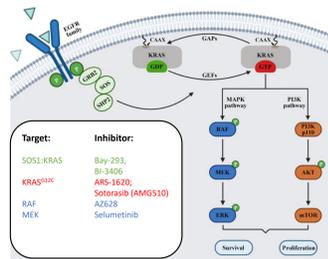


## Introduction

The KRas/RAF/MEK signalling axis has been identified to play a critical role in the formation of cancer, resulting in several investigational new drugs targeting this pathway<sup>1</sup>.



Adapted from Zhu et al., (2021) Mol. Cancer 20: 143 ff

Identification of appropriate drugs is hampered, however, by the assumption that a three-dimensional setting may be required to observe significant inhibitory effects, possibly due to alternate gene expression and protein activity in such a "closer-to-physiology" setting. In order to explore that topic, in the current study we have analysed the activity of above indicated inhibitors in our 140 cell lines (CL)-2D proliferation assay (3 days incubation) and our 100CL-3D Soft Agar panel (> 7 days incubation). Selected settings have been tested in a 3D spheroid assay. Comparative analysis of overlapping cell lines of both panels clearly supports the notion that potencies tend to be higher in the 3D situation when targeting this pathway.

## Method

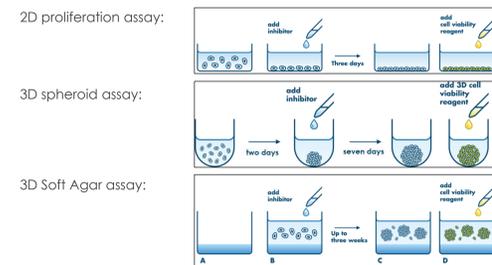
### Principle of the 2D and 3D cellular assays

**2D proliferation assay:** Cells were seeded in white clear-bottom 384-well plates. Next day, compounds were added using a nanodrop-dispenser (Tecan D300E). After incubation for 3 days, the CellTiter-Glo dye was added and luminescence signals measured as correlate of viability.

**3D spheroid assay:** Cells were seeded in clear round-bottom ultra-low attachment 384-well plates. After spheroid formation (2 days), compounds were added using a nanodrop-dispenser (Tecan D300E). After 7 days incubation, spheroids were imaged and then lysed with 3D-CTG before transfer to white plates for luminescence measurement.

**3D Soft Agar assay:** Cells were seeded in 0,4% Soft Agar in clear 96 plates pre-coated with 0,6% Soft Agar. Next day, compounds were added by nanodrop-dispension. After incubation for up to three weeks, Resazurin was added and metabolism-associated fluorescence measured as indirect quantification of colony growth.

IC50 calculation was performed using GraphPad Prism software with a variable slope sigmoidal response fitting model using 0% viability as bottom constraint and 100% viability as top constraint unless otherwise stated.



## References

- Zhu et al., (2021) Mol Cancer 20: 143 ff
- Lanman et al., (2020) J Med Chem 63: 52 ff
- Hofmann et al., (2021) Cancer Discov 11:142 ff

## Comparison of the IC50 profile of KRas pathway inhibitors in 3D Soft Agar growth vs 2D proliferation

Cells from the 100 CL 3D Soft Agar and the 140 CL 2D proliferation panel were tested for their response to indicated KRas pathway inhibitors. For the 86 cell lines overlapping within both panels, the distribution of potency was plotted to compare data generated under both conditions. Results showed that inhibitors to different extent act more potently in 3D than in 2D, which was specifically pronounced with the RAF and MEK inhibitor. No significant potency difference was observed e.g. with proteasome inhibitor Bortezomib (data not shown). Cell lines indicated with an arrow were chosen for a close-up look in the next figure.

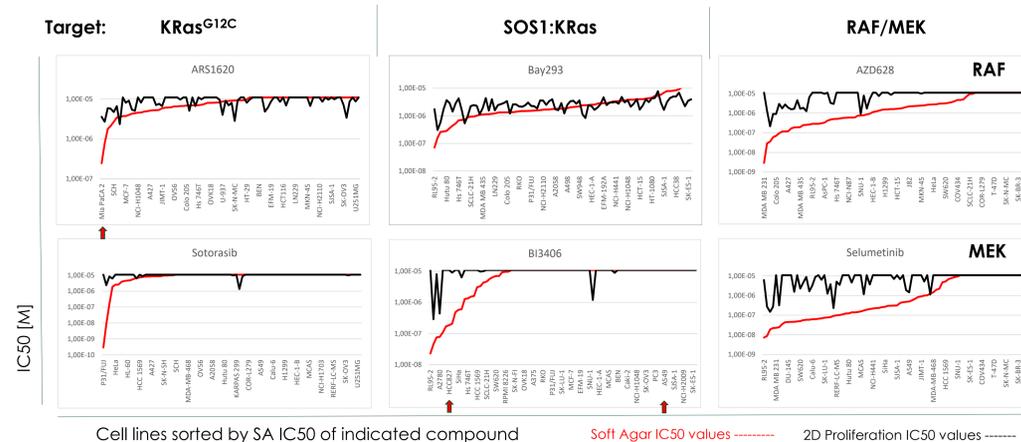


Fig. 1: Plotting the IC50 distribution in 3D Soft Agar vs 2D proliferation over the whole panel of cell lines.

Obtained IC50 values for the 86 cell lines were sorted from highest to lowest potency based on the Soft Agar results which are depicted as red line. The corresponding values for the 2D proliferation assay are shown as black line. The plots show that the red SA line is generally below the black 2D proliferation line, supporting the notion that KRas pathway inhibitors show better activity in a three-dimensional setting.

## Focus on cell lines MiaPaCa2, HCC827 and A549

Analysis of exceptionally sensitive cell lines of each KRas inhibitor target group, i.e. KRas<sup>G12C</sup> mutated MiaPaCa2 pancreatic tumor cells<sup>2</sup> or EGFR<sup>delE746-A750</sup> mutated HCC827 NSCLC tumor cells<sup>1</sup> as target for SOS1:KRas inhibitors revealed pronounced susceptibility of these cell lines in 3D only to the respective inhibitor group, but not to the other (see yellow boxes). In contrast to literature<sup>3</sup>, however, significant effects of SOS1 inhibitors on KRas<sup>G12S</sup> mutated A549 NSCLC tumor cells were reproducibly not observed. Therefore we explored these cells in another 3D system shown in the next figure.

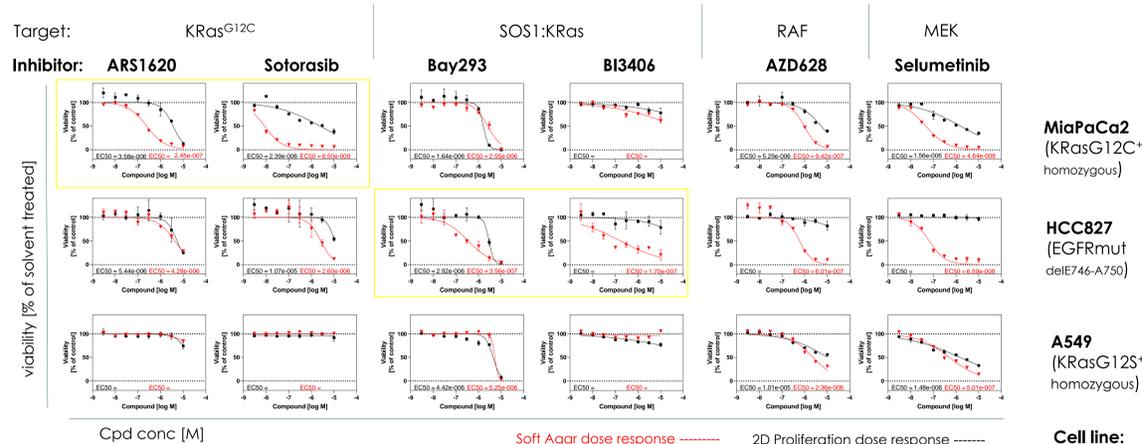


Fig. 2: Dose-response curves in overlay plots of the six inhibitors on indicated cell lines.

Raw luminescence data were converted into percent cell viability relative to the High and Low control, which were set to 100% and 0%, respectively. IC50 calculation was performed using GraphPad Prism software with a variable slope sigmoidal response fitting model using 0% viability as bottom constraint and 100% viability as top constraint. Shown are means +/- SD. Data obtained from 3D Soft Agar and 2D Proliferation experiments are shown in red and black, respectively.

## Impact of BI3406 and Sotorasib on A549 in 3D Spheroid assay

Analysis of A549 cells upon 2 days of spheroid formation and subsequent 7 days of compound treatment resulted in a partial but clear response to BI-3406, but not Sotorasib. These observations confirm data from literature<sup>3</sup> for the pronounced 3D activity of BI-3406 on A549 cells.

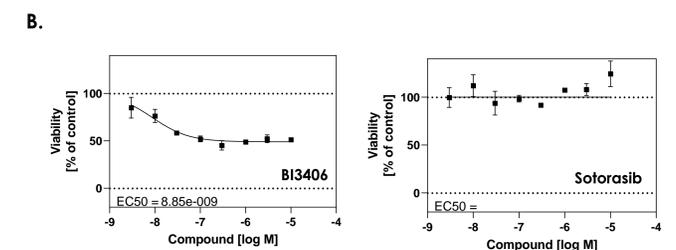
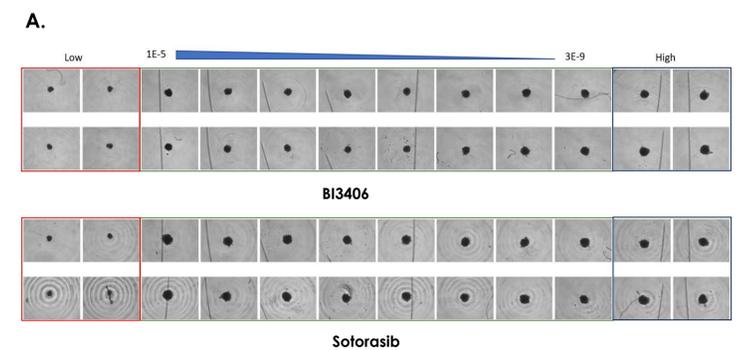


Fig. 3: Analysis of A549 3D spheroid growth in the presence of indicated inhibitors.

A549 cells were seeded in clear ultra-low attachment 384-round bottom plates and after spheroids had formed, compounds were added for another 7 days. Spheroids were photographed (A.) and then lysed with 3D-CTG before transfer to white plates for luminescence measurement. IC50 plots (B.) were determined as described in Figure 2 but without bottom constraints. Low = 1E-5M Staurosporine; High = 0,1% DMSO.

## Summary

Comparison of potencies for six KRas pathway inhibitors on 86 cell lines in 3D versus 2D cellular assays showed that:

- Overall, a higher potency is observed in 3D.
- This is broadly observed with RAF- and MEK kinase inhibitors
- In many cases, compounds indicate a trend of activity in 2D, which is more pronounced in 3D (e.g. Sotorasib or Selumetinib on MiaPaCa2 cells)
- In some cases, compounds appear inactive in 2D, but very potent in 3D (e.g. BI3406, Selumetinib or AZD628 on HCC827)
- Especially for moderate effects, testing different 3D assays may be of use to identify adequate inhibitors (e.g. BI3406 on A549 in Spheroid vs Soft Agar assay)
- Our results show that 3D growth analysis clearly supports the development of KRas/RAF/MEK pathway inhibitors.

## Contact Information

Jan E. Ehlert, PhD

Reaction Biology Europe  
Engesserstr. 4  
Freiburg, 79108, Germany

+49-761-769996-0  
j.ehlert@reactionbiology.de  
www.reactionbiology.com