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Identification by High-Throughput Screening of Viridin Analogs as Biochemical and Cell-Based Inhibitors of the Cell Cycle-regulated Nek2 Kinase

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SUPPLEMENTAL METHODS

NEK2A Centrosome-splitting assay

Cells were seeded at a density of 4000 cells/well in a black clear bottom Packard Viewplate (Perkin Elmer, Ma) and incubated overnight at 37°C, 5% CO_2. The following day, the cells were induced with 1 µg/ml Doxycycline (Sigma Chemicals Co, Poole)(as described in Faragher and Fry, 2003). The same volume of PBS was added to the control uninduced cells. Simultaneously, the cells were treated with test compound (diluted to 1X, 3X or 5X GI50, or in a dilution series to: 100 µM, 33 µM, 11 µM, 3.3 µM, 1.1 µM and 0.33 µM in 1% DMSO (v/v) final concentration). 1% DMSO (v/v) was added in all wells and used as a negative control. The cells were then incubated overnight at 37°C, 5% CO_2.

The media was removed and cells washed 1X with PBS before being fixed for 10 minutes on ice with cold 100% methanol (-20°C). The methanol was removed by washing 3X with PBS and 50 µl of primary antibody added per well. This was a rabbit polyclonal antibody raised to the centrosomal marker, pericentrin (Abcam, Cambridge), and added at 1:500 dilution, in 5% BSA/PBS. The plate was incubated with a slight agitation for 1 hour at room temperature. Following a further wash with PBS the secondary antibody; goat anti-rabbit Alexa-488 (1:1000 dilution; 2 µg/ml) and the nuclear stain DAPI (0.1 µg/ml) (both Invitrogen, Paisley) were added in 5% BSA/PBS and incubated for 1 hour at room temperature. The plate was then washed with 3X PBS and refrigerated until ready to image.

In Cell Analyser 1000 acquisition procedure

The assay plates were read on the In Cell Analyser 1000 using the Workstation 1000 acquisition software (GE Healthcare). The instrument was equipped with x20 dry Nikon objective, a D360/40X DAPI excitation filter, a HQ480/40X FITC excitation

filter, a HQ460nm/40M bandwidth emission filter and a HQ535nm/50M bandwidth FITC emission filter. The exposure times were consistently between 100-150 ms in both the DAPI and FITC channels, and the levels min/max were between 100/600 (DAPI) and 100/900 (FITC). 3 fields of view were imaged in all wells so that average well data could be calculated.

In Cell Analysis Algorithm

The MultiTarget Analysis (MTA) module in the In Cell Investigator suite (GE Heathcare, Cardiff) was used to analyse the assay data. The algorithm was designed to identify the DAPI-stained nuclei, and then segment the cells setting a radius of 100 μm as the cell 'collar'. The pericentrin-positive centrosomes, present only within the 100 μm cell radius, were counted by adding another segmentation parameter (organelles). The size of the objects that the algorithm detected was optimised to be between1-4 μm, with a pixel intensity (or sensitivity) set to 40-45. A threshold filter was added to the algorithm in order to further classify the number/percentage of cells which had 1 centrosome or >1 centrosome.