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Title
Drug Combinations to Harness Metabolic Stress in the Therapy of Neuroendocrine Tumors

Background/Significance
As of 2021 the identification of novel therapies for neuroendocrine cancers (NECs) remains a challenge but represent a unique opportunity to develop anticancer therapies. The spectrum of NECs from low to high grade, and of various histological differentiation patterns, reflect a spectrum of intrinsic tumor biology that confer varying aggressiveness on a common background but with as-yet unknown pathways to oncogenesis. Yet, several vulnerabilities suggest opportunities for targeting antineoplastic therapy. Our objective is to exploit metabolic vulnerabilities to develop therapies to take to the clinic, Disrupting the intrinsic biology that is supported by high energy metabolism could render more aggressive NECs particularly vulnerable.

Material and Methods
We performed a human neuroendocrine tumor (NET) screen using over 4500 compounds from two pharmaceutical libraries: (1) the **NCGC Pharmaceutical Collection (NPC),** and (2) the **Mechanism Interrogation Plate library (MIPE).**
Our cell line panel included small cell lung cancer cell lines (DMS-79, NCI-H146, NCI-H526, NCI-H69, NCI-H82, and SHP-77), neuroblastoma cell lines (CHP-126, Kelly, and NH6) and lung carcinoid cell lines (NCI-H720, NCI-H727, NCI-H835, and UMC-11). The NAMPT inhibitors, GMX-1778 and STF-1188804 were purchased from APExBIO (Houston, TX). Romidepsin (depsipeptide) was purchased from Sigma-Aldrich (St. Louis, MO). Western blotting was carried using standard protocols and signal was quantitated using the Odyssey CLx imaging system. For the annexin V assays apoptosis was measured using the annexin V fluorescein isothiocyanate (annexin V-FITC) Apoptosis Detection Kit (BD Biosciences, San Diego, CA). Total RNA was isolated from cell lines was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) and assessed on Agilant Bioanalyzer (Agilent Technologies). Global gene expression analysis used the Affymetrix GeneChipR Human Genome U133 Plus 2.0 Array covering +47.000 transcripts. Microarray gene expression data were summarized by applying the RMA (robust multi-array average) and quantile normalization workflow. Differentially expressed genes (DEG) were identified by ANOVA, and significance was adjusted for multiple testing by estimating false discovery rates (FDR). Cell viability was assessed using the Cell Titer Glo assay (Promega, Madison, WI). Synergy of the combinations was calculated using the Excess Over Bliss methodology

Results
With a goal of identifying novel drug classes to pursue as therapeutic options, we began a collaboration with investigators at the National Center for Advancing Translational Sciences (NCATS, National Cancer Institute in Bethesda, MD) to screen compounds for potential repurposing. We performed a human neuroendocrine tumor (NET) screen using over 4500 compounds from two pharmaceutical libraries: (1) the **NCGC Pharmaceutical Collection (NPC),** a library of about 2750 small molecules that have been approved for use by the USFDA or regulatory agencies in other countries; and (2) the **Mechanism Interrogation Plate library (MIPE),** that includes 1920 mechanistically annotated agents prioritized for clinical relevance. We began the screen with three commonly used neuroendocrine cell lines – NCI-H69, DMS 79 and NCI-H727 – and then used an extended set in the validation screen. There were hits that selectively killed NCI-H727 cells, a KRAS mutant line, and others that killed NCI-H69 and DMS-

79 cells. To avoid compounds particularly active in the setting of KRAS mutation, we pursued compounds selectively cytotoxic to H69 and DMS-79 cells. For the validation run, in addition to an expanded set of cell lines, drugs classes identified in the first run were expanded and "pan-actives" identified. Three classes of agents emerged from this screen: HDAC, NAMPT and proteasome inhibitors. Identification of HDAC inhibitors was consistent with prior observations including work by Califano at Columbia University, in which gene expression profiles from neuroendocrine tumors were used to infer the functional state of key master regulators (MRs). Agents able to reverse the activity of the MRs identified in NETs were considered candidates for drug development. As in our screen and validation runs, HDAC inhibitors were particularly active in these studies. Additionally, NAMPT inhibitors emerged as active agents in our screens and when their impact on TCA intermediates was examined, reduced levels of pyruvate, citrate, alpha-ketoglutarate, and succinate were demonstrated, findings that were not unexpected given the inhibition of NAD synthesis that occurs with these agents. Parallel data demonstrated a profound impact of HDAC inhibitors on the critical TCA precursor, acetyl-CoA, and suggested a novel combinatorial approach that led to marked synergy when HDAC and NAMPT inhibitors were combined even at very low sublethal concentrations.

Conclusion

We have identified two novel drug classes with agents that have either regulatory approval (HDAC inhibitors) or are in clinical development (NAMPT inhibitors). For NAMPT inhibitors, their metabolic consequences that we have begun to explore, made their initial development difficult. However new agents targeting NAMPT are now in clinical trials, such that identification of a metabolic vulnerability in NECs is now particularly relevant. For the HDAC inhibitors, multiple lines of investigation have pointed to activity in NECs. Although not successfully realized in early clinical trials, these were small pilot studies that could have missed activity in a subset of patients. Combining low doses of NAMPT inhibitors with low doses of HDAC inhibitors to induce cell death implies the type of synergistic activity that can be very successful clinically and provides support for the development of a rational combination whose synergy exceeded expectations in the laboratory.

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