Targeting RAS and downstream signaling in high-grade serous ovarian carcinoma with novel RAS inhibitors

DO NOT POST

Mattox TE[1], Norton TS[2], Keeton AB[1], Ward AB[1], Maxuitenko YY[1], Berry KL[1], Zhu B[1], Musiyenko A[2], Gavin E[2], Ramirez-Alcantara V[1], Chen X[1,3], Valiyaveettil J[1], Scalici J[2], Rocconi RP[2], Piazza GA[1,3*], Madeira da Silva L[2]**

[1]Drug Discovery Research Center, Mitchell Cancer Institute, University of South Alabama, Mobile, AL; [2]Gynecologic Oncology Research Division, Mitchell Cancer Institute, University of South Alabama, Mobile, AL; [3]ADT Pharmaceuticals, Inc., Orange Beach, AL

** gpiazza@health.southalabama.edu; **lsilva@health.southalabama.edu*

Background/Significance

High grade serous ovarian carcinoma (HGSOC), which accounts for 70-80% of ovarian cancer deaths, is mainly characterized by *TP53* mutations and about half of these tumors present defects in the homologous recombination (HR) DNA repair pathway genes, including BRCA1/2 [1]. Poly(adenosine diphosphate-ribose) polymerase (PARP) inhibitors have now become FDA-approved for the treatment of recurrent and BRCA-associated ovarian cancer. Despite providing one of the biggest advances in ovarian cancer treatment in recent years, acquired and *de novo* resistance to PARP inhibitors is often encountered and new treatment strategies must be devised to effectively fight these malignancies [2].

Oncogenic *RAS* and constitutively active RAS signaling feed through multiple downstream cascades to increase cell proliferation, survival, and malignant transformation. Despite the absence of *RAS* mutations, upregulation in the *RAS* pathway is prevalent in HGSOC, which suggests that RAS and its signaling components represent key, yet relatively under-explored, inhibition targets for the treatment of ovarian cancer. Further bolstering the importance of RAS pathway inhibition are recent reports on cytotoxic synergistic effects for the combination of mitogen-activated protein kinase (MAPK) kinase (MEK) and PARP inhibition in ovarian cancer models [3-4].

The development of inhibitors directly targeting RAS has been largely hindered by the lack of suitable surfaces on the structure of the protein for small molecule binding and its high affinity for GTP binding [5-6]. We developed a novel series of indene derivatives that showed highly selective growth inhibitory activity in tumor cells harboring constitutively active RAS versus tumor cells with low levels of active RAS (**Fig. 1**). Chemical optimization resulted in series of compounds that potently and selectively inhibit RAS-dependent tumor cell growth by blocking GTP binding to RAS.

Here we examined the effects of RAS inhibition by two novel compounds, MCI-059 and MCI-062, in a panel of HGSOC cell lines with variable baseline levels of active RAS.

DISCOVERY STRATEGY

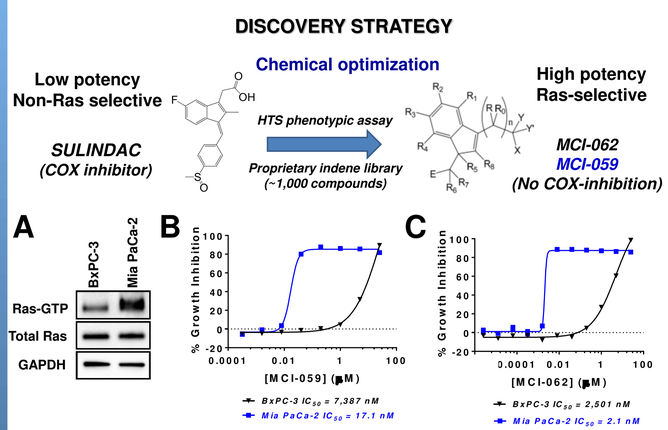


Figure 1: A novel class of RAS inhibitors potently and selectively inhibits the growth of pancreatic cancer cells with high levels of active RAS. (A) Active RAS pull-down assays were performed using GST-RAF1-RBD/glutathione agarose to pull-down active RAS from cell lysates. Levels of active RAS were detected by western blotting. **(B-C)** Growth inhibitory activity of MCI-059 (B) and MCI-062 (C) was assessed after 96 hours of treatment, using the CellTiter-Glo luminescence assay.

RAS in ovarian cancer

High grade serous ovarian cancer (HGSOC) is invariably characterized by the key driver mutation in *TP53*, but other mutational drivers such as mutations or altered methylation of BRCA1 and BRCA2, cyclin E1, PIK3CA and AKT1/2 amplifications, and loss of NF1, RB1 and PTEN are also commonly found. Conversely, RAS mutations are usually associated with low grade serous ovarian carcinoma (LGSOC) and mucinous ovarian tumors.

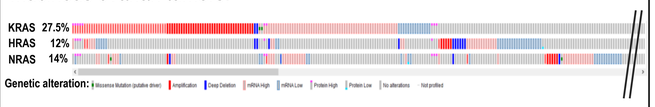


Figure 2: cBioPortal data analysis for RAS proteins in ovarian serous cystadenocarcinoma (TCGA, Provisional). Gene amplifications, deletions, mutations, and mRNA and protein expression data (Z-score>1.5 or <-1.5). Alterations found in 233 (39%) of 594 sequenced patients (591 total).

References

[1] Domcke S *et al.* (2013) Nat Commun 4: 2126.
[2] Pilié PG *et al.* (2019) Nat Rev Clin Oncol 16(2): 81-104.
[3] Sun C *et al.* (2017) Sci Transl Med 9:eaal5148.
[4] Kalimutho *et al.* (2017) Molec Oncol 11: 470-490.
[6] Keeton AB *et al.* (2017) Cancer Research 77: 221-226.
[5] Vigil D *et al.* (2010) Nature Reviews Cancer 10: 842-857.

Please check these other abstracts:

Poster #345: Mattox *et al.* MCI-062 in PDAC

Talk #2707: Keeton *et al.* MCI-062 in colon cancer

Results

High levels of constitutive RAS activation were observed in 5 (OVCAR5, OVCAR8, ES2, KURAMOCHI, and IGROV1) out of 12 ovarian cancer cell lines tested, as measured by the active RAS pull-down assay, comparable or higher than those of MIA PaCa-2 pancreatic cancer cells, which harbors the activating mutation G12C on *KRAS*. The 7 remainder cell lines tested (A2780, SKOV3ip, CAOV3, OVCAR4, OVCAR3, OVSAHO, and OV90) had levels of active RAS comparable or lower than BxPC-3 pancreatic cells, which lacks constitutively active RAS (**Fig. 3**).

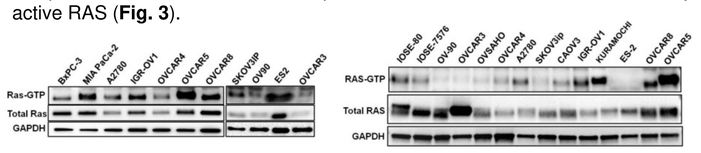
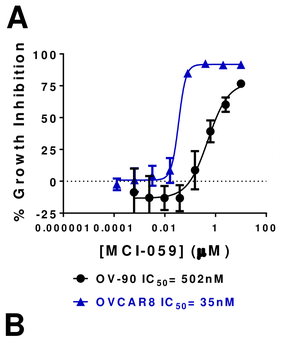


Figure 3: Variable levels of active RAS in HGSOC cell lines. Total protein lysate (500µg) was extracted from each cell line and immediately used in the active RAS pull-down assay, which was performed using GST-RAF1-RBD/glutathione agarose to pull-down active RAS. Levels of active RAS were detected by western blotting with anti-Ras antibodies (RAS-GTP). Total RAS and GAPDH were probed in the total lysate.

We observed potent cancer cell growth inhibitory activity for these novel inhibitors in most of the 15 ovarian cancer cell lines tested (IC_{50}s approximately 25nM and 7nM for MCI-059 and MCI-062, respectively), with the lesser sensitivity in OV-90 cells (IC_{50}~150nM for MCI-059 and ~30nM for MCI-062) correlating with its lowest basal levels of active RAS measured by a pull down assay using GST-RAF1-RBD/ glutathione agarose beads (**Fig. 4**).



Cell line	Ras Pathway Gene Alteration	IC_{50} MCI-059	Active Ras Status
Kuramochi	KRAS ampl.	28.5nM	+++++
Ovsaho		44.1nM	++
OVCAR4		12.4nM	+++
CAOV3		17.9nM	+++
OVCAR3		21.3nM	+
ES2	$BRAF^{mut}$	26.3nM	++++++
OV-90		502.1nM	+
OVCAR8	$ERBB2^{mut}$, $KRAS^{mut}$	35.2nM	+++++
SKOV3	$PI3KC^{mut}$, ERBB2 ampl.	19.3nM	++
SKOV3ip	$PI3KC^{mut}$, ERBB2 ampl.	21.5nM	++
A2780	$PI3KC^{mut}$, $PTEN^{mut}$, $BRAF^{mut}$	27.1nM	+++
IGROV1	$PI3KC^{mut}$, $PTEN^{mut}$	39.0nM	++++
IOSE-80		33.8nM	+++
IOSE-7576		24.4nM	+++
OVCAR5	ERBB2 overexpression	81.8nM	++++++

++ levels comparable to BxPC-3
+++++ levels comparable to Mia PaCa-2

Cell line	Ras Pathway Gene Alteration	IC_{50} MCI-062	Active Ras Status
Kuramochi	KRAS ampl.	6.9nM	+++++
Ovsaho		12.4nM	++
OVCAR4		5.1nM	+++
CAOV3		9.0nM	+++
OVCAR3		5.0nM	+
OV-90		346.5nM	+
OVCAR8	$ERBB2^{mut}$, $KRAS^{mut}$	4.9nM	+++++
SKOV3	$PI3KC^{mut}$, ERBB2 ampl.	6.7nM	++
A2780	$PI3KC^{mut}$, $PTEN^{mut}$, $BRAF^{mut}$	6.1nM	+++
IGROV1	$PI3KC^{mut}$, $PTEN^{mut}$	11.6nM	++++
OVCAR5	ERBB2 overexpression	8.4nM	++++++

++ levels comparable to BxPC-3
+++++ levels comparable to Mia PaCa-2

Figure 4: Growth inhibitory activity of RAS inhibitors in ovarian cancer cell lines. Representative 96 hr IC_{50} curves, IC_{50} values, and relative levels of active RAS (measured by GST-RAF1-RBD pull-down assays) in tested ovarian cancer cell lines for **(A)** ADT-006 and **(B)** DC070-547. *HGSOC classification from Domcke S *et al.* (2013) [1]; other: cell lines not shown in Ref [1]. IOSE cell lines are immortalized ovarian surface epithelial cells kindly provided by Dr. Auersperg (OVCARE, Canada).

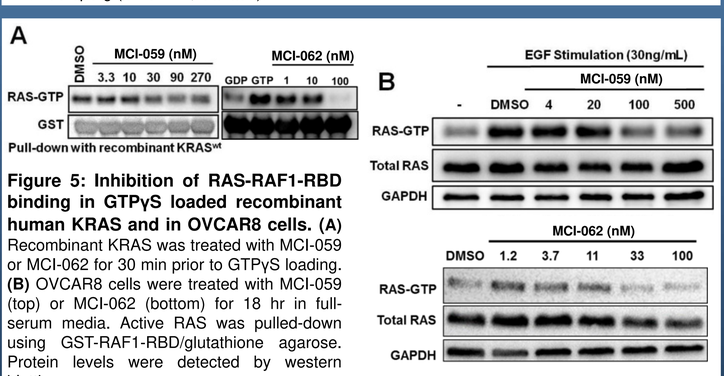


Figure 5: Inhibition of RAS-RAF1-RBD binding in GTPγS loaded recombinant human KRAS and in OVCAR8 cells. (A) Recombinant KRAS was treated with MCI-059 or MCI-062 for 30 min prior to GTPγS loading. **(B)** OVCAR8 cells were treated with MCI-059 (top) or MCI-062 (bottom) for 18 hr in full-serum media. Active RAS was pulled-down using GST-RAF1-RBD/glutathione agarose. Protein levels were detected by western blotting.

Acknowledgements

Dr. Madeira da Silva is a recipient of the 2019 AACR Minority and Minority-Serving Institution Faculty Scholar in Cancer Research. We thank Dr. Sharon Campbell at the University of North Carolina- Chapel Hill for providing the recombinant K-RAS. This work was supported by internal funds at the Mitchell Cancer Institute and by the National Cancer Institute of the National Institutes of Health under Award Numbers R01CA131378, R01CA197147 and 3R01CA155638-04S1. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Time-course experiments revealed that MCI-062 induces apoptosis beginning at 18-24 hours as measure by western-blotting for cleaved PARP and caspase-3 (**Fig. 6**). Furthermore, both MCI-059 and MCI-062 inhibited: i) RAS-RAF1-RBD binding in GTPγS loaded recombinant human KRAS (**Fig. 5A**); ii) RAS-RAF1-RBD binding in OVCAR8 and SKOV3ip cells under normal culture growth conditions (not shown) or under serum starvation overnight followed by EGF-stimulation (**Fig. 5B**); iii) EGF-stimulated activation of the RAF-MEK-ERK and PI3K-AKT cascades in OVCAR8 and SKOV3ip cells (**Fig. 7**). We also confirmed that MCI-062 decreases several components of the RAS downstream signaling pathway via Reverse Phase Protein Array (RPPA) analysis of OVCAR8 cells treated with DMSO *vs.* 20nM MCI-062 for 24 hours or 48 hours (**Fig. 8**).

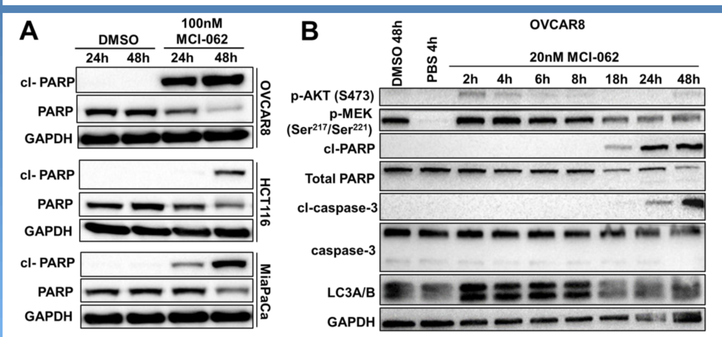


Figure 6: MCI-062 induces apoptotic cell death in OVCAR8, HCT116 and MiaPaCa cells. (A) Cells were treated with MCI-062 inhibitor for 24 or 48h hr in complete growth medium and harvested for WB. (B) OVCAR8 cells were treated with 20nM MCI-062 in complete growth medium for a time-course and then harvested for WB. We noticed an increase in the autophagy marker LC3A/B in early time points (2-8h), while apoptic markers c-PARP and cleaved-caspase-3 are induced at later time-points (18-48h, depending on the cell line).

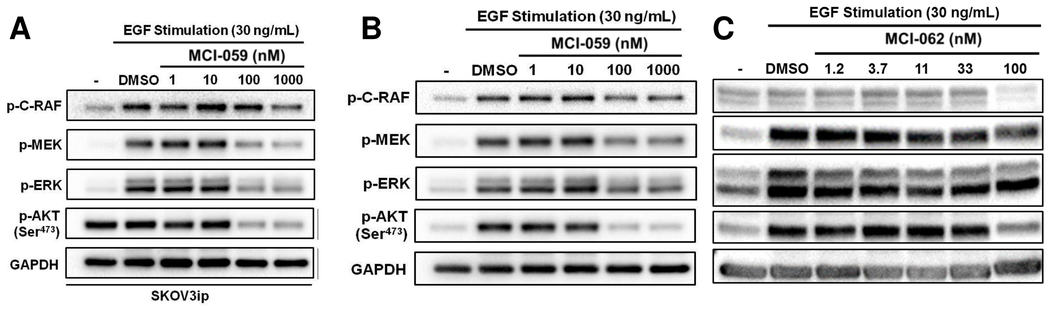


Figure 7: MCI-059 and MCI-062 inhibit EGF stimulation-induced RAF/MAPK and PI3K/AKT signaling in SKOV3ip (A) and OVCAR8 cells (B-C). Cells were treated with noted inhibitor for 18 hr in serum-free media prior to 10 min of EGF stimulation.

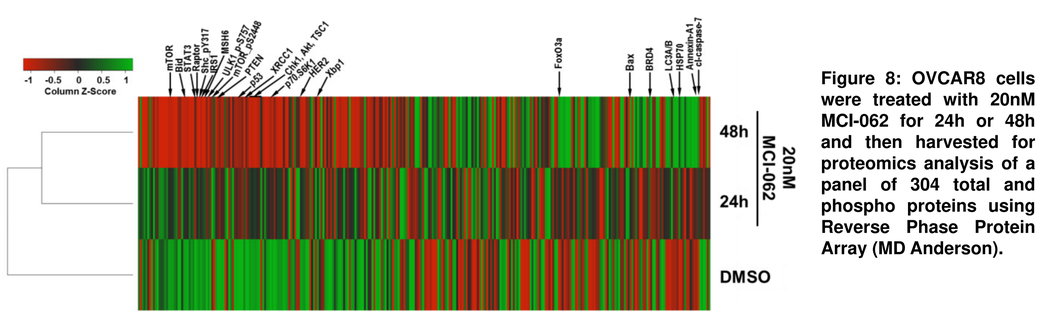
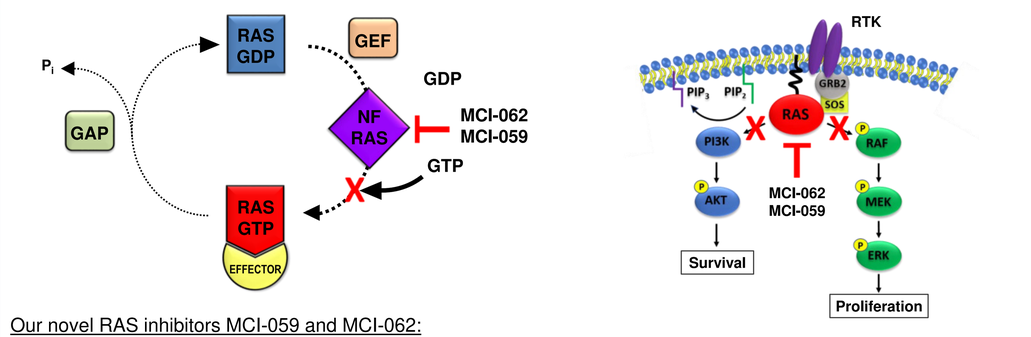


Figure 8: OVCAR8 cells were treated with 20nM MCI-062 for 24h or 48h and then harvested for proteomics analysis of a panel of 304 total and phospho proteins using Reverse Phase Protein Array (MD Anderson).

Conclusions

Figure 9: Proposed mechanism of action of MCI-059 and MCI-062.



Our novel RAS inhibitors MCI-059 and MCI-062:

- ❑ Potently inhibit growth of HGSOC cells with constitutively active RAS, while showing diminished growth inhibitory activity in a cell line (OV-90) with very low levels of active RAS (**Fig. 4**).
- ❑ Block RAS-RAF1-RBD binding in recombinant human K-RAS and intact OVCAR8 and SKOV3ip cells (**Fig. 5**).
- ❑ Induce apoptosis in OVCAR8 cells (**Fig. 6**).
- ❑ Inhibit RAF/MAPK and PI3K/AKT signaling at concentrations that inhibit growth, which is consistent with a mechanism involving direct inhibition of active RAS (**Fig. 7**).
- ❑ Mattox *et al.* (Poster #345 and Talk #2707) showed that MCI-062 reduces 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. Therefore, ***our proposed mechanism of action for these compounds MCI-062 inhibits RAS-driven tumor cell growth by blocking GTP loading of RAS (Fig. 9).***
- ❑ In summary, our results demonstrate that MCI-059 and MCI-062 inhibit HGSOC cell growth by blocking RAS-effector interactions, and support further evaluation of our novel RAS inhibitors for the treatment of ovarian cancer. Future work will consist in evaluating the efficacy of MCI-062 in xenograft and syngeneic animal models of ovarian cancer.