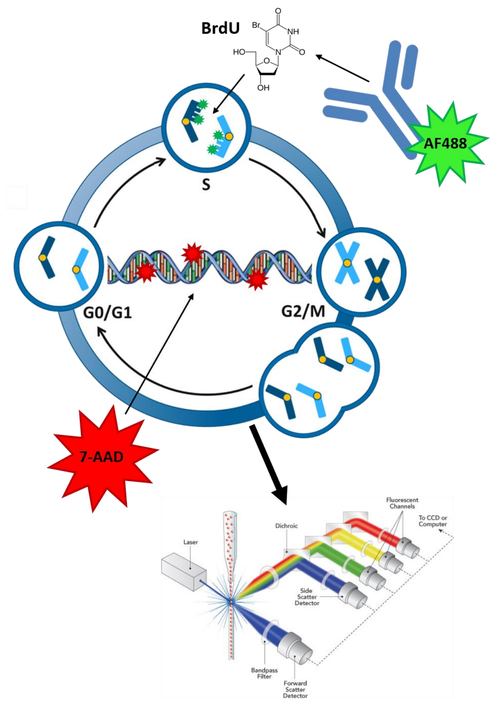


Summary and Conclusions

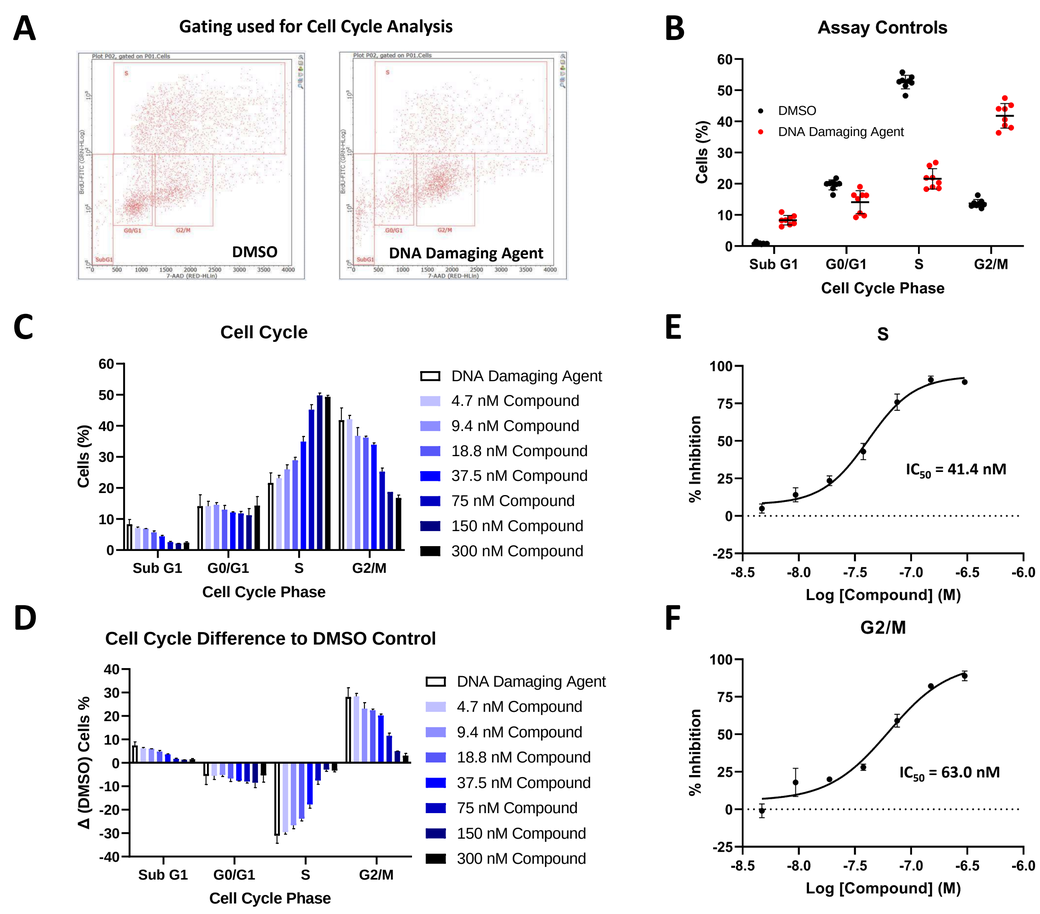
- The NanoBRET™ Target Engagement assay enables us to screen compounds against our protein target of interest and determine selectivity over a related off-target protein. These assays are performed in cells with full-length protein constructs and thus we do not see dramatic reductions in reported IC_{50} values in downstream functional assays which can be typical of other binding assays technologies.
- Cytotoxic compounds are identified using the CellTiter-Glo® assay, enabling non-toxic testable dose ranges to be determined for downstream functional assays, minimising outliers and miscellaneous results.
- The flow cytometry based TUNEL assay effectively enables the functional activity of compounds to be determined in a biologically relevant mechanistic assay with excellent reproducibility and robustness.
- Active compounds in the TUNEL assay are then confirmed in the orthogonal cell cycle flow cytometry assay. The cell cycle assay assesses the ability of compounds to reverse the effect of a DNA damaging agent to induce G2/M arrest, due to the activity of our protein of interest.

Overall, the design, optimisation and implementation of these biologically relevant assays has resulted in the generation of a robust screening cascade which is capable of identifying inhibitors of a DNA damage response protein.

Cell Cycle – On-Target Functional Response (Orthogonal Readout)



It is essential in any screening cascade to confirm the results of active compounds in an orthogonal assay, which uses a different assay method, to control against the potential for false positives. We therefore established a cell cycle assay to confirm the activity of compound hits. Cells are treated with compounds and the DNA damaging agent for 48h prior to pulse staining with BrdU, fixation and permeabilisation. Cells are then treated to a limited DNase digest to expose BrdU-labelled epitopes before being stained with an anti-BrdU-FITC antibody and 7-AAD. As a cell traverses its cell cycle the amount of DNA present in a cell will increase from G0/G1 phase, to G2/M phase, with an intermediate amount of DNA in S phase when DNA replication occurs. 7-AAD binds DNA stoichiometrically and so is proportional to the amount of DNA within each cell, whereas BrdU is incorporated only in cells actively replicating their DNA in S phase.



Using the combination of BrdU-FITC and 7-AAD staining, cells can be gated **(A)** as being in G0/G1, S or G2/M phase, whilst apoptotic cells (undergoing DNA cleavage)are gated as SubG1. Treatment with a DNA damaging agent **(A and B)** induces G2/M arrest, a decrease in the % of cells in S phase and a small increase in the % of cells in SubG1. The effect of compound treatment on DNA damaging agent-induced G2/M arrest can be displayed for the whole cell cycle **(C)** or as the difference to DMSO treated cells **(D)**. In both displays **(C and D)** we can clearly observe a dose-dependent inhibition of the effect of the DNA damaging agent. We can also plot % inhibition values (generated by normalising the data to the controls) for the effect on S phase **(E)** and G2/M **(F)** phase cells. Here we observe complete compound inhibition curves and correlation in the IC_{50} values with those observed in the Target Engagement and TUNEL assays.