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Inhibition of mutant IDH enzymes reduces production of 2-HG but does not restore wild-type IDH activity *in vitro* or *in vivo*

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BACKGROUND

- Mutations in isocitrate dehydrogenase (IDH) 1 and 2 occur in a variety of malignancies, including >70% of low-grade gliomas, ~20% of acute myeloid leukemias, and up to 25% of intrahepatic cholangiocarcinomas.
- These mutations lead to neomorphic enzymatic activity that results in the production of the oncometabolite (D)-2-hydroxyglutarate (2-HG).[1,2]
- Ivosidenib (IVO; AG-120) and vorasidenib (VOR; AG-881) are potent, orally available, mutant IDH (mIDH) inhibitors; IVO inhibits mIDH1, and VOR inhibits both the mIDH1 and mIDH2 enzymes (**Table 1**).
- IVO and VOR also inhibit wild-type IDH *in vitro* (wtIDH) (**Table 1**); however, the physiological relevance of this inhibition is unknown.
- It is unknown whether IVO and VOR inhibit or restore wtIDH activity in mIDH cells.
- An understanding of the cellular effects of mIDH inhibitors on wtIDH activity is critical, as some synthetic vulnerabilities in mIDH cells have been hypothesized to be due, in part, to reduced wtIDH function in those models.[3,4]

Table 1. Activity of IVO and VOR against mIDH and wtIDH in an enzymatic assay

	Test agent	mIDH1 1 hr IC_{50}, μM	mIDH2 1 hr IC_{50}, μM
Mutant IDH[a]	IVO	0.002	NA
	VOR	0.006	0.118
		wtIDH1 1 hr IC_{50}, μM	**wtIDH2 1 hr IC_{50}, μM**
Wild-type IDH[b]	IVO	0.071	NA
	VOR	0.190	0.374

[a]mIDH enzyme was incubated with NADPH and test agent for 1 hr in 150 mM NaCl, 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 0.05% BSA, and 2 mM β-mercaptoethanol before the reaction was initiated with 2-oxoglutarate
[b]wtIDH enzyme was incubated with NADP+ and test agent for 1 hr in 150 mM NaCl, 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 0.05% BSA, and 2 mM β-mercaptoethanol before the reaction was initiated with isocitrate
NA = not applicable

OBJECTIVES

- We developed an assay that couples stable isotope tracing with mass spectrometry to monitor wtIDH flux in cells and *in vivo* to address the following questions:
  - What is the physiological relevance of wtIDH inhibition by IVO and VOR?
  - How does the presence of an m*IDH1* allele impact wtIDH activity at baseline or upon treatment with IVO and VOR?

METHODS

Cell-based assay development

Which IDH activity to monitor: oxidative or reductive wtIDH activity?

- The stable isotope tracing approach is shown in **Figure 1**.
- To determine whether wtIDH inhibition in cells could be monitored with this approach, HCT-116 cells were incubated with 2 mM $^{13}C_5$-glutamine at t=–3 hr, and when isotopic steady state was reached, cells were treated with 100 nM IVO or VOR.
- VOR robustly reduced the product of reductive wtIDH ($^{13}C_5$-citrate) relative to dimethyl sulfoxide (DMSO), whereas the oxidative wtIDH product ($^{13}C_5$-α-ketoglutarate [α-KG]) was only mildly affected, suggesting that IDH3 is the major contributing isoform for the oxidative wtIDH activity (**Figure 2**).
- The reductive wtIDH activity was used to further characterize wtIDH activity in cells.

Estimating wtIDH flux from a single time-point readout

- VOR was used to optimize a single time-point readout for the combined inhibition of the reductive flux of wtIDH1+2.
- Single time-point parameters measured 15 min post $^{13}C_5$-glutamine addition afforded consistent IC_{50} values compared with the flux-based IC_{50} (δ$^{13}C_5$-cit/δt; **Figure 3**).
- The $^{13}C_5$-citrate/$^{13}C_4$-citrate ratio after 15 min incubation with $^{13}C_5$-glutamine was used to assess the percentage inhibition of wtIDH1+2.

Compound incubation time

- To determine whether short-term incubation with IVO or VOR was sufficient to achieve maximal inhibition, cells were treated for 3 or 48 hr.
- VOR increased in potency over time in both cell-based and enzymatic assays (**Figure 4**).
- Assays were performed after 48 hr of incubation with compound.

In vivo assay development

- To determine an optimal time point to capture wtIDH activity with our stable isotope tracing method *in vivo*, ^{13}C incorporation into citrate was monitored after a bolus injection of $^{13}C_5$-glutamine (**Figure 5**).
- Glutamine enrichment reached its maximum 10 min and 15 min post $^{13}C_5$-glutamine bolus in plasma and tumor, respectively (**Figure 5A-B**).
- Differential kinetics between plasma and tumor $^{13}C_5$-citrate enrichment demonstrate that the $^{13}C_5$-citrate measured in the tumor is produced by the tissue and not taken up from the plasma (**Figure 5C-D**).
- For the *in vivo* wtIDH assay, mice were sacrificed 10 min post intraperitoneal (IP) injection of $^{13}C_5$-glutamine to capture the initial kinetics of $^{13}C_4$-citrate and $^{13}C_5$-citrate production in the tumor.

RESULTS

Cell-based wtIDH assay demonstrates physiological relevance for wtIDH inhibition by IVO and VOR

- We validated a cell-based assay to measure wtIDH1+2 activity. Cell-based inhibition of wtIDH by IVO and VOR qualitatively matched enzymatic predictions and demonstrated the physiological relevance of this activity.
- In the cell-based assay, VOR demonstrated potent pan-wtIDH1/2 inhibition with an IC_{50} of 40 nM after 48 hr of incubation (**Figure 4**).
- In the cell-based assay, IVO partially inhibited wtIDH activity with an IC_{50} of 7 μM after 48 hr of incubation (**Figure 4**).
- Cells bearing a knock-in mIDH1-R132H mutation (HCT-116$^{+/R132H}$) have less wtIDH activity than wild-type cells (HCT-116$^{+/+}$) (**Figure 6B**).
- IVO inhibits wtIDH activity only in wild-type cells, suggesting its inhibitory effects are specific for wtIDH1 (**Figure 6B**, **Figure 7B**).
- wtIDH activity is not restored upon treatment with IVO or VOR (**Figure 6B**, **Figure 7B**).

In vivo wtIDH activity inhibition by IVO and VOR

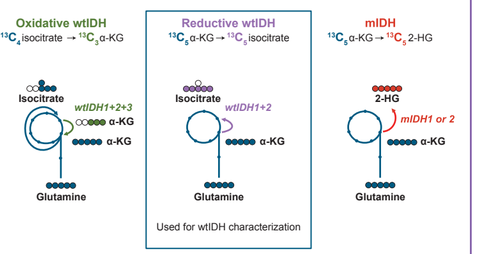
- Predicted plasma exposures to achieve 90% inhibition of wtIDH *in vivo* were estimated to be 10,493 ng/mL for IVO in HCT-116$^{+/+}$ tumors and 55 and 1866 ng/mL for VOR in HCT-116$^{+/+}$ and HCT-116$^{+/R132H}$ tumors, respectively (**Table 2**).
- IVO plasma exposure in mice at clinically matched area under the curve (AUC) values is insufficient to achieve the IC_{50} of wtIDH in HCT-116$^{+/+}$ or HCT-116$^{+/R132H}$ tumors. VOR plasma exposures are within the IC_{50}s of wtIDH activity at clinically matched AUCs (5–50 mg/kg once daily) in HCT-116$^{+/+}$ tumors, but not in HCT-116$^{+/R132H}$ tumors (**Figure 8**).
- The $^{13}C_5$-glutamine bolus time course demonstrated optimization of wtIDH activity measurements *in vivo* (**Figure 5**).
- Both IVO and VOR reduced 2-HG levels in mutant tumors in a dose-dependent manner but not in wild-type tumors (**Figure 9A**).
- Both IVO and VOR reduced wtIDH activity in HCT-116$^{+/+}$ tumors (**Figure 9B**).
- Consistent with the cell-based model (**Figure 7B-C**), IVO and VOR were less potent against wtIDH activity in HCT-116$^{+/R132H}$ tumors (**Figure 9B**).

Table 2. *In vivo* dose projections from a cell-based assay to achieve IC_{90} wtIDH inhibition based on plasma concentrations at C_{min}

Cell type	mIDH inhibitor	IC_{50}, μM	Total C_{min} predicted to achieve IC_{90} for wtIDH	Dose and regimen to maintain C_{min} above IC_{90}
HCT-116$^{+/+}$	IVO	6	10,493	≈450 mg/kg BID
	VOR	0.044	55	5 mg/kg QD
HCT-116$^{+/R132H}$	IVO	No fit	NA	>>450 mg/kg BID
	VOR	1.5	1866	50 mg/kg QD

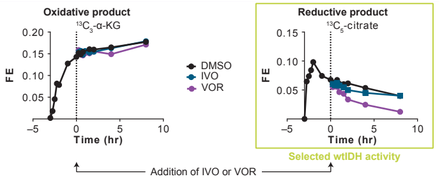
BID = twice daily; C_{min} = minimum concentration; QD = once daily

Figure 1. Stable isotope tracing approach to monitor IDH activity



Metabolite isotopologue patterns produced by the oxidative and reductive wtIDH or mIDH activity upon incubation with $^{13}C_5$-glutamine are shown.

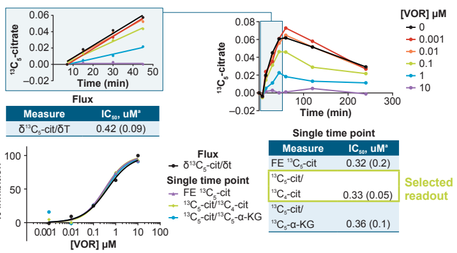
Figure 2. Reductive wtIDH flux provides the sensitivity and selectivity to measure wtIDH1+2 activity



HCT-116 cells were incubated with 2 mM $^{13}C_5$-glutamine (t=–3 hr). Once metabolites reached isotopic steady state, cells were treated with 100 nM IVO or VOR (t=0 hr). VOR reduced $^{13}C_5$-citrate enrichment relative to DMSO-treated cells. $^{13}C_4$-α-KG was only mildly affected, suggesting IDH3 contributes the majority of flux through oxidative wtIDH activity, but not to the reductive wtIDH flux.

FE = fractional enrichment

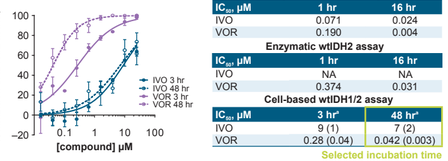
Figure 3. VOR treatment can eliminate reductive wtIDH activity in cells



HCT-116 cells were incubated with VOR for 3 hr followed by the addition of $^{13}C_5$-glutamine. Glutamine incorporation into $^{13}C_5$-citrate was monitored over time. wtIDH flux was estimated by the initial linear rate of $^{13}C_5$-citrate production (δ$^{13}C_5$-cit/δt from 0 to 45 min). **A.** VOR inhibited reductive wtIDH flux with an IC_{50} of 0.4 μM. **B.** To achieve a higher throughput readout, parameters measured from a single time point were assessed against the flux data. The $^{13}C_5$-citrate/$^{13}C_4$-citrate ratio after 15 min $^{13}C_5$-glutamine incubation was further used for wtIDH activity characterization.

*Values are mean (SD)
FE = fractional enrichment

Figure 4. Cell-based assay validates enzymatic wtIDH inhibition by IVO and VOR



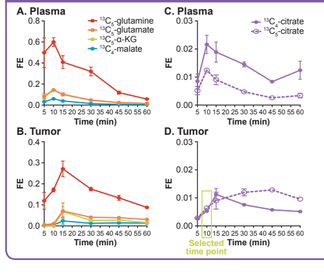
Enzymatic wtIDH1 assay		
IC_{50}, μM	1 hr	16 hr
IVO	0.071	0.024
VOR	0.190	0.004
Enzymatic wtIDH2 assay		
IC_{50}, μM	1 hr	16 hr
IVO	NA	NA
VOR	0.374	0.031
Cell-based wtIDH1/2 assay		
IC_{50}, μM	3 hr*	48 hr*
IVO	9 (1)	7 (2)
VOR	0.28 (0.04)	0.042 (0.003)

Selected incubation time

HCT-116 cells were treated with IVO or VOR for 3 or 48 hr and then 2 mM $^{13}C_5$-glutamine for 15 min. Both compounds inhibited wtIDH. VOR was more potent and showed a higher top percentage inhibition than IVO, correlating with the isoform specificity observed by enzymatic assay. The potency of VOR increased with incubation time, validating the slow-on effect observed by enzymatic assay.

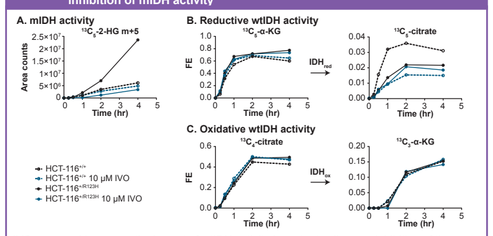
*Values are mean (SD)

Figure 5. $^{13}C_5$-glutamine bolus time course demonstrates that wtIDH activity in tumors can be measured *in vivo*



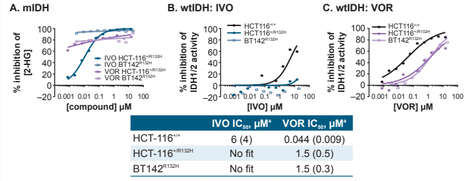
Tumor-bearing Nu/Nu mice received an IP bolus of 350 mg/kg $^{13}C_5$-glutamine. ^{13}C incorporation over time into downstream metabolites was monitored by mass spectrometry. The initial linear kinetics of $^{13}C_4$-citrate and $^{13}C_5$-citrate production in the tumor were captured 10 min post IP injection, making it a suitable time point to assess wtIDH activity *in vivo*.

Figure 6. mIDH1 cells have lower wtIDH reductive flux, which is not restored upon inhibition of mIDH activity



IVO was used to assess the impact of wtIDH activity in mutant cells upon inhibition of the neomorphic enzymatic activity of mIDH1. Isogenic wild-type (HCT-116$^{+/+}$) and mIDH1 (HCT-116$^{+/R132H}$) cells were incubated with 10 μM IVO for 48 hr and then $^{13}C_5$-glutamine. **A.** Treatment with IVO in HCT-116$^{+/R132H}$ reduced $^{13}C_5$-2-HG production to levels measured in the parental wild-type cells. **B.** HCT-116$^{+/R132H}$ cells have less wtIDH activity than HCT-116$^{+/+}$ ($^{13}C_5$-citrate), and wtIDH is not restored upon treatment with IVO. **C.** IVO does not affect oxidative wtIDH activity ($^{13}C_5$-α-KG), suggesting wtIDH1 does not contribute significantly to the oxidative wtIDH flux in these cells.

Figure 7. IVO and VOR are less potent against wtIDH in cell lines bearing mIDH1

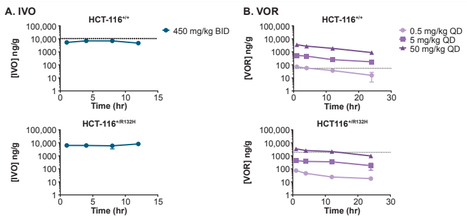


	IVO IC_{50}, μM*	VOR IC_{50}, μM*
HCT-116$^{+/+}$	6 (4)	0.044 (0.009)
HCT-116$^{+/R132H}$	No fit	1.5 (0.5)
BT142^{R132H}	No fit	1.5 (0.3)

HCT-116 wild-type cells (HCT-116$^{+/+}$), HCT-116 knock-in mIDH1 cells (HCT-116$^{+/R132H}$), or mIDH1 BT142 neurospheres that have lost wtIDH1 expression (BT142^{R132H}) were treated with IVO or VOR for 48 hr and incubated with 2 mM $^{13}C_5$-glutamine for 15 min. **A.** IVO or VOR inhibited mIDH1 activity, as measured by the reduction in 2-HG. **B.** IVO only inhibits wtIDH in HCT-116$^{+/+}$ cells, suggesting it is specific for wtIDH1. **C.** VOR inhibits wtIDH in both mutant and wild-type cells, suggesting it is a pan-wtIDH1/2 inhibitor. It is less potent against wtIDH in cells expressing mIDH1. This is because wtIDH2 contributes all the reductive flux in these cells (**Figure 5B**) and VOR is less potent against wtIDH2 relative to wtIDH1 (**Table 1**).

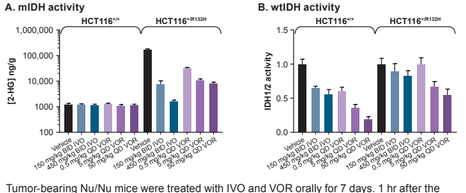
*Values are mean (SD)

Figure 8. Total plasma exposure of VOR but not IVO shows potential for wtIDH inhibition in both mutant and wild-type IDH tumors



ICR SCID mice were injected subcutaneously with 5 × 10^6 HCT-116 cells. Mice were dosed with **A.** IVO or **B.** VOR for 15 days. Plasma drug exposures are shown with the estimated IC_{50} for wtIDH inhibition marked by the dotted lines. IC_{50} for IVO in HCT-116$^{+/R132H}$ could not be calculated.

Figure 9. IVO and VOR inhibit wtIDH *in vivo* but are less potent against the wtIDH activity in HCT-116$^{+/R132H}$ tumors



Tumor-bearing Nu/Nu mice were treated with IVO and VOR orally for 7 days. 1 hr after the last dose, the mice received an IP bolus of 350 mg/kg $^{13}C_5$-glutamine 10 min before being sacrificed. **A.** HCT-116$^{+/R132H}$ tumors produced 2-HG *in vivo* (180,000 ng/g vs 1100 ng/g 2-HG in mutant vs wild-type tumors, respectively). IVO and VOR reduced 2-HG in HCT-116$^{+/R132H}$ tumors in a dose-dependent manner. 150 and 450 mg/kg BID IVO reduced 2-HG by 96 and 99%, respectively. 0.5, 5, and 50 mg/kg QD VOR reduced 2-HG by 82, 94, and 95%, respectively. **B.** IVO and VOR reduced wtIDH activity *in vivo* in a dose-dependent manner. In HCT-116$^{+/+}$ wild-type tumors, 150 and 450 mg/kg BID IVO reduced wtIDH activity by 35 and 44%, respectively. VOR was more potent against wtIDH *in vivo*. In HCT-116$^{+/+}$ tumors, 0.5, 5, and 50 mg/kg QD VOR reduced wtIDH activity by 39, 64, and 80%, respectively. IVO and VOR also inhibited wtIDH in HCT-116$^{+/R132H}$ mutant tumors, but with reduced potency. 150 and 450 mg/kg BID IVO reduced wtIDH activity by only 10 and 17%, respectively. 0.5, 5, and 50 mg/kg QD VOR reduced wtIDH activity by 0, 33, and 45%, respectively.

CONCLUSIONS

- IVO and VOR inhibit wtIDH1 and wtIDH1+2 activity, respectively, in physiological conditions both *in vitro* and *in vivo*.
- Knock-in IDH1-R132H mutation reduces wtIDH1 activity despite the presence of the wild-type allele.
- The treatment of mIDH tumor cells with IVO and VOR does not restore wtIDH activity.
- Synthetic vulnerabilities induced by the reduction of wild-type activity in mIDH tumors[5] will persist upon mIDH1/2 inhibitor treatment.

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