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ACTIVATION OF GCN2 BY HC-7366 RESULTS IN SIGNIFICANT ANTI-TUMOR EFFICACY AS MONOTHERAPY AND IN COMBINATION WITH VENETOCLAX IN AML MODELS

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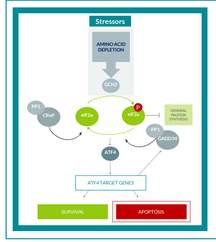
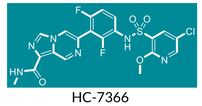
ABSTRACT

The integrated stress response pathway (ISR) is an adaptive signaling pathway that cells utilize to respond to a wide range of extrinsic and intrinsic stresses, many of which are important for tumorigenesis. Activation of ISR plays a dual role in cell fate decisions; while ISR promotes survival, prolonged activation of ISR induces apoptosis. Activation of general control nonderepressible 2 (GCN2), an ISR kinase that senses and responds to nutrient stress conditions has been shown to result in antitumor effect. We are developing HC-7366, a first-in-class, first-in-human GCN2 activator and are currently evaluating it in a phase 1 clinical trial in solid tumors (NCT05121948). In this study, we present the characterization of the antitumor effect of HC-7366 in acute myeloid leukemia (AML).

Higher expression of GCN2 and ISR markers such as ATF4 has been observed in primitive or minimally differentiated AML cells, suggesting that AML may be particularly sensitive to HC-7366. Encouragingly, *in vivo* efficacy studies in MOLM-16 and KG-1 tumor models showed 100% complete response and 100% tumor growth inhibition, respectively. Analysis of tumors by IHC demonstrated activation of ISR as evidenced by increased expression of the ATF4 targets ASNS and PSAT1, confirming that HC-7366 is functioning as a GCN2 activator. In MV4-11 model, a differentiated subtype of AML that shows limited response to venetoclax, the combination of HC-7366 and venetoclax produced strong benefit resulting in 26% tumor regression. Enhanced activation of ISR pathway was again observed when HC-7366 was combined with venetoclax. HC-7366 also impacted possible venetoclax resistance mechanisms by increasing PUMA and reducing S100A8/A9 proteins. To investigate the effect of the compound on primary AML, we performed an *ex vivo* screen of HC-7366 in cells from AML patients where it also showed a remarkable decrease in cell proliferation. Furthermore, we tested HC-7366 in a xenotransplantable model of patient-derived AML and found that HC-7366 significantly reduced mature myeloid (CD33+) AML cells in bone marrow compared to standard of care (SOC) agents, including venetoclax. Investigating the mechanism of action using GCN2 CRISPR-knockout cells, we confirmed that the HC-7366 mediated activation of ISR, reduction of cell viability, and inhibition of protein synthesis was dependent on GCN2. In addition, HC-7366 reduced mitochondrial respiration in MOLM-16 cells, demonstrating the effect of HC-7366 on cellular bioenergetics. Metabolomics analyses of AML xenograft tumors showed that HC-7366 significantly altered metabolites associated with amino acid metabolism, urea cycle and oxidative stress. Together, our *in vitro* and *in vivo* results demonstrate that HC-7366 is a potent GCN2 activator with strong antitumor activity in AML as a single agent as well as in combination with venetoclax supporting investigation of HC-7366 in clinical trial for AML patients.

BACKGROUND





HC-7366

Figure 1: GCN2 in the Integrated Stress Response (ISR). GCN2 responds to AA deprivation. Activated GCN2 phosphorylates eIF2α, attenuating global translation but enhancing translation of the transcription factor ATF4, resulting in survival or apoptosis depending on context.

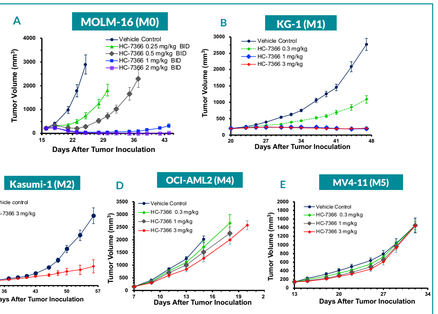


Figure 2. HC-7366 shows potent single agent activity in AML models. A. HC-7366 shows complete eradication of MOLM-16 xenograft tumors at 2 mg/kg whereas other doses show tumor growth inhibition. **B**. HC-7366 at 1 and 3 mg/kg shows tumor stasis in KG-1, 100% TGI. **C**. HC-7366 at 3 mg/kg exhibited 73% TGI in Kasumi-1. **D**. HC-7366 at 3 mg/kg showed about 38% TGI in OCI-AML2. **E**. HC-7366 did not show tumor growth inhibition in MV4-11. The FAB classification of each AML is shown (M0-M5). HC-7366 showed robust responses in M0-M2 subtypes of AML. All treatments were administered twice daily by oral gavage and tolerated well as measured by body weight.

RESULTS

KG-1 (M1)

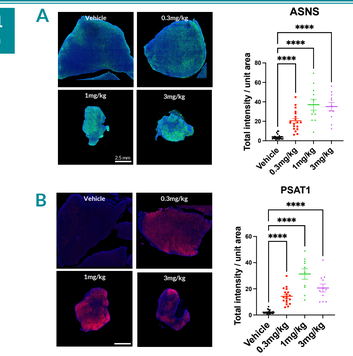


Figure 3. HC-7366 potently induces ISR in KG-1 tumors. HC-7366 treated tumors were evaluated for ISR markers by IHC at the end of study (day 27). HC-7366 potently induced ATF4 targets ASNS (**A**) and PSAT1 (**B**). The highest induction was observed at 1 and 3 mg/kg dose of HC-7366. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA.

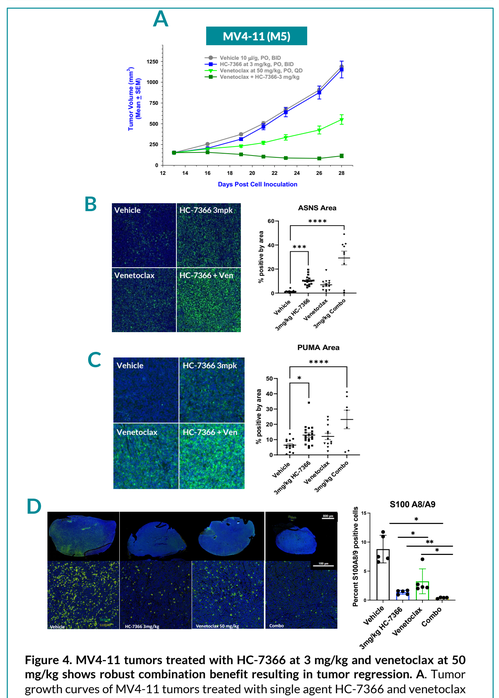


Figure 4. MV4-11 tumors treated with HC-7366 at 3 mg/kg and venetoclax at 50 mg/kg shows robust combination benefit resulting in tumor regression. A. Tumor growth curves of MV4-11 tumors treated with single agent HC-7366 and venetoclax or combination. HC-7366 did not affect tumor growth as a single agent. Venetoclax monotherapy showed 62% TGI. However, HC-7366 at 3 mg/kg shows 26% tumor regression when combined with venetoclax. ISR marker analysis of the end of study tumors showed that HC-7366 at 3 mg/kg caused potent activation of ISR markers such as ASNS and PUMA, which were further increased with venetoclax combination suggesting hyperactivation of ISR (**B&C**). **D**. S100A8/A9 levels were significantly reduced with single agent treatment of both HC-7366 and venetoclax but the combination of venetoclax and HC-7366 robustly decreased S100A8/A9 levels further. PSAT1 levels did not change (data not shown).

RESULTS

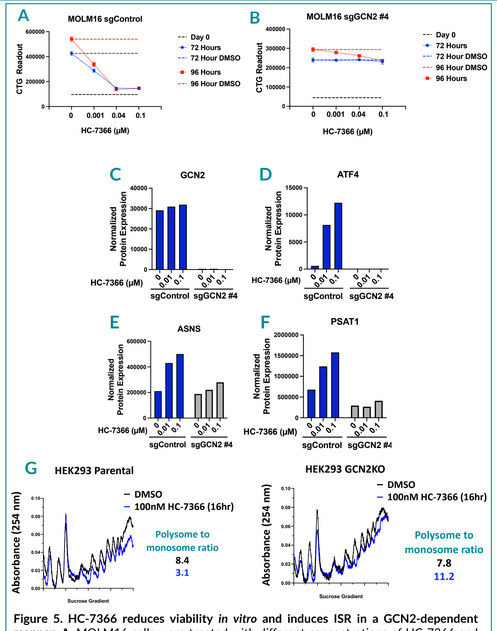


Figure 5. HC-7366 reduces viability *in vitro* and induces ISR in a GCN2-dependent manner. A. MOLM16 cells were treated with different concentrations of HC-7366 and viability was assessed by Cell Titer Glo (CTG) assay after 72 and 96 hours of treatment. HC-7366 potently reduced viability. **B.** This response was reversed in GCN2 CRISPR knockout cells. **C.** Protein expression of GCN2 in WT and CRISPR knockout cells as measured by JESS at 24hr. HC-7366 mediated activation of ISR was assessed by looking at ATF4 and its downstream targets ASNS and PSAT1 at 24hr which showed a GCN2-dependent increase (**D-F**). The effects of HC-7366 on protein synthesis was evaluated by polysome profiling of GCN2 WT and CRISPR knockout HEK 293 cells. **G.** HC-7366 reduced polysome to monosome ratio which reflects reduced translation in GCN2 WT cells but not in GCN2 knockout cells.

PDX MODEL	HC-7366 EC_{50} (µM)
CTG-2456*	31.8
CTG-2229	34.0
CTG-2455*	35.7
CTG-2236	36.5
CTG-2227	39.9
CTG-2775	43.4
CTG-3440	55.2
CTG-2234	65.5
CTG-3673	65.6
CTG-2232	84.3
CTG 2774	96.9
CTG-3441	101.7
CTG-2233	101.7
CTG-2235	103.1
CTG-3679	106.7
CTG-2238	122.8
CTG-3674	128.6
CTG-2453	179.5
CTG-3438	196.2
CTG-3680*	230.8
CTG-2457*	247.8
CTG-2700	333.1
CTG-3439	349.4
CTG-3667*	924.9
CTG-2239	1166
CTG-2228	1771
CTG-2240	2220
CTG-2704	2253
CTG-2454*	>10000
CTG-2701	ND

Sensitive responders
Moderate responders
Resistant responders
Non-responders

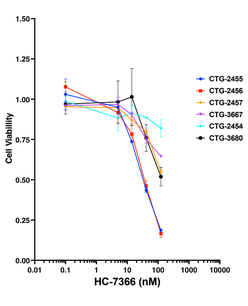
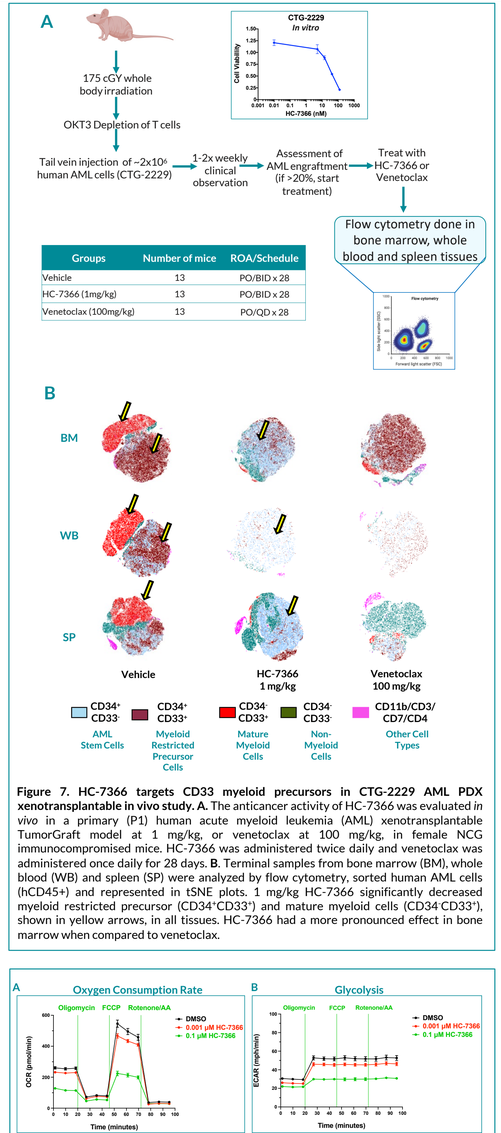


Figure 6. HC-7366 potently reduces viability of primary AML PDX cells. An ex vivo screen was performed with HC-7366 in 30 primary AML PDX models. Viability was assessed by CTG after 6 days of treatment with HC-7366. 11 PDX models were sensitive responders with EC_{50} values < 100 nM. 12 PDX modes were moderate responders with EC_{50} values of 100-350 nM. 5 PDX models were resistant responders with EC_{50} values of 924-2253 nM. Only two models showed no response to HC-7366. EC_{50} values were calculated relative to 100 nM. *Representative viability curves are shown.

RESULTS



Groups	Number of mice	ROA/Schedule
Vehicle	13	PO/BID x 28
HC-7366 (1mg/kg)	13	PO/BID x 28
Venetoclax (100mg/kg)	13	PO/QD x 28

Figure 7. HC-7366 targets CD33 myeloid precursors in CTG-2229 AML PDX xenotransplantable in vivo study. A. The anticancer activity of HC-7366 was evaluated *in vivo* in a primary (P1) human acute myeloid leukemia (AML) xenotransplantable TumorGraft model at 1 mg/kg, or venetoclax at 100 mg/kg, in female NCG immunocompromised mice. HC-7366 was administered twice daily and venetoclax was administered once daily for 28 days. **B**. Terminal samples from bone marrow (BM), whole blood (WB) and spleen (SP) were analyzed by flow cytometry, sorted human AML cells (hCD45+) and represented in tSNE plots. 1 mg/kg HC-7366 significantly decreased myeloid restricted precursor ($CD34^+CD33^+$) and mature myeloid cells ($CD34^-CD33^+$), shown in yellow arrows, in all tissues. HC-7366 had a more pronounced effect in bone marrow when compared to venetoclax.

Figure 8. HC-7366 reduces oxygen consumption rate (OCR) and glycolysis. A. MOLM-16 cells were treated with HC-7366 (0.001 and 0.1 µM) for 16 h and OXPHOS was analyzed by Seahorse XF Analyzer. HC-7366 significantly reduced oxygen consumption rate and glycolysis (**B**) with the strongest effect being at 0.1 µM. Notably, the reduction in mitochondrial oxygen consumption and glycolysis resulted in reduced ATP production (data not shown).

RESULTS

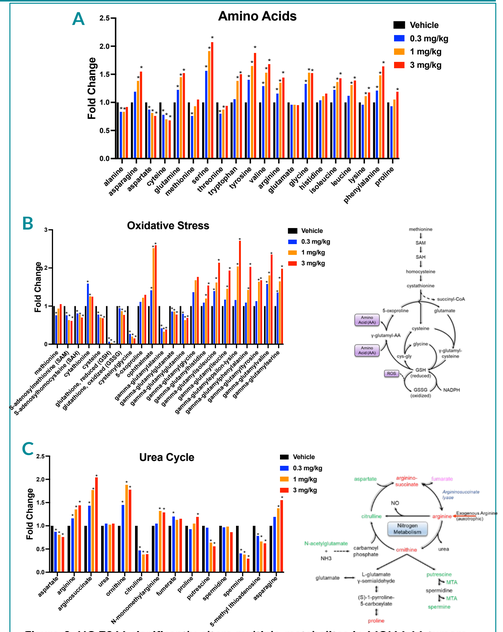


Figure 9. HC-7366 significantly alters multiple metabolites in MOLM-16 tumors. MOLM-16 tumors were treated with HC-7366 at 0.3, 1 and 3 mg/kg for 4 days and collected for metabolite analysis (n=12). Graphs show metabolite levels normalized to vehicle control. **A.** HC-7366 significantly elevated levels of most amino acids. Some amino acid levels were decreased with HC-7366, including aspartate and cysteine. **B.** HC-7366 significantly reduced glutathione levels (GSH and GSSG) and enhanced numerous γ-glutamyl-amino acids suggesting that HC-7366-treated tumors are attempting to increase glutathione production, potentially in response to increased oxidative stress in the cells. **C.** HC-7366 significantly reduced aspartate metabolism and polyamine-related metabolites (putrescine, spermine, 5-methylthioadenosine (MTA)) at all doses. Generally, the most changes in metabolites were occurring at 1 and 3 mg/kg doses. *Statistically significant ($p \leq 0.05$) increases or decreases.

SUMMARY/CONCLUSIONS

- The GCN2 modulator HC-7366 has potent monotherapy efficacy in multiple AML models, with greatest efficacy displayed in undifferentiated AML subtypes
- HC-7366 treatment leads to ISR activation as evidenced by clinically translatable PD markers (e.g., ASNS)
- HC-7366 shows significant combinatorial benefit with venetoclax in MV4-11 (FLT3 mutant) model, which showed limited response to HC-7366 monotherapy
- Combination of HC-7366 and venetoclax induces ISR hyperactivity leading to activation of cell death programs, suggestive of the potential for reversal of venetoclax resistance
- HC-7366-mediated activation of ISR, reduction of cell viability, and inhibition of protein synthesis is dependent on GCN2
- HC-7366 potently reduces viability of 30 AML PDX models in ex vivo screen and significantly decreased myeloid restricted precursor ($CD34^+CD33^+$) and mature myeloid cells ($CD34^-CD33^+$) in vivo
- HC-7366 reduces both mitochondrial oxygen consumption rate and glycolysis and significantly altered several metabolites involved in amino acid metabolism, oxidative stress, and urea cycle
- These data support further investigation into treating AML patients with HC-7366 and the combinatorial benefit of HC-7366 and venetoclax

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