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Ras Inhibitors

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Selective Inhibition of Mutant Ras Protein through Covalent Binding

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As one of the first oncogenes to be discovered and one of the most prevalently mutated tumor genes, *Ras* is one of the most (in)famous genes for cancer researchers and clinicians alike. Yet, three decades after identification of the Ras protein, no effective treatments for Ras-mutant tumors are available despite large research investments.

The Ras proteins function as GDP-GTP regulated binary switches in many signal transduction pathways that govern cell growth and differentiation and can signal downstream only in the GTP-bound "on state". Key enzymes that regulate the activation state of Ras are the guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP by GTP, and the GTPase activating proteins (GAPs), which terminate the "on state" by catalyzing the hydrolysis of bound GTP to GDP. Most of the mutant Ras proteins found in human cancers have lost their sensitivity to the GTP-hydrolysis-promoting action of GAPs, thus rendering Ras chronically active.[1]

Three isoforms comprise the Ras family, H-Ras, N-Ras, and K-Ras, with mutations of the K-Ras isoform particularly prevalent in human cancers and occurring in 60% of pancreatic, 34% of colorectal, and 16% of lung cancers (numbers from the COSMIC database,[2] although higher frequencies have been seen in other studies). Glycine 12, which is involved in nucleotide binding and positioning of the γ-phosphate moiety of GTP for hydrolysis, is the most frequently mutated residue in K-Ras, with G12D most commonly detected in colorectal and pancreatic cancer and G12C most commonly detected in lung cancers (see Table 1).[2]

Over the last 30 years scientists have explored a wide array of strategies to disrupt the oncogenic function of K-Ras, from targeting the Ras protein surface directly to less direct approaches such as inhibiting post-translational modification of Ras, its membrane localization, or targeting downstream Ras effector proteins. To date, none of these strategies have led to clinically satisfactory outcomes, frequently because of compensatory mechanisms abrogating the efficacy of the approaches chosen. Inhibition of Ras effector proteins, such as Raf and PI3K, once thought to hold great promise, will likely only be effective as part of combination therapies, and this is currently the subject of intense clinical investigation.[3]

Table 1: Cancer types most frequently associated with K-Ras mutations and their most commonly observed mutants.[a]

	Colorectal cancer [%]	Pancreatic cancer[%]	Lung cancer [%]
K-Ras mutations	33.6	59.8	16.3
of which			
G12D	35.9	50.3	17.6
G12V	23.3	29.3	20.9
G12C	8.4	2.9	41.9

[a] Data from COSMIC database.[2]

The Ras structure has a relatively flat surface, and apart from the nucleotide binding groove, there are no cavities clearly suitable for the binding of small-molecule ligands. Nevertheless, in the past two decades several research groups succeeded in the identification of functionally active small-molecule binders.

In the mid-1990s, a group at Schering identified phenylhydroxylamine-containing Ras binders, such as SCH-54292, with inhibitory effects on GEF-catalyzed nucleotide exchange.[4] Recently, groups at Genentech and Vanderbilt independently conducted NMR-based fragment screens that yielded the first examples of cocrystal structures of complexes formed between small-molecule ligands and Ras. The binding of these compounds was weak (>100 μM), but nevertheless imparted inhibitory effects in nucleotide exchange assays.[4] Last year, another research group identified weak Ras binders that disrupted the Ras-Raf interaction.[5] Furthermore, the first structurally characterized example of an allosteric inhibitor approach, trapping of a Ras-GTP conformational state with low affinity for effector proteins by means of Zn^{2+}- or Cu^{2+}-cyclen, was recently reported.[4,6]

By using a tethering approach to discover drug binding pockets on Ras, a team led by Shokat took advantage of the fact that the G12C mutation found in many lung cancers creates a nucleophilic acceptor.[7] Screening a library of disulfide-containing molecules led to the identification of a compound series that selectively bound K-Ras G12C over wild-type K-Ras (even though the latter contains three cysteine residues), and bound K-Ras preferentially in the

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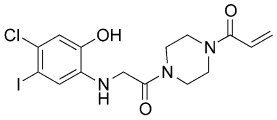
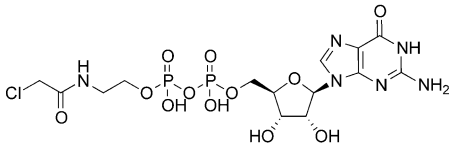
GDP-bound state. As shown by X-ray crystallography, the tethered compounds lie in a pocket extending from Cys-12 in the opposite direction from the nucleotide binding site, created by the movement of residues from the switch II region. This pocket, termed "Switch II Pocket", is not apparent in other published structures of Ras, but, interestingly, was suggested to be at least partially occupied by SCH-54292 almost two decades ago.[4]

By using different compounds with vinyl sulfonamide or acrylamide electrophilic groups (e.g. "compound 12"), the authors showed that although EDTA-promoted exchange of



"Compound 12"
(Shokat et al., 2013)



SML-8-73-1
(Gray et al., 2013)

GDP to GTP is compromised approximately threefold, GEF-mediated exchange is almost completely abolished. They also demonstrated that the binding of Ras effectors BRaf and cRaf was compromised in K-Ras G12C-expressing cells treated with these compounds. This could either be due to the binding of inhibitor-bound K-RasGTP to effectors with lower affinities, or preferential GDP-loading caused by the inhibitor. Moreover, the inhibitor promoted increased apoptosis and decreased viability in four K-Ras G12C-expressing lung cancer cell lines compared to three lung cancer cell lines with a K-Ras G12D mutation, an N-Ras Q61K mutation, or no Ras mutations.

Ras and other small G-proteins possess a nucleotide-binding pocket that accommodates GTP and GDP, and the presence of the γ-phosphate moiety in GTP-bound Ras forms the basis for maintaining the protein in an activated form. Hence, the strategy of developing GTP mimics, by analogy to ATP mimics for kinase inhibitors, has frequently been raised, but largely been discounted because of unfavorable thermodynamics: guanine nucleotides have picomolar affinity for Ras, and GTP is abundant in the cytosol (close to millimolar concentrations).

Gray and co-workers recently recognized the opportunity stemming from the G12C mutation adjacent to the active site, near the position of the γ-phosphate moiety of the native GTP, and envisioned that formation of a covalent bond with a GTP mimic equipped with a reactive functional group would overcome the thermodynamic hurdle to nucleotide displacement.[8] Using structure-guided design, the team synthesized SML-8-73-1, a GDP analogue linked to a terminal electrophilic α-chloroacetamide moiety. Mass spectrometry demonstrated exclusive covalent labeling of the GTP binding site of K-Ras G12C, while hydrogen-exchange mass spectrometry studies, as well as functional biochemical assays, suggested that this compound stabilizes an inactive form of K-Ras G12C with lowered affinity for effector proteins.

Since SML-8-73-1 cannot pass through cell membranes because of its two negatively charged phosphate groups, the β-phosphate unit was capped with an alanine ester phosphoramidate, designed to be removed intracellularly through enzymatic cleavage. Cellular "target engagement" assays with this structural analogue, SML-10-70-1, indeed demonstrated penetration into cells and effective competition for binding in the GTP-binding pocket. Antiproliferative effects with this compound in various tested cell lines were relatively weak though (EC_{50} values >20 μM), and indiscriminant of the Ras G12C mutation status.

These studies certainly support the potential utility of these approaches to disrupt the function of oncogenic K-Ras, although some important questions remain unanswered. For example, the in vitro studies were conducted with unmodified Ras, and it would be of interest to know if the effects can be recapitulated with modified Ras. For compounds that preferentially bind GDP-loaded Ras, the time course of GTP hydrolysis may be critical. The reported half-life of Ras (ca. 24 h) is not much longer than the rate of hydrolysis of the mutant Ras GTP (8–17 h with $k_{cat} \approx 1–2 \times 10^{-3}\ min^{-1}$), and newly synthesized Ras would preferentially bind the more abundant cellular GTP. What is the specificity of these compounds for K-Ras compared to the cellular proteome? While the compounds reported by Gray and co-workers did not show discrimination for K-Ras G12C-expressing cells, the compounds reported by Shokat and co-workers did appear to show some selectivity, although a larger panel of cell lines expressing K-Ras G12C should be tested to determine if selective inhibition of K-Ras G12C-mutant cancer cell lines is observed consistently. Nevertheless, we believe that the concepts explored by the authors are compelling, and that current limitations may be overcome with further optimization.

Covalent modification was proposed many years ago as a strategy to overcome the fundamental thermodynamic problem of disrupting protein–protein interactions with small molecules,[9] and this approach, elegantly applied by the teams of Shokat and Gray, seems warranted, especially for conditions associated with a substantial unmet medical need, such as Ras-mutant cancers. While the number of K-Ras G12C-expressing tumors only represents about 10% of K-Ras mutations overall, 7% of all lung, as well as 3% of pancreatic and colorectal cancer patients would be predicted to express this mutation, and therefore, might benefit from a therapy that takes advantage of this approach. There are few examples of opportunities for such mutation-selective targeting in cancer, and further exploration of these promising starting points, therefore, seems highly warranted. The study by Shokat and co-workers is also another reminder of the dynamic nature of Ras and poses a challenge to the common assumption that Ras does not present any binding pockets apart from the nucleotide binding groove. Time will tell if this or other transient binding pockets can be leveraged for the binding of small molecules even without the help of an

adjacent nucleophilic center, potentially broadening the K-Ras targeting opportunity beyond G12C mutations.

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