CONTROLLING RAS/MAP KINASE SIGNALING BY GRB2 CONFORMATION STABILIZING COMPOUNDS

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ABSTRACT

The growth factor receptor tyrosine kinase (RTK) family is a potent inducer of cellular growth. Ligand binding to cognate receptors on plasma membrane induces receptor dimerization and activation, which in turn recruits intracellular molecules for downstream signal transduction. A key signaling pathway activated by GF receptors is the Ras signaling cascade that ultimately leads to cell proliferation. Activation of RTKs leads to the recruitment of adapter protein Grb2, which is constitutively bound to the mammalian homologue of drosophila *son of sevenless* (SOS), and a guanine exchange factor (GEF) for Ras. The recruitment of Grb2-SOS complex by activated RTK places the GEF at close proximity to Ras and exchange of Ras-GDP to Ras-GTP and the initiation of the mitogen-activated protein (MAP) kinase cascade. All RTKs recruit Grb2 either directly or indirectly to their early signaling complex as a means to activate Ras-MAP kinase signaling. Virtual drug screening has identified several dimer interface binding drug-like molecules bridging the two Grb2 protomers which severs the link to SOS that is critical to Ras/MAP kinase activation. The design and synthesis of novel analogues to enhance binding and stabilization of dimeric Grb2 is presented.

BACKGROUND

Structurally Grb2 comprised of one central Src Homology2 (SH2) domain sandwiched by two Src Homology3 (SH3) domains, one is N-terminal another one is C-terminal.[1]. When growth factor receptor such as epidermal growth factor receptor (EGFR) binds to extracellular receptor of receptor tyrosine kinases (RTKs), leads to dimerization as well as activation of kinases, resulting auto-phosphorylation of certain tyrosine residue in the intracellular domains[2]. Through SH2 domain Grb2 interacts with activated growth factor receptors either directly or indirectly with receptor associated proteins (e.g., Shc, FAK, Syp and IRS-1), by recognizing specifically a short pYXNX sequence motif [3] and this interaction translocate cytoplasmic Grb2-SOS complex to the cell membrane, which brings SOS in the vicinity of membrane anchored Ras and promote activation of Ras by changing Ras bound ADP for ATP[1]. The Grb2-SH2 domain basically demonstrates the presence of Grb2 as a dimeric state, whereas the characterization of Grb2 as a monomer exist at the SH3 domain through the proline-rich site [3]. The dissociate change of Grb2 dimerization state to monomeric version occur due to the increase of tyrosine kinase activities in cytosol, causing the reversible phosphatases actions which eventually led to the dissociation [3]. In addition, mutants at the active monomeric Grb2 formations are more stable than at the inactive Grb2 dimerization state(Fig 1), such as upon the phosphorylation of tyrosine on Grb2-SH2 (Y160) residue [3]. Under other conditions, the Grb2 dimerization state plays a vital role to initiate and amplify rapid signal through RTKs stimulation [1]. In this manner, the inhibition at the dimeric Grb2 can occur while the promotion activities of Grb2 monomeric via the mitogen activated protein kinase (MAPK) Pathway [2]. Thus, the synthesis of small non-peptide molecules to disable the reversible dissociation process to monomer can shut down the Ras-MAPK signaling pathway in cytosol.

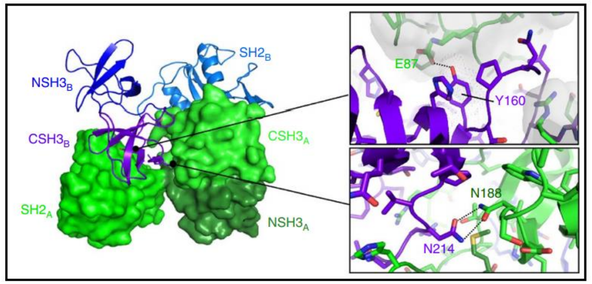


Figure 1: Green depicts homodimer with individual Grb2 protomers.[3]

METHODS

A virtual drug screen of 5000 compounds identified several dimer interface binding drug-like molecules. The top 100 scoring compounds were assayed which resulted in the identification of indolinone urea **1** as a Grb2 dimer stabilizer (Fig 2. A). To explore the binding interface of the protein dimer we prepared a series of ureas utilizing three closely related templates; indolinone **2a**, benzoxazolinone **2b**, benzimidazolone and **2c** (Fig 2. B). We have prepared a large set of diverse ureas **4** were prepared by asimplistic acylation of readily available **2** with isocyantes **3**. All the compounds were purified by column chromatography and confirmed by LCMS, H^1 and C^{13} NMR. Analogues were isolated in moderate to high yield (55% - 85%).

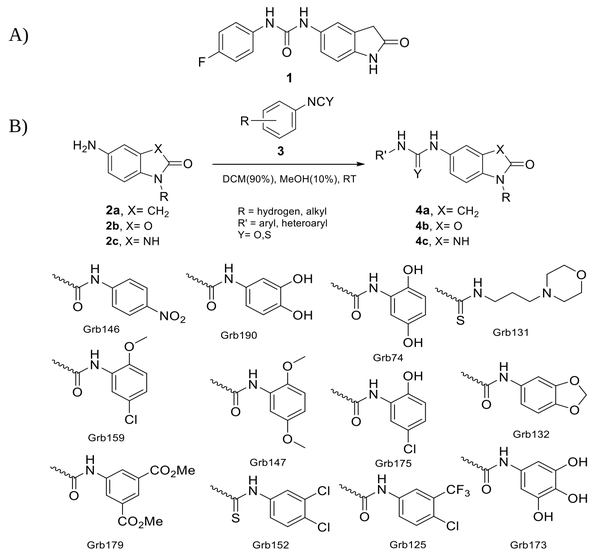
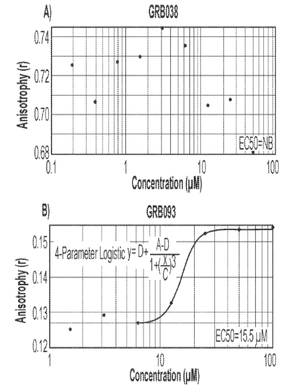


Fig 2: A) Screening hit **1**. B) Representative urea analogues **4** synthesized.

RESULTS AND DISCUSSION

All compounds **4** were tested in HEK293T and NIH-3T3 cell-lines and a number of cancer cell-lines including PC3, OVCAR-5 cells with G12V KRAS mutation. OVCAR-8 contains ERBB2 G776V, p53 Y126 to K132 deletion and a rare P121H KRAS mutation that display RAS/MAPK activation. Also found to be effective in triple negative MDAMB- 231 breast cancer (TNBC) cells, which bears a G13D KRAS as well as p53 mutations, and A549 lung cancer cells containing G12S KRAS mutation. Expectedly, found that the stabilization of dimeric Grb2 leads to inhibition of EGF-stimulated Ras/MAP without hampering the receptor tyrosine kinase activity. Since dimeric Grb2 yields higher anisotropy value than the monomeric Grb2, reflecting higher size of the molecule, the fluorescence anisotropy assay was employed to measure the Grb2 dimerization.

100 nM Atto488 labeled Grb2 was used to identify compounds that induce dimerization, where EC_{50} values represents corresponding Fluorescence anisotropy assay data of the compound that induces dimerization function as opposite to binding affinity. Fig. 3 A shows a representative anisotropy measurement of no-binding (NB) data for compound Grb038. However, Fig. 3 B shows a representative anisotropy measurement with Grb093 inducing Grb2 dimerization at a EC_{50} of 15.5 µM, where NB represents compounds inability to induce Grb2 dimerization.



Compound ID	ECso (µM)	Compound ID	ECso (µM)
GRB085	1.1	GRB093	15.5
GRB086	1.5	GRB094	23
GRB087	4.6	GRB095	50.9
GRB088	8.3	GRB096	NB
GRB089	9.1	GRB097	NB
GRB090	13	GRB098	NB
GRB091	13.3	GRB099	NB
GRB092	13.8	GRB100	NB

Table 1. EC_{50} anisotropy data for dimeric Grb2 stabilizers. NB = No Binding; ND = Not determined.

Figure 3: A) NB anisotropy data for Grb038. B) Grb2 dimerization anisotropy data for Grb093 at EC_{50} of 15.5 µM.

CONCLUSIONS

In conclusion, our work suggests Grb2 dimer stabilizers may be useful chemical tools to study EGFR and KRAS driven cancers. Interestingly, TNBC cells shows greater sensitivity to the Grb2 dimer stabilizers and correlates with Ras/MAPK signal transduction, paving the way for developing a novel strategy towards cancer therapy.

REFERENCES

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3. Ahmed, Z., Timsah, Z., Suen, K., Cook4, N., Lee, G., Lin, C.C., Gagea, M., Marti, A.A., & Ladbury, J.E.(2015). Grb2 monomer–dimer equilibrium determines normal versus oncogenic function. Nat. Com. 6,7354.