IDCIFICATION OF ALLOSTERIC MODULATORS OF KRAS USING BIODESY’S SECOND HARMONIC GENERATION TECHNOLOGY

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To screen for conformational modulators of oncogenic K-Ras (G12D), we built and optimized, unbiased SHG assay, which we validated using antibody controls. From an initial ~2700 fragment library screen, we identified several KRas conformational modulators. We believe these fragments will reveal novel allosteric binding pockets for drug targeting and lead compounds to interrogate the relationship between KRAs conformation and its biological functions.

Introduction

- KRAs is the most frequent oncogenic alteration found in human cancers.
- KRAs has been typified as 'undrugable'.
- Second harmonic generation (SHG) is a biophysical technique that can sense and discriminate a molecule’s resulting conformation elicited upon binding that can be screened in a throughput manner.
- We optimized and validated a 384-well high-throughput SHG-based assay to identify allosteric modulators of oncogenic KRas.
- A 1.8% hit rate (i.e. CRI > 3) for the whole screen) was observed from an initial 2,714 fragment primary screen performed in singlicate under stringent selection conditions.
- Initial follow-up validated hits and revealed dose-dependent and nucleotide-specific effects.

More than 30% of all human cancers are driven by alterations in Ras, a structural family of GTPases that regulate cell growth and differentiation outcomes.

Biochemical attempts to target Ras by conventional drug discovery schemes has largely been unsuccessful for lack of a structural parameter that can both be monitored and experimentally challenged. To overcome this challenge, Biodesy has developed a second harmonic generation (SHG) platform to enable the real-time measurement of protein conformational change in physiological conditions.

SHG is advantageous in primary screens because it can predict the mechanism of action elicited by specific allosteric modulators. Recent findings suggest that Ras contains transient and dynamic allosteric pockets that so far could only be detected with time and resource intensive structural techniques.

Methods

- N-terminal His-tagged KRAS(G12D) (1-166) was purified from E. coli with Hi-IDA beads and further purified by size exclusion chromatography.
- KRAs nucleotide exchange was carried out in vitro in the presence of 300X molar excess GMP-PNP. Exchange was quantified by RP-HPLC.
- SHG probe conjugation to the amine (SE)-reactive dye was carried out according to the manufacturer’s instructions and incorporation was confirmed by mass spectrometry.

Definitions

- Conformational response (CRS) was calculated as Δ[SHG] (SHG-SHGi)/SHGi
- Ligand Efficiency (LE): -logEC50/H atoms
- Lipophilic Ligand Efficiency (LLE): -logIC50/LogP

Results

- Work Flow

- Fig. 1. KRAs®[SHG] SHG Assay Schematic. (A) When a labeled protein fragment (G12D) is bound to a surface coated with the G12D probe, a portion of the incident infrared light is scattered to non-species and a portion is scattered to species, which is detected by a detector. The second harmonic signal is highly dependent on the orientation of the dye probe relative to the surface, and is sensitive to changes in the time- and space-averaged orientation of the dye probe that are indicative of conformational changes. A conformational change that alters the orientation of the label in relation to the surface normal increases or decreases the signal. When the SHG signal shifts away from the signal from the label, the signal is decreased. (B) GMP-PNP KRas®[SHG] G12D probe with V-shape. (C) SHG probe conjugation strategy.

- Fig. 2. SE-KRAs®[SHG] Assay Development, GMP-PNP G12D conformationally altered by binding to Ras antibodies as monitored in the presence of 10 µM buffer-buffered media (mean ± SD, n = 3). (B) Both GMP-PNP and GMP-PNP KRAs®[SHG] conformational changes are specifically altered by binding to Ras antibodies as monitored in the presence of 10 µM buffer-buffered media (mean ± SD, n = 3). (C) Conformational change in the presence of 15 µM buffer-buffered media (mean ± SD, n = 3).

- Fig. 3. SE-KRAs®[SHG] Primary Screen. (A) 2,714 Fragment primary screen was carried out at 50 µM and 250 µM against 'GTP' KRaS®[SHG]. Follow-up Screening. (B) 490 compounds (greater than ±10% GMP-PNP KRas®[SHG]) were selected for secondary screening at 100 µM and 200 µM against ‘GTP’ KRas®[SHG] SHG assay (mean ± SD, n = 3).

- Fig. 4. SE-KRAs®[SHG] Primary Screen. (A) Bin 3 includes 27 GPPnhp compounds selected for showing a complete DRC and low maximum EC50 values; columns are color coded according to their magnitude; (B) Examples of the typical sigmoidal dose response curves are shown.

Conclusions

- We successfully built and validated SHG-based screens against ‘GTP’ and ‘GDP’ bound oncogenic KRAs®[SHG].
- We observed a 1.4% hit rate in a ~2700 fragment screen against ‘GTP’ KRAs®[SHG] (1-166).
- Secondary screening at lower concentrations confirmed > 50% of the hits and revealed dose-response and nucleotide-specific effects.
- ~90 fragments (23 lead compounds plus ~70 lower priority) will be selected for further SHG characterization (EC50 values and kinetic traces).
- Selected fragments are concurrently being validated and characterized in binding and structural assay (SPR, 2D NMR).

- Compound activity and SAR will be explored by biochemical and cellular assays.