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Drugging the Ral GTPase

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The RAL GTPases have emerged as important drivers of tumor growth and metastasis in lung, colon, pancreatic and other cancers. We recently developed the first small molecule inhibitors of RAL that exhibited antitumor activity in human lung cancer cell lines. These compounds are non-competitive inhibitors that bind to the allosteric site of GDP-bound RAL. The RAL inhibitors have the potential to be used in combination therapy with other inhibitors of the RAS signaling pathway. They also provide insights toward directly targeting other GTPases.

Keywords: allosteric, GTPase, metastasis, personalized medicine, precision medicine, Ral, Ras, small molecule, targeted therapy

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The RAS superfamily of small GTPases consist of more than a hundred proteins and can be divided into 6 main subfamilies: Ras, Rho, Rab, Ran, Arf, and Rad.[1] Each subfamily has been shown to play different physiological roles in human cells. The RAS subfamily is the most studied and has a well-established role in human cancer with the 3 RAS genes (HRAS, KRAS, and NRAS) being the most common oncogenes in human cancer. Activating RAS mutations are found in about one third of all human tumors and are especially common in pancreatic, lung, and colorectal cancer.[2] For more than 3 decades, there have been intensive efforts to inhibit RAS and related GTPases with no clear clinical success.[3] In contrast, inhibitors targeting the downstream effectors of RAS signaling including inhibitors of the RAF-MEK-ERK kinase pathway and the PI3K-AKT-mTOR kinase pathway[4,5] have been developed. A third effector arm of RAS signaling, mediated by RAL (Ras-like) has emerged in recent years as critical driver of RAS oncogenic signaling and has not been targeted until very recently.[6,7] RAL belongs to the RAS branch of the RAS superfamily and shares a high structure similarity (50% sequence identity) with RAS.[8] In human cells there are 2 isoforms RALA and RALB, both of which have been shown to play roles in the proliferation, survival and metastasis of a variety of human cancers including lung, colon, pancreatic, skin and bladder cancers.[7]

Recently our lab discovered the first small-molecule inhibitors of RAL using a structure-based approach.[7] As a RAS subfamily GTPases, RAL shares similar domain architecture: a highly conserved G-domain for nucleotide binding and a C-terminal sequence for membrane targeting. The two loop regions in the G-domain namely switch I and switch II change conformation during GDP-GTP cycling and are involved in the binding with both activators and effectors.[8] We analyzed the structure of RALA in both the GDP- and GTP-bound forms and identified a potential binding pocket on the surface of inactive GDP-bound form of RALA, which is absent in the GTP bound active form. This site is located near the switch II region and close to the nucleotide binding site. We hypothesized that small molecule compounds could bind to this allosteric site and lock the GTPase in its inactive state and prevent the conformational change induced by GTP exchange and subsequent effector binding. I*n silico* virtual screening of a 500,000 compound library and identified 88 potential hits that were tested in cellular and biochemical assays. Three of these were found to be potent inhibitors of RAL; and two of these shared the same chemical scaffold. Based on this chemical scaffold, we synthesized a series of derivatives and eventually identified compound BQU57 as a potent inhibitor of RAL activity and tumor growth both *in vitro* and *in vivo*.

Numerous efforts have tried to directly target RAS and related GTPases. Direct targeting of the nucleotide binding site of the GTPase has been unsuccessful because of its high affinity (pico-molar) for GDP/GTP and the high concentration (millimolar) of these nucleotides in cells. Furthermore, as the nucleotide binding site is highly conserved across the RAS superfamily targeting this site would be nonspecific. In contrast, allosteric inhibitors have the potential to be both potent and specific. However, this requires establishing the presence of "druggable" sites and identifying compounds that bind to these sites with the required affinity all of which are challenges. In our studies we were able to successfully use the crystal structures of RALA for modeling and virtual screening, and subsequently confirm that these drugs can also target the same site in RALB using NMR spectroscopy.

Not all small GTPases have a "druggable" allosteric site. In our study, we also performed computational analysis using the crystal structures of other small GTPase including RAS, RAP, and RHEB; however, we did not identify an allosteric site comparable to that seen in RALA (unpublished data). Nevertheless, there are several reported attempts to identify potential binding pockets on the surface of RAS. One of the first candidate inhibitors of RAS, SCH-54292 was shown to weakly bind to a hydrophobic pocket near the switch II region of RAS and inhibited nucleotide exchange.[9] Sulindac and analogs were shown to bind to RAS at the RAF-binding site (switch II region) and inhibited the RAS-RAF interaction.[10] More recently, using fragment-based screening, 2 small molecule compounds DCAI and VU0460009 were discovered as KRAS inhibitors and shown to bind to the same pocket located near the switch II region of KRAS and inhibited SOS1-mediated activation.[11,12] In another structure-based approach, 2 KRAS inhibitors Kobe0065 and Kobe2062 both bind to a site near the switch II region and inhibited downstream effector signaling.[13] However, there are different types of activating mutations for RAS including G12C, G12D, G12V, G13D, and Q61L and each mutation type could have a different impact on the protein structure. Therefore structural determination and searching for druggable binding pockets on the protein surface will have to be performed for each mutation type.

The compounds we developed are the first of their kind to use the concept of targeting the inactive form of a small GTPase. This approach takes advantage of the continuous GDP-GTP cycling in cells and the fact that all RAL protein molecules will eventually cycle back to the inactive state. For this approach to be effective the binding between the inhibitor and the protein, although noncovalent, has to be tight enough to outcompete the conformational change induced by GTP binding. This approach will not work for those tumors that have activating mutations as these mutations generally function to maintain the protein in the GTP-bound state. In the case of RAL, and other Ras like family members, mutations are extremely rare (<1 %) in human cancer (https://tcga-ata.nci.nih.gov/tcga and http://cancer.sanger.ac.uk/cosmic). Rather, the wild-type protein is commonly overexpressed in patient tumors and this is often correlated with poor patient outcome. This suggests that our approach has the potential to be used in targeting Ras driven signaling in multiple tumors irrespective of whether this is driven by Ras mutation, overexpression or enhanced activity of signaling partners upstream of Ras.

In future work it would be interesting to determine if RAL inhibitors offer additional effects in combination with other inhibitors of the RAS signaling pathways such as those inhibiting RAF-MEK-ERK kinase pathway and the PI3K-AKT-mTOR kinase pathway.[3] In principle, such synergistic inhibition would offer the greatest potential to inhibit RAS oncogenic signaling. In addition to Ral inhibitors, it is also reasonable to speculate that inhibitors of RALGEFs would also act to inhibit RAL and may in some cases, depending on the stimulus be more potent. Finally, RAL can also be activated in cancer cells independent of RAS via phosphorylation by kinases such as Protein kinase C and Aurora kinase A.[14–16] Therefore, inhibitors of Aurora-A and PKC could also contribute to the inhibition of RAL signaling pathway in cases where RAL is only minimally or not activated directly by RAS.

In conclusion, our work suggests that direct targeting of inactive small GTPases that are not mutated and cycle between active and invective states holds promise for the development of novel cancer therapeutics. Blockade of RAS signaling can now be contemplated as reagents directed against the primary 3 downstream signaling pathways are available.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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