Developing a DIPG cell line viability assay

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Aim - To develop a reliable method to assay the growth or viability of DIPG cell lines for use during screening of pre-clinical compounds.

Methodology

The design of this assay was informed as yet unpublished viability assayed used by other DIPG focused groups, as well as my own optimisation (outlined below). The assay consists of adherent growth of the DIPG cell line followed by measurement of end-point cell viability using the luciferase based CellTiter-Glo® assay (Promega) that produces luminescence in proportion to the amount of ATP present in the cells within the well.

Growth media

Tumour Stem Medium – Base (TSM-B) and Tumour Stem Medium – Complete (TSM-C) were made up as previously described (Grasso et al., 2015), and outlined in a previous upload (https://doi.org/10.5281/zenodo.1172048).

Experimental details

The following describes the latest design of the assay. Changes made in progressive iterations are noted were relevant.

Laminin coating plates

For HSJD-DIPG-07 cells to grow adherently, 96-well tissue culture plates (Greiner cat. no. 655090) (recommended for use with Celigo image cytometer) must be coated with laminin (Engelbreth-Holm-Swarm murine sarcoma, Sigma, cat. no. L2020) as follows:

- Dilute laminin 1:100 in sterile water
- Add 45µLof laminin to each well*
- Incubate for 2h at 37°C
- Remove laminin**
- Wash well with PBS
- Store plate with coated wells in PBS at 4°C
- Before plating cells remove PBS and allow wells to dry

* Excluding outer wells, as these are more likely suffer from evaporation, or to be out of focus in the Celigo

** This can be stored frozen for one additional use

Cell seeding

Procedure for splitting cells was described in a previous upload (https://doi.org/10.5281/zenodo.1172048).

Seed cells using a repeater pipette (for higher accuracy) in 100µL TSM-C as follows:

X	X	X	X	X	X	X	X	X	X	X	X
X	3000							6000			X
X								3000			X
X								1500			X
X								750			X
X								375			X
X								0			X
X	X	X	X	X	X	X	X	X	X	X	X

In the first iteration of this assay the main wells were seeded with 3,000 cells, however this number of cells became overgrown and began detaching from the well bottom before the desired time point (i.e. at least 3 days in the presence of the compound to be tested). Therefore, in all following iterations only 2,000 cells were seeded. The outer wells are filled with PBS to reduce evaporation within the plate and to avoid focus issues when using the Celigo.

The titration of cells seeded in the right three columns initially served as a means to record the growth kinetics of HSJD-DIPG-07 cells growth adherently, act as untreated (vehicle only) controls.

Compound treatment

Three days after cell seeding (i.e. if cells are seeded on Friday, add compounds on Monday) compounds are added at the following final concentrations (2 per plate):

X	X	X	X	X	X	X	X	X	X	X	X
X	**10uM**	**5**	**1**	**0.5**	**0.1**	**0.05**	**0.01**	DMSO			X
X											X
X											X
X	**10**	**5**	**1**	**0.5**	**0.1**	**0.05**	**0.01**				X
X											X
X											X
X	X	X	X	X	X	X	X	X	X	X	X

All compounds are made up at 500x concentration in DMSO and diluted into 100µLTSM-C at 2x concentration. Therefore, all vehicle controls are treated with a final concentration of 0.2% of DMSO in TSM-C.

In later repeats propidium iodide (PI) was simultaneously added at a final concentration of 1:1000 to mark dead cells. This allows the differentiation of reduced proliferation and increased cell death. End point readings were taken on the ClarioSTAR although the Celigo can also record PI signal and allow the recording of death over the whole treatment period.

Data collection and processing

Confluency can be measured daily using the Celigo to produce growth curves for all treatments (and can allow identification of outliers).

After 4 days record the confluency of each well with Celigo “confluency 1” algorithm, and then record cell viability by adding 100µLof CellTiter-Glo® 3D Cell Viability Assay solution (Promega) and incubating according to manufacturer protocol. Record the luminescence of each well (with the ClarioSTAR plate reader record a 3x3 matrix of each well, with gain of 3500 and recording time of 1s).

Data is normalised to the untreated control (or with PI readings, the treated repeat with the highest signal) and plotted in GraphPad Prism. IC50 values are generated using the [Inhibitor] vs. normalised response, robust fit settings.

Results

The first iteration of this assay allowed the recording of adherent confluency over time **(Fig. 1)**. This allowed the selection of the correct number of cells to seed for future iterations.

A starting density of 2,000 cells was selected, allowing a 4-day treatment with the drug. Other groups produced an 8-day time-point, however these observations indicate that these cells cannot be grown exponentially over that period of time without additional medium changes. Thus far 3 biological repeats have been completed for the two ALK2 inhibitors K03841* and K00991** (that have been previously assayed by other groups). K03841 did not appear to inhibit drugs in this concentration range with a four-day time point, however earlier tests completed with a three-day time point did show a reduction in viability (Fig. 2, 3). It may be the case that this drug is metabolised rapidly and needs to be administered more than once over the time course. On the other hand, a mean IC50 of 1.00 µM (3 s.f.) was recorded for K00991 at four days (Fig. 3). The full set of end point readings has been uploaded as a separate spreadsheet.

* also known as LDN-213844

** also known as LDN-193189

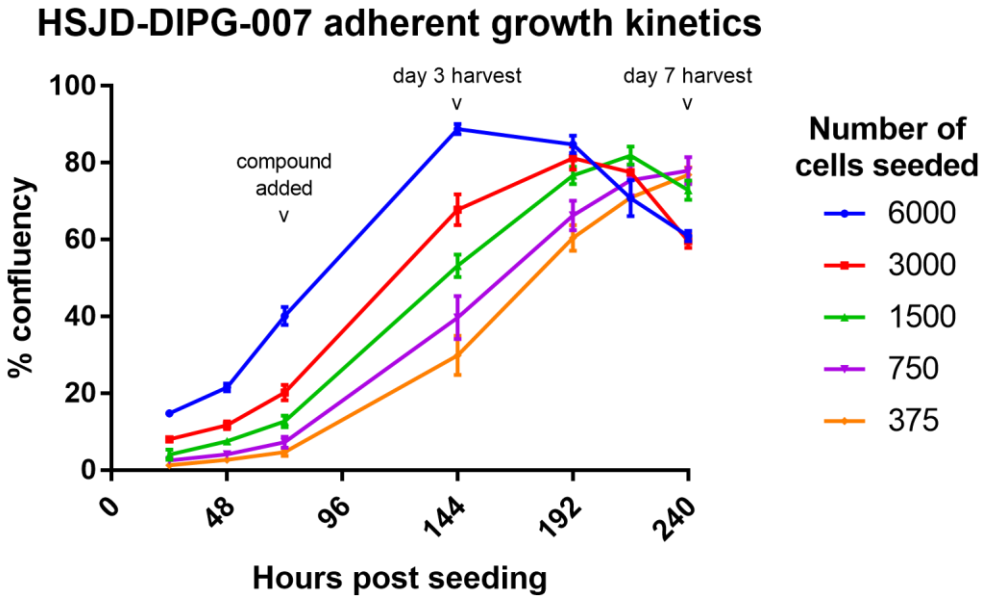


Figure 1 – *Graph illustrating the growth of HSJD-DIPG-007 cells when seeded adherently in laminin coated plates.*

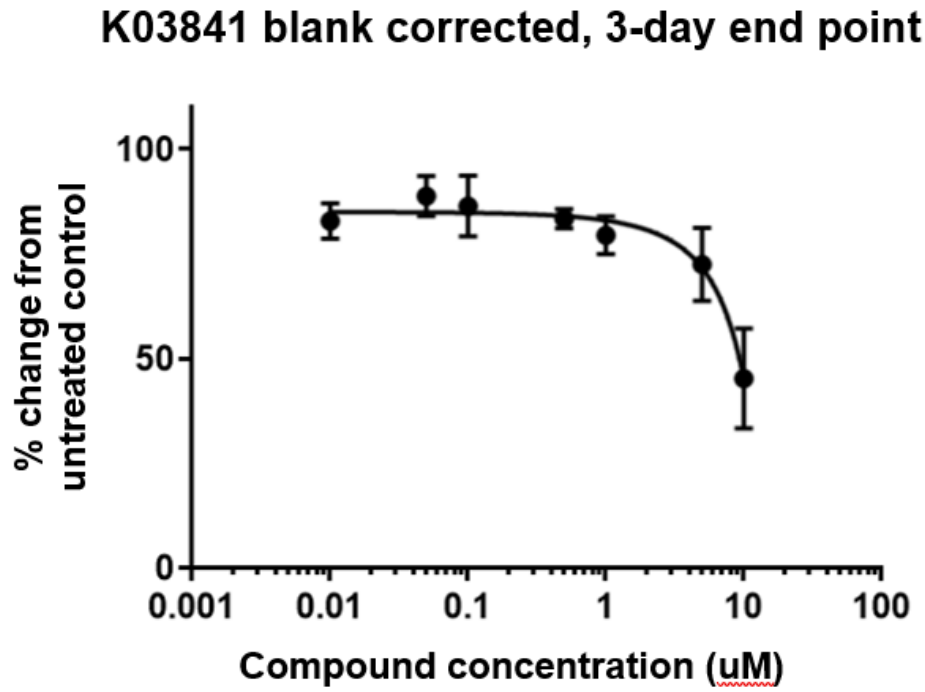
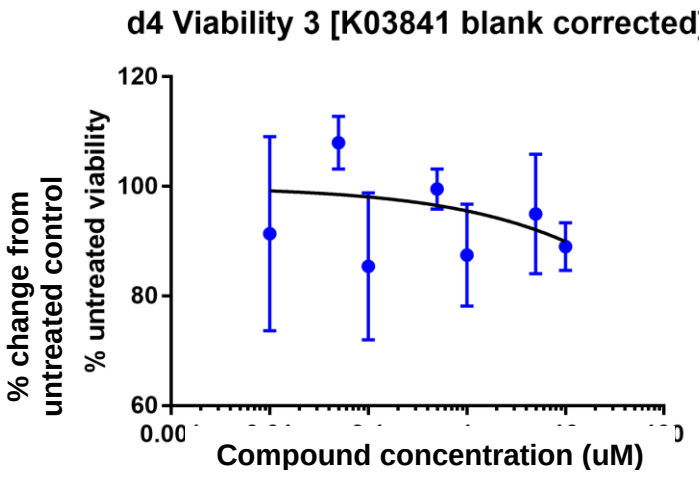
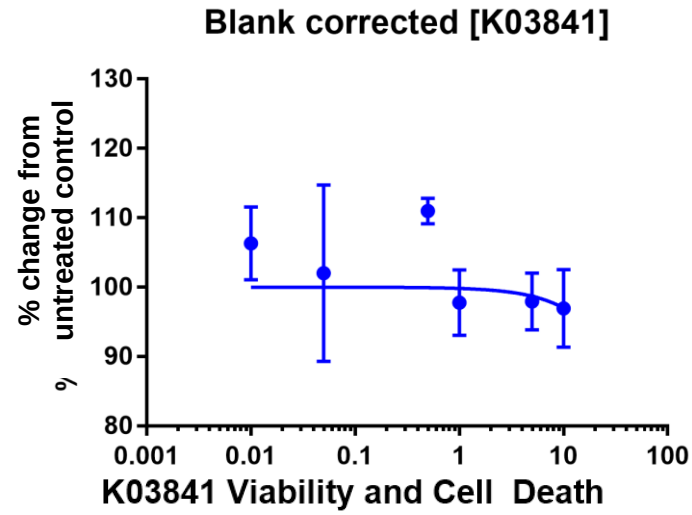
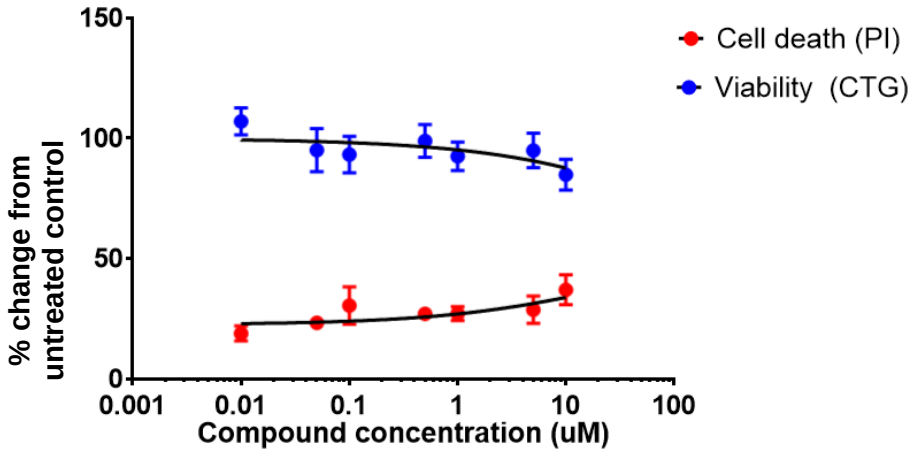


Figure 2 *- HSJD-DIPG-007 cells are grown adherently for three days and then treated with the indicated concentrations of compound. After a 3 day incubation cell viability is quantified using CellTiter-Glo. Each point represents 3 technical repeats (with mean and standard deviation plotted). All values are normalised to untreated controls. Compare the drop in viability at this time point to that observed at the four day time point recoreded in Figure 3.*

Figure 3 *– HSJD-DIPG-007 cells are grown adherently for three days and then treated with the indicated concentrations of compound. After a 4 day incubation cell viability is quantified using CellTiter-Glo, and cell death using PI. Each graph represents one biological repeat, and each point represents 3 technical repeats (with mean and standard deviation plotted). All values are normalised to untreated controls (or in the case of PI readings, the technical triplet with the highest signal).*







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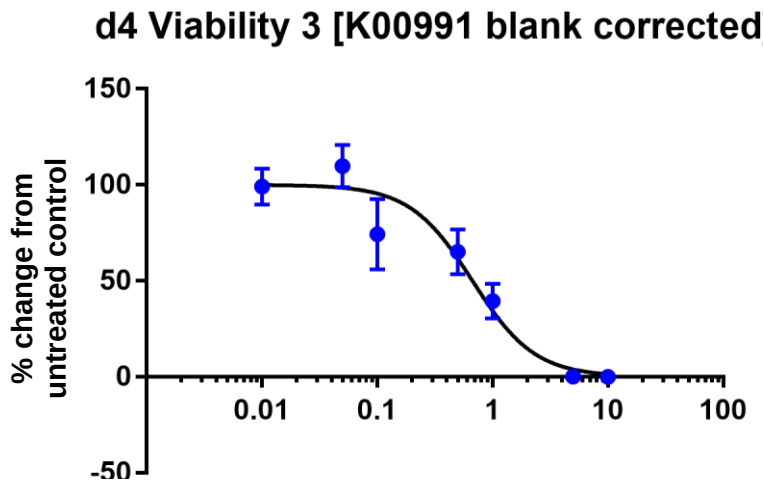

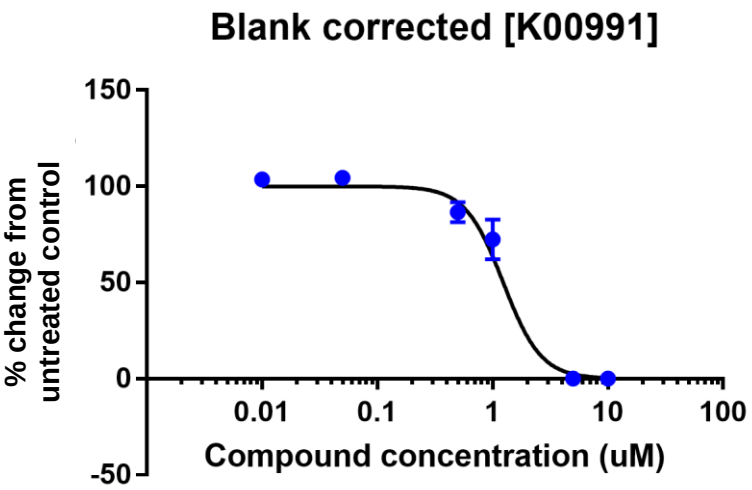

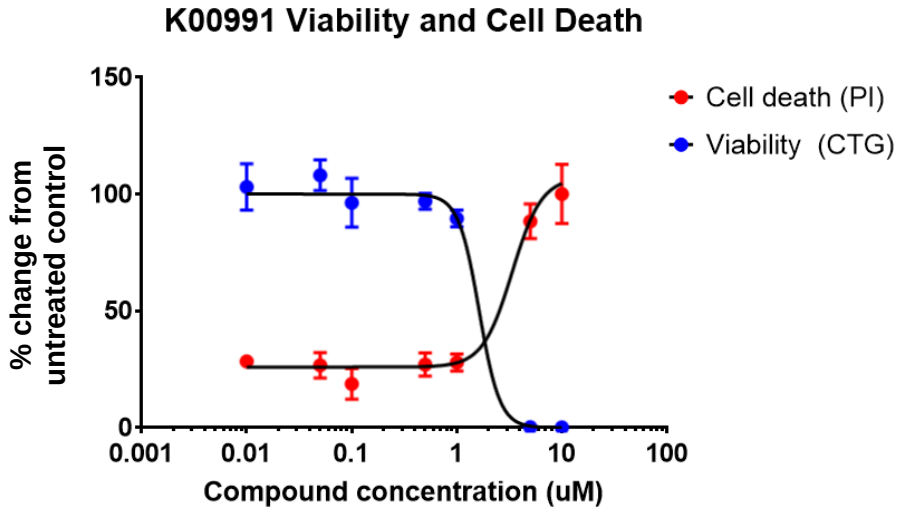

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