TOOLS FOR THE DISCOVERY OF KRAS PATHWAY INHIBITORS

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Introduction

The small GTPase, KRAS, is a known oncogene that is frequently mutated in a large percentage of cancers and is associated with poor disease prognosis. Mutated KRAS is locked in the activated GTP bound state and facilitates enhanced Ras signaling in cancer cells. While being a desirable target, absence of good druggable binding pockets has made modulator compound discovery challenging and unsuccessful.

Recent identification of a unique binding pocket (1) and successful inhibition of the KRAS G12C mutant by covalent chemical modifiers (2, 3) has led to the resurgence of interest in the design of KRAS inhibitors. Alternative efforts are directed at inhibition of interactions with exchange factors (4) and effector proteins.

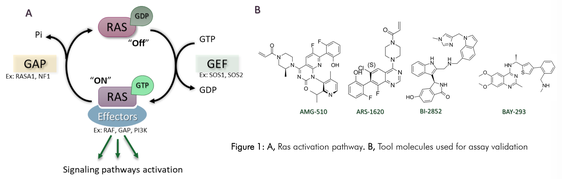


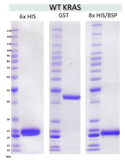
Figure 1: A, Ras activation pathway, B, Tool molecules used for assay validation

At Reaction Biology we have produced proteins and validated a set of assays for the discovery and characterization of compounds that target different steps of Kras pathway.

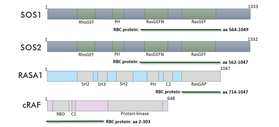
Recombinant Proteins

KRAS wt, G12C, G12D and G12V

Figure 2: Kras protein and regions. Various KRAS recombinant constructs available at RBC. BSP, biotin ligase substrate peptide; Site specific, in vivo biotinylated protein



Factors and Effectors



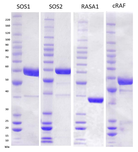


Figure 3: Factors and effector proteins and domains. Partial recombinant proteins are available at RBC. Note: domain sizes are not to scale

Nucleotide exchange assay

Nucleotide exchange assay (NEA) monitors SOS1/2 mediated exchange of fluorescently labeled GDP (GDP*) to GTP. The main application of the assay is to identify compounds that lock KRAS in inactive "OFF" state by preventing GTP binding. Several types of fluorescently labelled GDP molecules can be used in NAE.

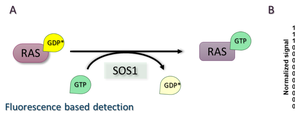
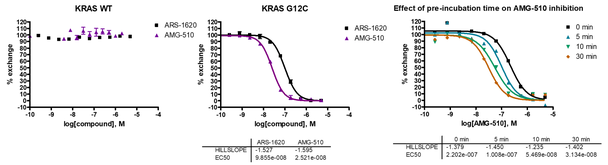
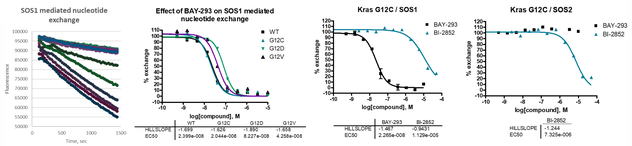


Figure 4: A, schematic of the nucleotide reaction. B, decrease in bodipy-GDP fluorescence is observed upon its dissociation from Kras. Presence of inhibitor, ARS-1620 shown in this example, reduces the exchange rate and the total change in fluorescence.

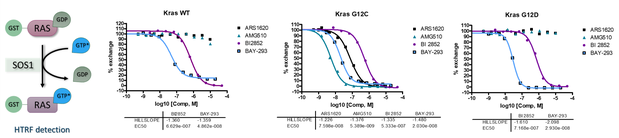
In addition to selectivity profiling between WT and mutants, NAE can be used for time-dependence analysis of compound inhibition.



At reduced SOS1 concentrations, NEA is well suited for characterization of SOS1 specific compounds as well as inhibition of Kras interaction with exchange factor proteins.



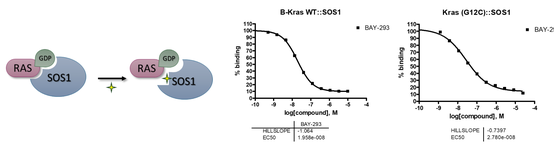
Alternative nucleotide exchange assay format utilizes GTP labelled with DY-647P1 and monitors the increase in HTRF signal observed upon GTP* binding to Kras. The assay is performed at lower GTP concentrations compared to the standard NEA and can evaluate various modes of nucleotide exchange inhibition.



Disruption of protein interactions

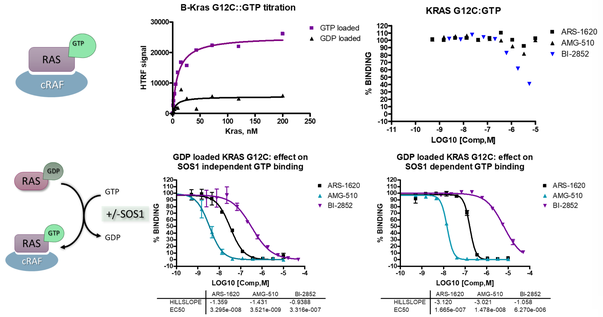
KRAS::SOS1

Disruption of SOS1 binding to Kras can be used as an orthogonal method for studying SOS1 specific compounds. Assay uses HTRF based detection of interaction.



KRAS::cRAF

cRAF recognizes the GTP bound form of KRAS. cRAF binding assay can be used for the identification of disruptors of interaction between Kras and cRAF, as well as quantification of nucleotide exchange reaction. This assay can be used as an alternative to the regular NEA with optional examination of SOS1 independent GTP binding. Assay uses HTRF based detection of interaction.

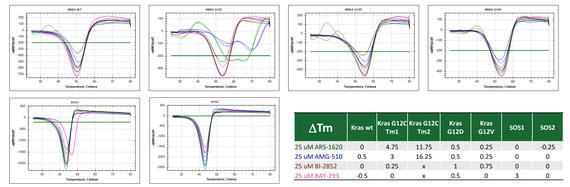


Evaluation of kinase inhibition

Reaction Biology has the largest collection of kinase assays, offering over 700 proteins for selectivity profiling and screening using radioisotope based miniaturized assay. Many of the Kras downstream kinases are available for testing.

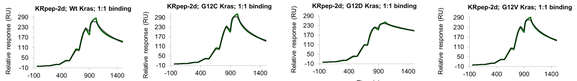
Direct binding assays

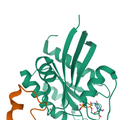
Thermal shift assays are used to assess the effect of compounds on protein stability. Selectivity of G12C compounds (ARS-1620 and AMG-510) is clearly shown among KRAS wt and mutants as well as selectivity of BAY-293 for SOS1.



ΔTm	Kras wt	Kras G12C Tm1	Kras G12C Tm2	Kras G12D	Kras G12V	SOS1	SOS2
25 uM ARS-1620	0	4.75	11.75	0.5	0.25	0	-0.25
25 uM AMG-510	0.5	3	16.25	0.5	0.25	0	0
25 uM BI-2852	0	0.25	x	1	0.75	0	0
25 uM BAY-293	-0.5	0	x	0.5	0	3	0

Surface Plasmon Resonance (SPR) is used to quantify binding affinity of the molecule as well as binding kinetics. Comparison between WT and mutant proteins can be performed to determine selectivity.





Binding Model	Target	Analyte	Chi² (RU²)	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)
1:1 binding	Wt Kras	KRpep-2d	3.59e+1	1.05e+6	2.75e-3	**2.62e-9**	270.7
1:1 binding	G12V Kras	KRpep-2d	1.92e+1	1.08e+6	2.85e-3	**2.65e-9**	282.9
1:1 binding	G12D Kras	KRpep-2d	8.60e0	6.86e+5	5.01e-4	**7.31e-10**	244.2
1:1 binding	G12C Kras	KRpep-2d	2.30e+1	1.05e+6	3.83e-3	**3.64e-9**	305.8

KRpep-2d: Ac-Arg-Arg-Arg-Arg-Cys-Pro-Leu-Tyr-Ile-Ser-Tyr-Asp-Pro-Val-Cys-Arg-Arg-Arg-Arg-NH2 (Ref 5)

References

1 Kessler, D., M. Gmachl, et al. (2019). Proc Natl Acad Sci U S A 116(32): 15823-15829.; **2**: Canon, J., K. Rex, et al. (2019). Nature 575(7781): 217-223; **3**: Janes, M. R., J. Zhang, et al. (2018). Cell 172(3): 578-589 e517; **4**: Hillig, R. C., B. Sautier, et al. (2019). Proc Natl Acad Sci U S A 116(7): 2551-2560.; **5**: Sogabe, S., Y. Kamada, et al. (2017). ACS Med Chem Lett 8(7): 732-736.

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