Novel RAS inhibitor, MCI-062, potently and selectively inhibits the growth of *KRAS* mutant pancreatic tumor cells by blocking GTP loading of RAS

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Abstract Number 345

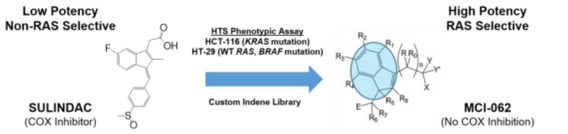
Background and Significance

Oncogenic mutations in *RAS* genes result in an intracellular elevation of active RAS and increased signal propagation through downstream signaling pathways responsible for tumor cell growth and survival. Mutations in *KRAS* drive over 90% of pancreatic ductal adenocarcinomas (PDACs), presenting promising therapeutic potential for a RAS inhibitor. However, many have deemed RAS "undruggable" as a result of its relatively smooth structure and its high affinity for guanine nucleotide substrates. As a result of these challenges, an FDA-approved inhibitor of RAS does not currently exist. We have identified a novel series of indene derivatives that potently and selectively inhibits the growth of tumor cells with high levels of active RAS, while having minimal effects on tumor cells lacking constitutively active RAS.

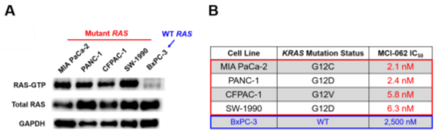
Here we report that our lead compound MCI-062:

- Potently and selectively inhibited the growth of *KRAS* mutant PDAC cells with IC_{50} values of approximately 5 nM and greater than 300-fold selectivity over BxPC-3 PDAC cells that lack constitutively active RAS
- Completely inhibited colony formation of a panel of PDAC cell lines with various KRAS mutations at low nanomolar concentrations
- Depleted RAS-GTP levels in basal and EGF-stimulated conditions
- Reduced active levels of all three RAS isoforms (K-RAS, N-RAS, and H-RAS)
- Inhibited downstream MAPK and AKT signaling, induces cell cycle arrest, and induces apoptosis
- Reduced RAS-RAF-RBD binding when recombinant K-RAS is treated in a nucleotide-free state, but not when K-RAS is treated in a nucleotide-bound state
- Inhibited binding of MANT-GTP to nucleotide-free recombinant K-RAS
- Inhibited growth of 3D tumor spheroids at the same low nanomolar concentrations that inhibit tumor cell growth in 2D monolayer culture
- Inhibited tumor growth, reduced RAS-GTP levels, inhibited MAPK pathway activation, and activated anti-tumor immunity in *KRAS* mutant mouse tumor xenograft models

Discovery Strategy



Results



B

Cell Line	*KRAS* Mutation Status	MCI-062 IC_{50}
MIA PaCa-2	G12C	2.1 nM
PANC-1	G12D	2.4 nM
CFPAC-1	G12V	5.8 nM
SW-1990	G12D	6.3 nM
BxPC-3	WT	2,500 nM

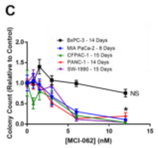


Figure 1. A novel RAS inhibitor potently and selectively inhibited the growth of PDAC cells with high levels of active RAS. A) Active RAS pull-down assays were performed using GST-RAF1-RBD/glutathione agarose to pull-down active RAS from cell lysates. Levels of active RAS were detected by western blotting. **B)** Growth inhibitory activity of MCI-062 was assessed after 96 hours of treatment using the CellTiter-Glo luminescence assay. **C)** MCI-062 was tested for inhibition of colony formation. The assay was terminated for each respective cell line when colonies in its vehicle control group reached a size of approximately 50 cells. One-way ANOVA; *p<0.05 vs. the vehicle (DMSO) control for each indicated cell line.

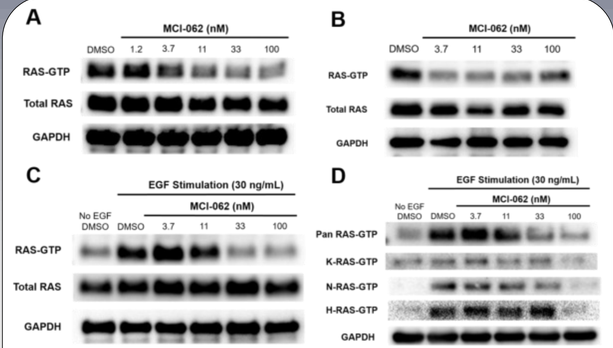


Figure 2. MCI-062 reduced RAS-GTP levels in MIA PaCa-2 pancreatic tumor cells. RAS-GTP levels after treatment were determined by the active RAS pull-down using GST-RAF1-RBD/glutathione agarose and detection by Western blotting. **A)** Cells were treated with vehicle or MCI-062 for 24 hours in complete growth media (10% FBS). **B)** Cells were treated with vehicle or MCI-062 for 24 hours in serum-free growth media. **C)** Cells were treated with vehicle or MCI-062 for 24 hours in serum-free media and subsequently stimulated with 30 ng/mL EGF for 10 minutes. **D)** Cells were treated with vehicle or MCI-062 for 24 hours in serum-free media and subsequently stimulated with 30 ng/mL EGF for 10 minutes.

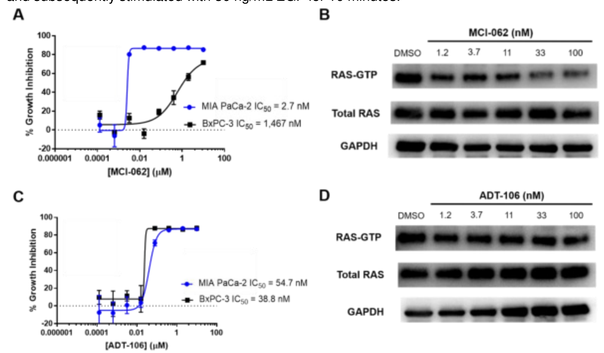


Figure 3. ADT-106, a structurally related but inactive analog of MCI-062, lacked RAS selective growth inhibitory activity and the ability to reduce RAS-GTP levels. A) The growth inhibitory activity of MCI-062 was assessed after 96 hours of treatment using the CellTiter-Glo luminescence assay. **B)** MIA PaCa-2 cells were treated with MCI-062 for 24 hours in complete growth media. RAS-GTP levels after treatment were determined by the active RAS pull-down using GST-RAF1-RBD/glutathione agarose and detection by Western blotting. **C)** The growth inhibitory activity of ADT-106 was assessed after 96 hours of treatment using the CellTiter-Glo luminescence assay. **D)** MIA PaCa-2 cells were treated with ADT-106 for 24 hours in complete media. RAS-GTP levels after treatment were determined by the active RAS pull-down using GST-RAF1-RBD/glutathione agarose and detection by Western blotting.

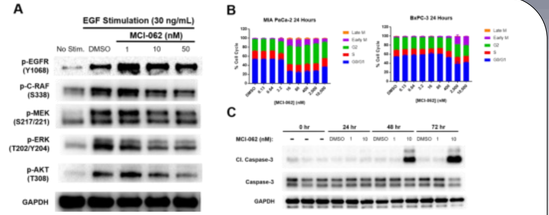


Figure 4. MCI-062 inhibited activation of downstream RAS signaling and induced cell cycle arrest and apoptosis. A) Cells were treated with vehicle (DMSO) or MCI-062 for 24 hours in serum-free media and subsequently stimulated with 30 ng/mL EGF for 10 minutes. Detection of phospho-protein levels was performed by Western blotting. **B)** Cells were serum-starved to synchronize them to the G0/G1 phase of the cell cycle. Cells were subsequently treated for 24 hours with MCI-062 with simultaneous stimulation of cell cycle progression with 5% FBS. The phase of cell cycle arrest at the conclusion of treatment was determined by staining with DAPI and phospho-histone H3B and quantification of staining intensity with a high-content imaging system. **C)** Cells were treated with vehicle (DMSO) or MCI-062 in complete growth media for their respective duration of treatment. Changes in protein levels were determined by Western blotting.

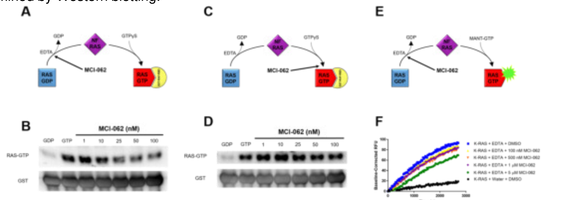


Figure 5. MCI-062 inhibited GTP loading of recombinant RAS in cell-free biochemical assays. A) Treatment before GTPγS addition to incubate compound with nucleotide-free recombinant RAS and assess potential inhibition of GTP loading. **B)** MCI-062 treatment was performed before GTP addition to assess potential inhibition of GTP loading as described in (A). **C)** Treatment after GTPγS loading, but before the active RAS pull-down, to incubate compound with GTP-bound recombinant RAS and assess potential inhibition of RAS-effector binding. **D)** MCI-062 treatment was performed after GTP loading, but before the active RAS pull-down, to assess potential inhibition of RAS-effector binding as described in (C). **E)** Treatment before MANT-GTP addition to incubate compound with nucleotide-free recombinant RAS and assess potential inhibition of GTP loading. **F)** MCI-062 treatment was performed before GTP addition to assess potential inhibition of MANT-GTP loading as described in (E).

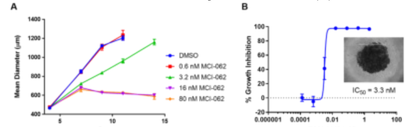


Figure 6. MCI-062 reduced the diameter and inhibited the growth of MIA PaCa-2 3D pancreatic tumor spheroids. A) Cells were plated and allowed 4 days to form spheroids prior to the start of treatment. Spheroids were treated for 10 days with vehicle (DMSO) or varying concentrations of MCI-062. **B)** The growth inhibitory activity of MCI-062 was assessed after 10 days of treatment using the CellTiter-Glo luminescence assay.

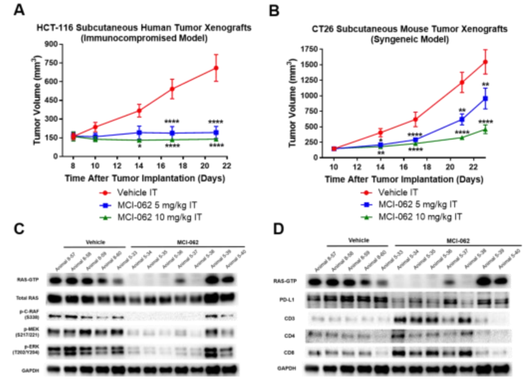
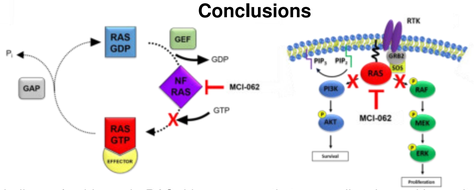


Figure 7. MCI-062 inhibited RAS-driven tumor growth, exhibited target engagement, and activated anti-tumor immunity. A) Mice were implanted in the right flank with 10 million HCT-116 tumor cells per mouse. MCI-062-treated mice received 5 or 10 mg/kg MCI-062 once daily by intratumoral administration. Vehicle-treated mice received 5% DMSO/5% cremophor EL/90% water once daily by intratumoral administration. N=8 mice for vehicle group, n=7 mice for 5 mg/kg MCI-062 group, n=4 mice for 10 mg/kg MCI-062 group; two-way ANOVA with Tukey's multiple comparisons test; *p<0.05, ****p<0.0001. **B)** Mice were implanted in the right flank with 1 million CT26 tumor cells per mouse. MCI-062-treated mice received 5 or 10 mg/kg MCI-062 once daily by intratumoral administration. Vehicle-treated mice received 5% DMSO/5% cremophor EL/90% water once daily by intratumoral administration. N=12 mice/group for vehicle group and 10 mg/kg MCI-062 group, n=11 mice for 5 mg/kg MCI-062 group; two-way ANOVA with Tukey's multiple comparisons test; *p<0.05, **p<0.01 ****p<0.0001. **C)** RAS-GTP levels in tumor lysates were determined by the active RAS pull-down and detection by Western blotting. Levels of MAPK proteins were determined by Western blotting with the whole tumor lysate. **D)** Levels of immune markers were determined by Western blotting with the whole tumor lysate.

Conclusions



Multiple lines of evidence in RAS-driven pancreatic tumor cells, along with results from cell-free biochemical assays using recombinant RAS, suggest that MCI-062 binds to nucleotide-free RAS and inhibits GTP loading. MCI-062-induced reductions of cellular RAS-GTP levels resulted in inhibition of downstream signaling and induction of cell cycle arrest and apoptosis. MCI-062 retained growth inhibitory activity in non-adherent pancreatic 3D tumor spheroid models. Additionally, MCI-062 inhibited RAS-driven tumor growth and showed evidence of RAS inhibition *in vivo*. Taken together, these results support further evaluation of MCI-062 for the treatment of pancreatic cancer, as well as other RAS-driven cancers.

Acknowledgements

This work is supported by grants from the National Cancer Institute: R01CA131378, R01CA148817, R01CA197147, and R01CA155638.