Characterization of a First-in-Class targeted inhibitor of the RALGDS oncoprotein

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Background

Ras is a small GTPase that exists in two states, a GTP-bound active state and a GDP-bound inactive state. In its active state, Ras is able to associate with several downstream effector proteins and activate multiple pro-growth signaling pathways. Ras is important because it is one of the most frequently mutated oncogenes in cancer. Mutations in Ras cause this GTPase to operate in a constitutively active state, sending uncontrolled proliferative messages throughout a cell regardless of external stimulus. In the human body, this can lead to tumorigenesis and metastasis.

Currently, there are no therapies that can directly target activated Ras. Therefore, the focus has shifted primarily to targeting Ras downstream effectors. Key effector components of the RAS/RAF/MAPK pathway and the RAS/PI-3 Kinase pathway have been targeted (Figure 1). However, clinical success has been limited. We felt that inhibition of the third mitogenic pathway, the RAS/RALGEF pathway is the missing component.

Using *in silico* screens followed by bioassay, we have now identified a First-in-class inhibitor of RALGEFS, designated C4. C4 binds directly to RALGEFS, such as RALGDS, thus preventing their interaction with RAS. This suppresses the ability of RAS to activate the RAL pathway and therefore drive tumorigenic transformation. Thus, targeting RalGDS with the small molecule inhibitor, C4, is a biologically relevant and innovative approach to treating cancer.

Objectives

The laboratory has identified a First-in-Class inhibitor of RALGEFs, important components of many cancers. The goal of this research project was to investigate the molecule's (designated C4) overall mechanism of action. We sought out to determine if C4 exhibited a titration dependent inhibition of RAL activation, how it suppressed 3D cell growth/survival and if we could detect inhibition of RAL signaling pathways, such as TBK1/AMPK.

Results

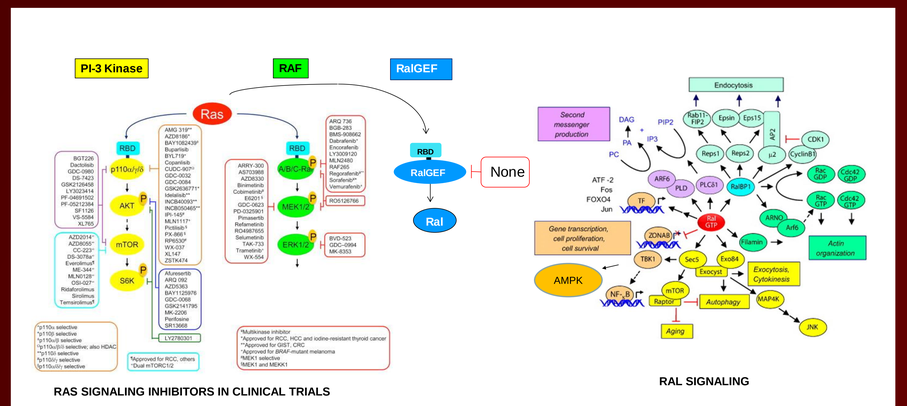

Figure 1– Ras Effector Signaling LEFT: Synopsis of attempts to inhibit the action of the RAS Oncoprotein in human cancer. Ras signals through three main mitogenic pathways the RAF/MAPK pathway, the PI-3K pathway and the RALGEF pathway. Multiple inhibitors for the MAPK and PI3K pathway have been developed. Their effectiveness has proved disappointing. No inhibitors of the third arm (RALGEF) have been described. **RIGHT:** The RALGEF pathway activates the RAL proteins which have multiple biological effects that are essential for tumorigensis and metastasis. Such as the activation of AMPK.

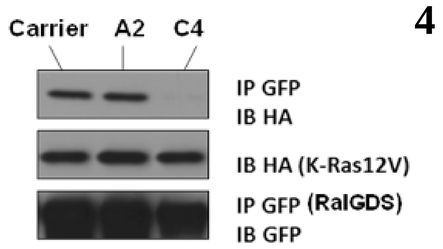

Figure 4 – C4 blocks the interaction of RAS and RALGDS. In overexpression studies. C4 almost completely disrupts the complex formation between RAS and RALGDS. Control molecule A2 has no such effect.

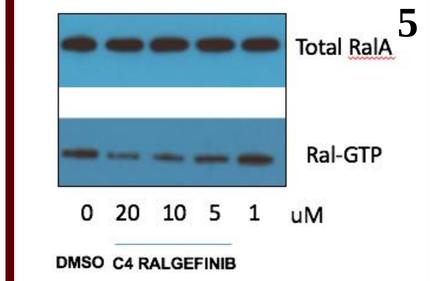

Figure 5- C4 inhibition of RalGDS is Dependent on Concentration: Increasing concentrations of C4 decrease the amounts of active Ral by binding to its coordinate GEF, RalGDS.

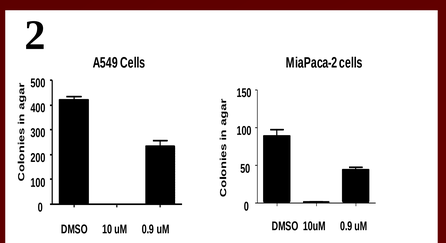

Figure 2 – C4 Decreases Tumor Formation in Soft Agar In silico screening via the JGBCC Molecular Modeling core identified a series of potential candidates. These were screened for the ability to inhibit the growth of RAS driven cell lines in soft agar. The most effective was selected fro further study. This was a 460 Mwt compound designated C4. C4 blocked the growth of multiple mutant RAS driven tumor cells in soft agar with an IC50 of around 1 uM.

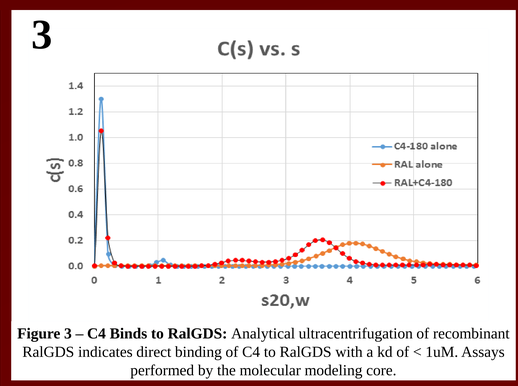

Figure 3 – C4 Binds to RalGDS: Analytical ultracentrifugation of recombinant RalGDS indicates direct binding of C4 to RalGDS with a kd of < 1uM. Assays performed by the molecular modeling core.

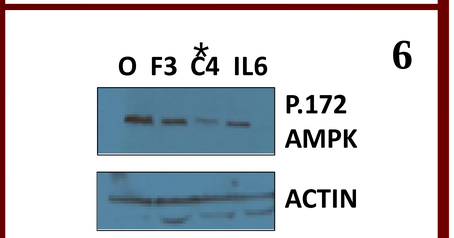

Figure 6 – C4 Blocks AMPK activation. One of the key RAL signaling pathways is the RAL-TBK1-AMPK pathways, which can be measured by phosphorylation at residue 172. Western analysis of C4 treated Mia-PaCa-2 pancreatic carcinoma cells (RAS mutant positive) showed that C4 suppresses AMPK activation. 0 = carrier (DMSO), F3 and IL^ are different drugs for other targets.

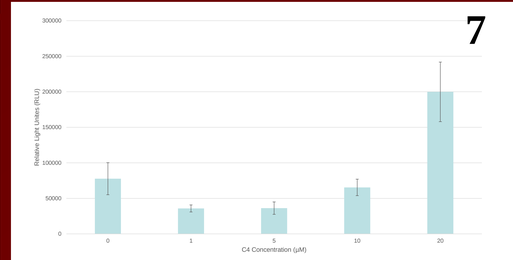

Figure 6 C4 induces Anoikis: Anokis (apoptosis induced by suspension culture) is suppressed by activated RAS, in part via the RAL pathway. Here we measured anoikis induced in MiaPaca-2 cells after treatment with C4 using a Caspase-Glo kit to measure caspase activation status. C4 induced anoikis after 5 hours of suspension with 20Um compound.

Acknowledgements

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Discussion

We have identified the first small molecule inhibitor for the RALGEF class of RAS effector. We have confirmed direct target binding and disruption of RAS/RALGEF effector complexes. The compound is active at low uM levels and exhibits a kd of < 1 uM. Additionally, this binding event in turn decreases levels of active RAL in cells. This observed decrease in RAL is in fact scaled, and depends on C4 concentration, with greater concentrations of C4 having more noticeable effects.

One of the major signaling pathways affected by activated RAL is the TBK1 pathway. In non-cancerous cells, TBK1 is a serine/threonine kinase that is involved in the inflammatory response. It performs several functions such as stimulating interferon regulatory factors and acting as an NF-kB effector. However, in cancerous cells, TBK1 has demonstrated an important role in suppressing apoptosis. This often leads to increased proliferation and may aid in initial transformation or sustained tumorigenesis. In cancer cells, TBK1 activates AMPK by phosphorylating it. AMPK is an important kinase that controls many aspects of cellular metabolism, especially metabolic responses to different stressors. Recently, it has also been shown to control apoptosis through unknown means. Activated AMPK works to suppress anoikis, a type of apoptosis that occurs with loss of anchorage to the ECM. When C4 binds to RALGDS, it prevents the activation of Ral, which furthermore prevents the activation of TBK1 and AMPK, respectively. In this state, AMPK can no longer suppress anoikis and cells undergo this form of programmed cell death the degree of which is dependent on C4 concentration, demonstrating C4's efficacy as a possible therapeutic agent. These results help explain our recent results showing strong anti-tumor activity of C4 against tumor models *in vivo.*