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Screening of Small Molecule Libraries for Identification of a Novel Inhibitor of ADAR1

David Guimond, Theodore Schiff, Gregory Joseph, Taylor Ismaili, Rachel Rowlands, Balaji Panneerselvam, Thomas Aicher, Xiao Hu, Nidhi Arora, Martin Rowbottom, Isharat Yusuf, Laura Carter
Gossamer Bio, Inc., San Diego, CA, USA

BACKGROUND AND TARGET VALIDATION

- Elevated double-stranded (ds) RNA within the cytoplasm triggers innate sensing by distinct pathways
  - Activation of melanoma-differentiation-associated gene 5 (MDA5) / retinoic-acid-inducible protein I (RIG-I) leading to elevated interferon stimulated gene (ISG) expression
  - Protein Kinase R/EIF2AK2 (PKR) activation leading to arrest of protein translation and cell lethality[1]
  - Adenosine Deaminase Acting on RNA (ADAR) 1 catalyzes an adenosine-to-inosine (A-to-I) conversion of dsRNA substrates and serves as a negative regulator of RNA sensing
- A subset of ADAR1-sensitive tumors overexpress the interferon-inducible p150 isoform to avoid the innate immune system and gain a survival advantage[2]
- Inhibition of ADAR1 in sensitive tumors is hypothesized to result in both on-target cell lethality and a more immunogenic tumor microenvironment due to elevated type I IFN[3,4]
- We developed a novel screening funnel to identify tractable small molecule inhibitors of ADAR1 for development of anticancer therapeutics by restoring dsRNA sensing

Figure 1. Role of ADAR1 in driving cancer progression

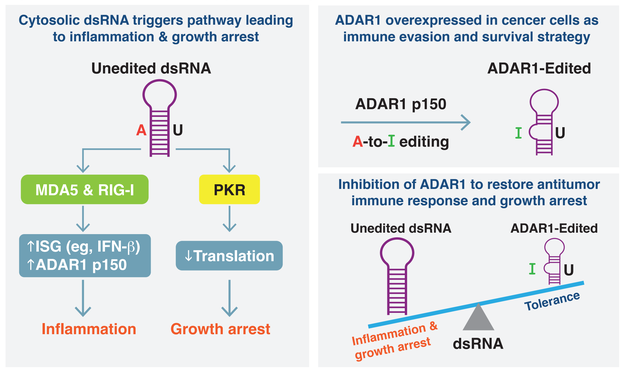
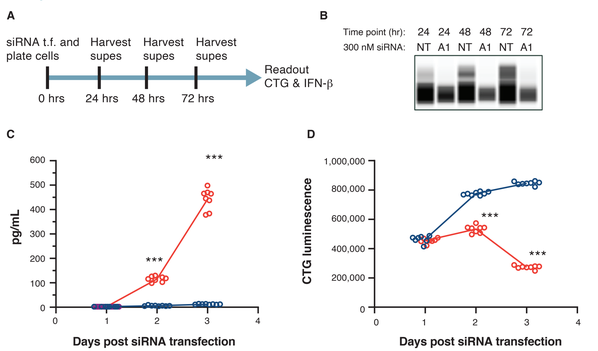


Figure 2. Target validation in ADAR1-sensitive OE21 cell line demonstrates dual phenotypes of elevated levels of secreted IFN-β and on-target cell lethality upon knockdown of ADAR1 p150 expression



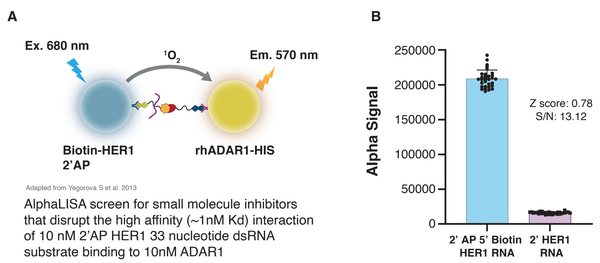
A. Workflow for siRNA transfections and dual readouts in ADAR1-sensitive OE21 cells. **B.** Kinetics of ADAR p150 protein knockdown following transfection with ADAR1-targeting vs. non-targeting (NT) siRNA. **C, D.** Elevated IFN-β in the supernatants of OE21 cells that is correlated with on-target cell lethality. ○, ADAR1-targeting transfectants; ○, NT transfectants CTG, CellTiter-Glo®

METHODS

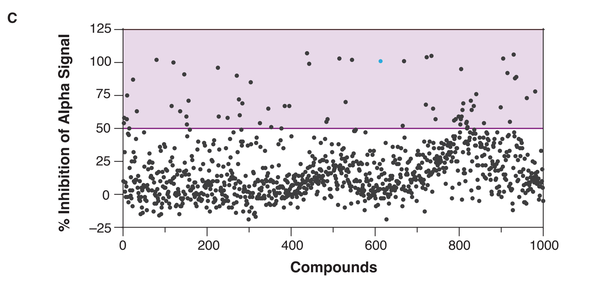
- Target validation was performed by knocking down expression of ADAR1 in the ADAR1-sensitive OE21 cell line to investigate the time-dependent correlation of cancer intrinsic cell lethality and secretion of type I interferons
- A compound testing funnel was devised that included two high-throughput screening (HTS) assays
- AlphaLISA assay to screen for small molecule inhibitors of HER1 dsRNA substrate binding to full-length ADAR1 p110
- HDV Nluc assay to screen for inhibitors of ADAR1 editing activity at a UAG stop codon within the HDV dsRNA substrate in HEK293 cells
- ~5,000 compounds run at 30 μM in HTS mode in both assays were additionally characterized in dose-response, followed by assays to rule out interference with the readouts for both assays
- In addition, lead compounds were assessed in an RNA binding and cell viability assays to rule out off-target effects

HER1:ADAR1 ALPHALISA SCREENING ASSAY

Figure 3. Identification of hits from small molecule libraries in AlphaLISA assay



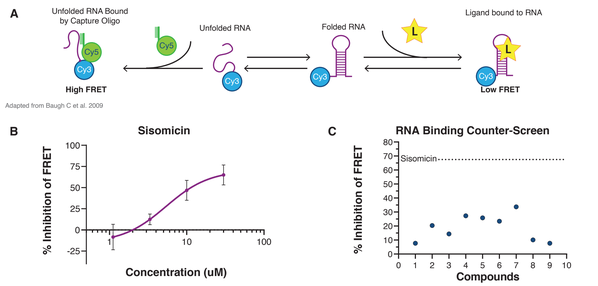
AlphaLISA screen for small molecule inhibitors that disrupt the high affinity (~1nM Kd) interaction of 10 nM 2'AP HER1 33 nucleotide dsRNA substrate binding to 10nM ADAR1



A. Diagram of AlphaLISA assay to identify compounds that disrupt binding of the 2-aminopurine substituted HMG2-Induced ER-Remodeling Protein 1 (2'AP HER1) dsRNA substrate to full-length recombinant ADAR1 p110[5,6]. **B.** Positive control for the assay using dsRNA HER1 substrate (blue bar) vs. substrate lacking the biotin required for conjugation to donor beads (purple bar), including assay statistics. **C.** Single-point (30 μM) screen of a small compound library in the AlphaLISA assay results in the identification of several hits defined as >50% inhibition (highlighted in purple shading). **D.** Characterization of a single-point hit in dose response results in compounds with sub-micromolar potency with the example of GB-10037 represented as blue data point in panel C

HER1:ADAR1 ALPHALISA SCREENING ASSAY (cont'd)

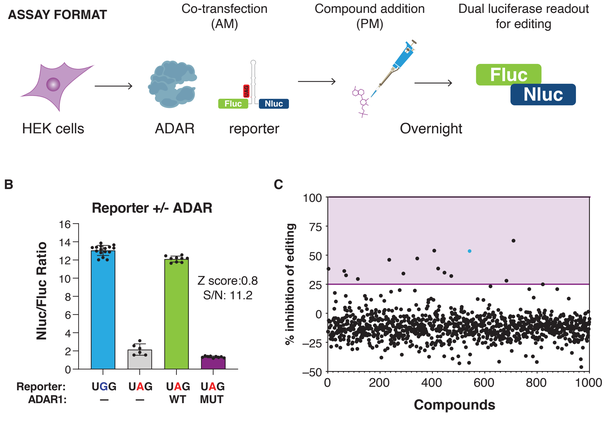
Figure 4. Counter-screen included in screening funnel rules out binding of compounds to dsRNA substrate rather than desired targeting of ADAR1



A. Diagram of counter-screening assay where a FRET signal results in the absence of compounds binding to dsRNA.[7] **B.** Dose response curve generated in dsRNA binding assay using a promiscuous RNA binder, sisomicin, sisomicin, that results in loss of FRET signal at increasing concentrations of compound. **C.** Characterization of subset of hits from AlphaLISA assay in RNA binding counter-screen indicating reduced RNA-binding activity as compared to sisomicin positive control compound

HDV NLUC REPORTER ASSAY

Figure 5. Identification and characterization of hits from small molecule libraries in cell-based HDV Nluc reporter assay



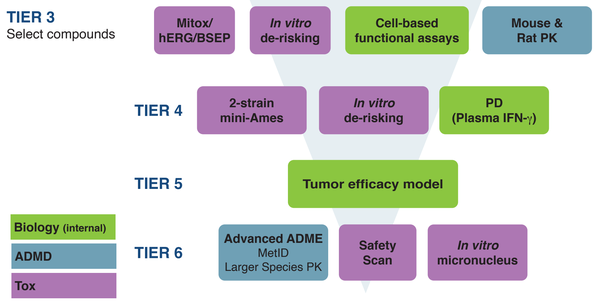
A. Diagram of HDV Nluc reporter assay where expression plasmids for ADAR1 p150 and an HDV Nluc reporter are co-transfected into HE293 cells followed by addition of compounds and readout of A-to-I editing occurring at a UAG stop codon upstream of the Nluc reporter.[8,9] **B.** Assay validation using positive control HDV reporter construct that contains a mutated stop codon ('G' in DNA plasmid equivalent to corresponding to A-to-I conversion in RNA, blue bar) as compared to transfection of the UAG-containing HDV reporter construct without overexpression of ADAR1 (gray bar); co-transfection of WT vs. catalytically inactive E912A mutant of ADAR1 shows expected results on Nluc reporter activity (green vs. purple bars). **C.** Screen of small molecule library with hit cutoff defined as >25% inhibition of Nluc/Fluc ratio (purple shading). **D.** Dose response characterization of GB-12250 indicated as blue data point in panel C showing partial activity in HDV Nluc reporter assay with further profiling of initial hits ongoing.

RESULTS

Table 1. Screening of small molecule libraries resulting in the identification of progressible chemical matter

Single-point HTS at 30 μM resulted in identification of a subset of hits that grouped into 10 clusters based on chemical structure, which was further refined upon dose response characterization to 2 unique chemical series.

Screening Stage	# of Cmpds	Hit Rate	Clusters
Initial #	5,113	N/A	N/A
Single-point hits	433	8.5%	10
DRC and counter-screens	86	1.7%	2



SUMMARY AND CONCLUSIONS

- ADAR1 knockdown in sensitive cancer cell lines results in elevated IFN-β in tissue culture supernatants in addition to on-target cell lethality
- Diverse compound libraries were screened in the AlphaLISA binding and HDV Nluc reporter assay resulting in the identification of progressable chemical matter from multiple chemical series
- Ongoing efforts aimed at improving physicochemical properties to design a first-in-class, selective inhibitor for use in immuno-oncology

REFERENCES

1. Chung H, Calis JJA, Wu X, Sun T, et al. *Cell* 2018; 172, 811-824 e814.
2. Lazzari E, Mondala PK, Santos, ND, et al. *Nat Commun* 2017; 8, 1922.
3. Gannon HS, Zou T, Kiessling MK, et al. *Nat Commun* 2018; 9, 5450.
4. Ishizuka JJ, Manguso RT, Cheruiyot CK, et al. *Nature* 2019; 565, 43-48.
5. Knutson SD, Korn MM, Johnson RP, et al. *Chemistry* 2020; 26(44):9874-9878.
6. Wang Y, Park S, Beal PA. *Biochemistry* 2018; 57, 1640-1651.
7. Baugh C, Wang S, Li B, Appleman JR, et al. *J Biomol Screen* 2009; 14, 219-229.
8. Fritzell K, Xu LD, Otrocka M, et al. *Nucleic Acids Res* 2019; 47, e22.
9. Sato S, Wong SK, Lazinski DW. *J Virol* 2001; 75, 8547-8555.
10. Yegorova S, Chavaroche AE, Rodriguez MC, et al. *Anal Biochem* 2013; 439(2):123-31.

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