Characterization of the selective inhibitory effect of KRas inhibitors in different cellular assay formats

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Introduction

In healthy cells, the small GTPase KRas functions as a finely regulated molecular switch for major physiological signaling pathways involved in cell proliferation, differentiation and survival.

Interestingly, it has been shown that KRas mutations are frequently present in different tumor entities, thereby being among the most common oncogenic drivers of tumorigenesis[1]. Missense mutations of KRas result in constitutive activation which enhances tumor-promoting downstream signaling pathways including the MAPK pathway. Most KRas mutations are located in exon 2 or 3 including the most frequently changed glycine 12 which is present in most pancreatic cancers as well as in colorectal cancers and lung adenocarcinomas[2].

Although direct inhibition of KRas is challenging due to its small size, smooth surface and limited druggable pockets on its surface, first promising drug candidates for selected KRas mutants have been developed in recent years[3].

It was the aim of this study to characterize the selective effects of KRas inhibitors in a panel of cancer cell lines harboring different KRas mutations like G12C, G12V or G12D.

References

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3. Moore, A. R., et al. Nat Rev Drug Discov 19, 533–552 (2020).
4. Zhu, G. et al. Mol Cancer 20, 143 (2021).
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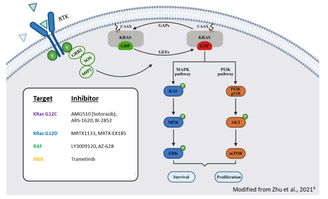
Material and Methods

Several KRas and MAPK pathway inhibitors were subjected to different cellular assays to characterize their selective inhibitory effects in a panel of cancer cell lines with different KRas status.

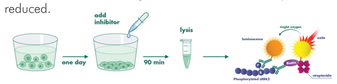


Modified from Zhu et al., 2021[4]

Cell line	KRAS status	Genotype	Tumor entity
PANC-1	G12D	Heterozygous	Pancreas
ASPC-1	G12D	Homozygous	Pancreas
MIA-PaCa-2	G12C	Homozygous	Pancreas
NCI-H358	G12C	Heterozygous	Lung
SW620	G12V	Homozygous	CRC
BxPC-3	wildtype	Homozygous	Pancreas
HT-29	wildtype	Homozygous	CRC

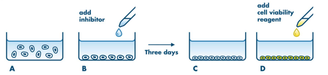
Cellular pERK Assay using the AlphaLISA Technology

Cells were plated in 96-well cell culture plates and treated with compounds for 1,5h (2D culture) or 3h (3D culture). After cell lysis, quantification of phospho-ERK was assessed using the AlphaLISA (amplified luminescent proximity homogeneous assay) Technology. In response to inhibition of KRas or components of the MAPK pathway, phosphorylation of the downstream target ERK1/2 at Thr202/Tyr204 is reduced.



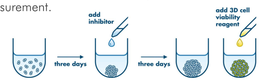
2D Cell Proliferation Assay

Cells were seeded in 384-well plates and incubated with compounds for 3 days. Promega's CellTiter-Glo dye was used to determine the number of viable cells by generation of a luminescent signal proportional to the amount of ATP present based on an ATP-dependent luciferase reaction.



3D Tumor Spheroid Assay

Cells were seeded in clear round-bottom ultra-low attachment multiwell plates. After spheroid formation (2 days), compounds were added and incubated for additional 6 days. Then spheroids were imaged, lysed with 3D-CellTiter-Glo and subjected to luminescence measurement.



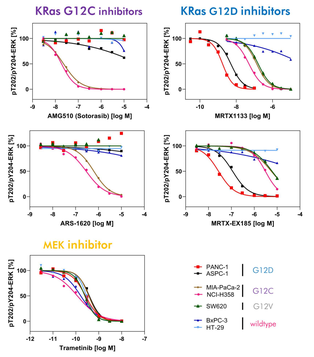
1. Analysis of the selective inhibitory effects of KRas inhibitors in cellular pERK phosphorylation assays

The proto-oncogene KRas is a well-described small GTPase that plays an important role in central physiological pathways such as proliferation and survival. To do so, KRas functions as a binary switch which can cycle between „ON" and „OFF" states which are characterized by the binding of GTP or GDP, respectively. It has been shown that missense single-base mutations such as G12C or G12D result in constitutive activation due to impaired GTP hydrolysis[5]. Amongst others the MAPK signaling cascade is regulated by KRas signaling leading to changes in the phosphorylation of downstream targets such as ERK1/2.

In this study, the inhibitory effect of selected KRas inhibitors was analyzed in a panel of cancer cell lines harboring different KRas mutations. To this end, eight inhibitors were subjected to a cellular pERK AlphaLISA phosphorylation assay in seven cell lines with different KRas status.

IC50 [M]		KRas G12C inhibitors			KRas G12D inhibitors		RAF inhibitors		MEK inhibitor
		AMG510	ARS-1620	BI-2852	MRTX1133	MRTX-EX185	LY3009120	AZ-628	Trametinib
KRas G12D	PANC-1	> 1,0E-05	> 1,0E-05	> 1,0E-05	1,8E-09	2,8E-08	n.d.	9,4E-07	2,6E-10
	ASPC-1	> 1,0E-05	> 1,0E-05	> 1,0E-05	5,0E-09	1,1E-07	1,0E-07	7,9E-08	3,9E-10
KRas G12C	MIA-PaCa-2	2,3E-08	6,3E-07	4,2E-06	1,3E-07	5,6E-06	n.d.	4,2E-07	3,7E-10
	NCI-H358	1,9E-08	2,9E-07	1,9E-06	5,0E-08	3,1E-06	2,9E-07	2,7E-07	1,2E-10
KRas G12V	SW620	> 1,0E-05	> 1,0E-05	5,5E-06	1,6E-07	6,0E-06	n.d.	n.d.	2,5E-10
wildtype	BxPC-3	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	n.d.	n.d.	1,6E-10
	HT-29	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	n.d.	n.d.	2,9E-10

Overview of IC50 values for three KRas G12C inhibitors, two KRas G12D inhibitors as well as two RAF inhibitors and a MEK inhibitor measured in the pERK1/2 (Thr202/Tyr204) AlphaLISA phosphorylation assay in seven cell lines with different KRas status.
IC50 values depicted in green indicate the strongest inhibitory effects in this study while red-colored values indicate no or only weak inhibitory effects in the tested concentration range for this inhibitor and cell line (n.d.: not determined).

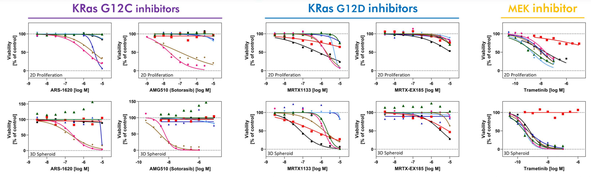


Dose-response curves for the KRas G12C inhibitors AMG510 (Sotorasib) and ARS-1620, the KRas G12D inhibitors MRTX1133 and MRTX-EX185 as well as the MEK inhibitor Trametinib measured in the pERK (Thr202/Tyr204) AlphaLISA phosphorylation assay in cell lines with different KRas mutations.

2. Analysis of inhibitory effects of KRas inhibitors in cellular 2D proliferation and 3D Tumor spheroid assays

KRas plays a central role in cellular proliferation and survival and thus the development of selective and potent KRas inhibitors can significantly impact cancer therapies. However, it has been challenging for decades to develop potent inhibitors for the "undruggable" target KRas. Furthermore, it has been shown for several KRas inhibitors that they exhibit more potent inhibitory effects in a 3D culture models as compared to 2D culture models. Most likely due to the fact that 3D growth assays mimic physiological conditions more closely than 2D settings.

In this study, the inhibitory effect of selected KRas inhibitors was analyzed in a 3D tumor spheroid model and compared to the results of the 2D proliferation assay.



Dose-response curves measured for the KRas G12C inhibitors AMG510 (Sotorasib) and ARS-1620, the KRas G12D inhibitors MRTX1133 and MRTX-EX185 as well as the MEK inhibitor Trametinib tested in 2D proliferation assay (upper panels) and 3D tumor spheroid assay (lower panels) in seven cell lines harboring different KRas mutations.

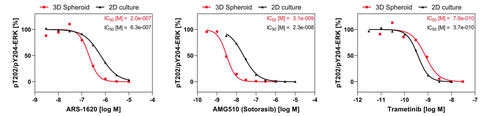
IC50 [M]		KRas G12C inhibitors						KRas G12D inhibitors				RAF inhibitors				MEK inhibitor	
		AMG510		ARS-1620		BI-2852		MRTX1133		MRTX-EX185		LY3009120		AZ-628		Trametinib	
		2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid	2D Prol.	3D Spheroid
KRas G12D	PANC-1	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	≥ 1,0E-05	9,4E-07	> 1,0E-05	6,0E-06	n.d.	1,1E-06	> 3,2E-05	n.d.	> 3,0E-09	3,2E-09
	ASPC-1	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	6,8E-07	6,4E-08	> 1,0E-05	3,0E-06	8,8E-07	2,2E-07	n.d.	1,2E-06	4,5E-08	5,9E-10
KRas G12C	MIA-PaCa-2	1,5E-08	2,5E-09	3,3E-06	2,6E-07	> 1,0E-05	> 1,0E-05	1,9E-06	1,3E-06	> 1,0E-05	> 1,0E-05	8,1E-07	n.d.	4,1E-06	1,3E-06	2,5E-08	4,3E-10
	NCI-H358	2,0E-08	4,4E-09	1,4E-06	2,8E-07	> 1,0E-05	> 1,0E-05	1,8E-06	1,5E-06	> 1,0E-05	> 1,0E-05	8,1E-07	8,1E-08	n.d.	4,9E-07	5,1E-09	3,0E-10
KRas G12V	SW620	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	9,7E-08	3,4E-06	2,0E-06	> 1,0E-05	> 1,0E-05	7,5E-07	n.d.	4,8E-06	n.d.	3,0E-09	2,5E-10
wildtype	BxPC-3	> 1,0E-05	> 1,0E-05	1,8E-06	6,3E-06	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	4,5E-07	7,2E-08	3,9E-06	8,0E-07	3,1E-08	3,0E-10
	HT-29	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	> 1,0E-05	5,1E-06	> 1,0E-05	> 1,0E-05	> 1,0E-05	2,3E-07	n.d.	9,0E-07	9,1E-08	2,0E-09	2,5E-10

Overview of IC50 values for three KRas G12C inhibitors, two KRas G12D inhibitors as well as two RAF inhibitors and a MEK inhibitor measured in the 2D proliferation and 3D tumor spheroid assay in seven cell lines with different KRas mutation status.
IC50 values depicted in green indicate the strongest inhibitory effects in this study while red-colored values indicate no or only a weak inhibitory effect in the tested concentration range for this inhibitor and cell line (n.d.: not determined).

3. Analysis of pERK levels in 3D tumor spheroids

Increased sensitivity of cells cultured in 3D setups to KRas inhibitors may also be reflected by the analysis phospho-ERK levels.

To this end, 3D spheroids from Mia-PaCa-2 cells were subjected to the pERK AlphaLISA phosphorylation assay. Finally, IC50 values were compared to the pERK assay data measured in a 2D setup.



Dose-response curves of MIA-PaCa-2 cells in 3D or 2D culture setup treated with the indicated inhibitors.
MIA-PaCa-2 cells were cultured in 3D spheroid or 2D standard format. Cells were treated for 1,5 hours (2D) or 3 hours (3D) with indicated inhibitors. After cell lysis, samples were subjected to the standard pERK1/2 (Thr202/Tyr204) AlphaLISA protocol.

Conclusion

KRas inhibition is a promising drug target, because activating KRas mutations have been found in approximately 25% of all cancers. Unfortunately, the development of direct KRas inhibitors has proven to be challenging. However, in recent years, there has been made significant progress in the development of selective and potent KRas inhibitors. In this study, some published candidates have been tested for their selective inhibitory effect in a panel of cancer cell lines with different KRas mutations. Phosphorylation of the downstream target pERK1/2 as well as 2D and 3D growth inhibition were used as experimental readouts for the evaluation of their selectivity and potency.

In this study it could be shown that:

- **Tested KRas G12C inhibitors show (highly) selective inhibitory effects in cell lines with KRas G12C mutation in the cellular pERK phosphorylation assay**
- **Tested KRas G12D inhibitors exhibit significantly increased potency in cell lines with the corresponding mutation in the pERK assay**
- **Selectivity of the tested KRas G12C and G12D inhibitors could be (partially) confirmed in 2D proliferation and 3D tumor spheroid assays**
- **Most KRas inhibitors are more efficient in 3D vs. 2D cell growth assays suggesting the application of not only 2D but also 3D growth studies to characterize the inhibitory potential of KRas inhibitors**
- **Increased efficiency of some KRas inhibitors could be confirmed by analyzing the inhibitory effect on ERK1/2 phosphorylation in 3D tumor spheroids using the AlphaLISA technology**
- **Cellular pERK1/2 AlphaLISA assay established for selected cancer cell lines could be a useful tool to screen newly developed KRas inhibitors for their selective inhibition of specific KRas mutants**