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Design of small molecules that compete with nucleotide binding to an engineered oncogenic KRAS allele

Yan Zhang¹, Marie-Hélène Larraufie², Leila Musavi², Hemanth Akkiraju^{2,3}, Lewis M. Brown^{2,3}, and Brent Stockwell^{1,2}

¹Department of Chemistry, Columbia University, Northwest Corner Building, 12th Floor, MC 4846, 550 West 120th Street, New York, NY 10027

²Department of Biological Sciences, Columbia University, Northwest Corner Building, 12th Floor, MC 4846, 550 West 120th Street, New York, NY 10027

³Quantitative Proteomics and Metabolomics Center, Columbia University, New York, NY 10027, USA

Abstract

RAS mutations are found in 30% of all human cancers, with *KRAS* the most frequently mutated among the three *RAS* isoforms (*KRAS*, *NRAS*, *HRAS*). However, directly targeting oncogenic *KRAS* with small molecules in the nucleotide-binding site has been difficult due to the high affinity of *KRAS* for GDP and GTP. We designed an engineered allele of *KRAS*, and a covalent inhibitor that competes for GTP and GDP. This ligand-receptor combination demonstrates that the high affinity of GTP/GDP for *RAS* proteins can be overcome with a covalent inhibitor and a suitably engineered binding site. The covalent inhibitor irreversibly modifies the protein at the engineered nucleotide binding site and is able to compete with GDP and GTP. This provides a new tool for studying *KRAS* function and suggests strategies for targeting the nucleotide-binding site of oncogenic *RAS* proteins.

INTRODUCTION

The *RAS* superfamily of small GTPases consists of more than 150 GTP-binding proteins that play key roles in the regulation of cell growth and survival¹. There is speculation that several members of this family, including *RAS*, *RAC1*, *RHOA* and *RHEB* proteins, could serve as effective targets in multiple diseases. *RAS* mutations are found in 30% of human cancers, with *KRAS* being the most frequently mutated (86%) among the three *RAS* isoforms (*KRAS*, *NRAS*, *HRAS*)². It has been postulated that *KRAS* is essential for tumor maintenance, but not for normal adult physiology, thus rendering inhibition of *KRAS* a “Holy Grail” in cancer therapy. Recently, the discoveries of a pan-*RAS* inhibitor³ and *KRAS*^{G12C} covalent inhibitors^{4–5} showed the feasibility of directly targeting *RAS* protein in

*Corresponding author: Brent R. Stockwell, Columbia University, Northwest Corner Building, 12th Floor, MC 4846, 550 West 120th Street, New York, NY 10027., bstockwell@columbia.edu; Tel: 212-854-2948; Fax: 212-854-2951.

Supporting Information

Supporting Information includes five figures, three tables, LC-MS data, and methods file.

some cases. However, pharmacological and genetic validation of the therapeutic index associated with targeting RAS and other small GTPases requires further investigation.

The KRAS protein functions as molecular switch, cycling between a GTP-bound active state and a GDP-bound inactive state. Oncogenic mutations in KRAS impair its intrinsic and GAP-mediated GTPase function, resulting in the accumulation of KRAS-GTP that constitutively activates KRAS downstream signaling. Pharmacological validation of KRAS and other small GTPases as therapeutic targets is difficult because of their picomolar affinity to GTP/GDP. Thus, creating a potent and selective small molecule inhibitor for these proteins is challenging.

Generating engineered, inhibitable alleles of proteins to validate targets has proved successful for kinases and other proteins^{6–15}. The essence of this strategy resides in the design of engineered alleles of the protein of interest that are sensitized to small-molecule inhibition, while being functionally indistinguishable from wild-type counterparts (Fig. 1A). Treatment with a complementary small-molecule probe can then provide selective, rapid, and dose-dependent inactivation of the protein of interest. More recently, Shah and co-workers reported a strategy for the selective inhibition and activation of an engineered H-Ras mutant by unnatural GDP and GTP analogues¹⁶. However, future applications of that study were limited by the use of GDP analogues as molecular probes, which are inherently non-cell-permeable and not selective. Moreover, that study did not address the feasibility of designing drug-like small molecule that target engineered small GTPases.

We aimed to address this issue by testing whether we could design a cell-permeable small molecule probe that would allow us to test the effects of pharmacological inhibition of suitably engineered small GTPases. We focused on the design of small molecules capable of inhibiting an engineered mutant allele of oncogenic KRAS (termed KRAS*). We were concerned that mutation of key conserved residues in KRAS might alter its natural nucleotide selectivity and important protein-protein interactions, making the mutant activated in a GEF-independent way and lose the switch function^{16–18}, as first suggested for RAS (N116I)¹⁹. Shah and co-workers have reported, however, that H-RAS (L19A, N116A) mutations remain fully functional as the wild-type enzyme¹⁶. We also demonstrated that the engineered inhibitable KRAS* allele we designed has an effective “on” and “off” switch mechanism and fully functions in cells. We report herein the computational design of a mutant engineered oncogenic allele of KRAS bearing an enlarged nucleotide binding site and a cysteine residue that can serve as a site for covalent targeting. We found that this mutant KRAS* was functionally indistinguishable from KRAS^{G12V}, but could be selectively inhibited by a computationally designed covalent inhibitor.

RESULTS

Rational design of KRAS allele

Small GTPases in the RAS family have a conserved binding site and share similar nucleotide-binding pockets²⁰ (Fig. 1B). Shah and co-workers demonstrated that mutation of L19 and N116, located on the backside of the HRAS GTP-binding site, to smaller alanine residues allowed access to a buried hydrophobic cavity. The crystal structure of KRAS^{G12V}

in the GDP-bound conformation (4TQ9²¹) was used as a template to model the influence of point mutations in the nucleotide-binding site. Prime and BioLuminate (Schrödinger) were used to calculate the change in structure and stability of the protein after introducing mutations (Table S1). Residues 19 and 116 were mutated *in silico* and all residues within 10 Å of either mutation were minimized. A new hydrophobic cavity 3 Å wide and 10 Å long appeared in the nucleotide-binding site (Fig. 1C). We next wished to introduce a cysteine residue in the hydrophobic pocket that could then be used as a handle for covalent inhibition³. Indeed, we envisioned that the use of covalent inhibitors might be key to be able to cope efficiently with the high cellular concentration of GTP (~1 mM)²². For that purpose, a cysteine residue (C114) that could be targeted by electrophilic moieties was installed in the backside of the engineered hydrophobic pocket. The stability of the engineered protein (KRAS*G12VL19AN116AV114C, termed KRAS*) was predicted to have decreased energy of 52.96 kcal/mol. The predicted structure of the engineered protein showed that C114 was orientated to perform a nucleophilic attack on electrophilic moieties in the pocket (Fig. 1C). Importantly, the three mutated residues in KRAS* are in a conserved site among the GTPases, so they could potentially be translated to other members of the family.

Function of engineered KRAS allele

To verify that the engineered mutant allele functions well as a “switch”, KRAS* was loaded with GDP or GTP and incubated with RAF1 RBD (RAS binding domain) protein attached to glutathione Sepharose beads. The unbound KRAS* was removed during the washing step, while the bound KRAS* was quantified by immunoblotting with anti-RAS antibody. As expected, RAF 1 RBD specifically bound to GTP-loaded KRAS* but not GDP-loaded KRAS* (Fig. 2A). To prove that the KRAS* is not nucleotide-free analog of RAS as suggested for HRAS(N116I), we tested the binding of GDP and GTP by KRAS* and KRAS^{G12V} protein (Fig. 2B and 2C). Both KRAS* and KRAS^{G12V} were nucleotide exchanged to BODIPY-GTP, a fluorescent analog of GTP which have high polarization value when binds with protein and low polarization value in unbound form. BODIPY-GTP was displaced by GDP or GTP in both KRAS* and KRAS^{G12V}, indicating KRAS* retains nucleotide selectivity. Compared with KRAS^{G12V}, the faster nucleotide exchange rate of BODIPY-GTP to GTP or GDP might come from the low binding affinity between KRAS* and BODIPY-GTP.

To further verify KRAS* function in cells, we used RAS-less MEFs. *K-Ras^{lox}(H-Ras^{-/-};N-Ras^{-/-};K-Ras^{lox/lox};RERT^{ert/ert})* mouse embryonic fibroblasts (MEFs) were stably transfected with KRAS* or KRAS^{G12V} alleles²³. The MEFs were generated to carry null *HRAS* and *NRAS* alleles along with a floxed *KRAS* locus and a knocked-in inducible Cre recombinase. After transfection, 4-hydroxytamoxifen was used to induce of Cre recombinase, resulting in the complete elimination of endogenous *KRAS* gene after two weeks (termed KRAS* MEFs and KRAS^{G12V} MEFs) (Fig. S1). The ability of KRAS* to activate RAF/MEK/ERK signaling was examined by measuring the abundance of phosphorylated ERK (pERK) and phosphorylated AKT (pAKT) in the transfected cell lines. Following EGF treatment, pERK and pAKT levels were increased in the transfected cell lines (Fig. 2D), demonstrating that the engineered mutations did not prevent KRAS* from activating effector proteins. The EGF-dependent signal in KRAS* MEFs also revealed that

its binding to GTP was not GEF-independent as suggested for RAS (D119N)¹⁸. KRAS mRNA levels were measured in the transfected cell lines by qPCR. We found that the *KRAS** mRNA was more abundant than the *KRAS*^{G12V} mRNA (Fig. 2E). Compared with the similar protein level of KRAS in *KRAS** MEFs and *KRAS*^{G12V} MEFs (Fig. 2A), that might because *KRAS** is less stable than *KRAS*^{G12V} (Fig. S1), which providing an advantage for rapidly inducing and removing this engineered protein from cells.

Design of small molecule ligands

A GDP-bound *KRAS*^{G12V} crystal structure (PDB: 4TQ9) was used to design compounds that covalently lock *KRAS** in the GDP-bound state, inactivating its signaling function. *KRAS*^{G12V} was mutated in silico to *KRAS**, and the engineered structure was refined by Schrödinger Protein Preparation Wizard for docking studies. A fragment-based design strategy was applied in the search for a covalent inhibitor²⁴ and two series of covalent inhibitors were designed and synthesized. 177,911 fragments (SI Software) were screened using the Schrödinger Glide program and the top-ranked fragments were used as scaffolds for further design. The first series of inhibitors (Fig. 3A, S2) harbor a carboxylic acid group, which is predicted to interact with the magnesium ion and multiple residues in the GTP-binding pocket, thus giving a high predicted binding affinity (as good as -12 for Glidescore). These inhibitors had high predicted binding affinity to the *KRAS** protein (Fig. S3), but low cellular activity (Table S3), making it difficult to study the *KRAS* therapeutic index in cells. Compound 7 had two carboxylic acid groups, thus likely could not penetrate cell membranes (Table S2). Esterification one carboxylic acid groups can make compounds more cell-membrane-permeable; the measured cellular accumulations of compound **6** and **8** were 3.9-fold and 15.6-fold (Table S2), respectively. The low cellular activity might thus derive from their inability to block *KRAS*-effector interactions in cells.

We then found that the conserved aspartate residue D119 is a key residue determining the specificity for GTP over other nucleotides through hydrogen bonding. We reasoned that the discovery of a compound that can form hydrogen bonds with D119 is thus important for a successful design. H-bond constraints to D119 were applied in fragment screens for a second inhibitor design series. Top-ranked fragments without carboxylic acid groups were tested in a fluorescence polarization assay to measure binding affinity to *KRAS**. We found that an indazole fragment **G**, with a docking score of -7.1, (Fig. 3B, S2) could compete with GTP binding for *KRAS* in vitro in the 100 micromolar range (Fig. S4), and was chosen as a scaffold for further design. As a next step, the hydrophobic pocket of the engineered allele was targeted by attaching a lipophilic group (Fig. 3B) to the appropriate site on fragment **G**. This produced compound **G***, which had a more favorable docking score of -9.1, compared to -7.1 for compound **G** (note that the Glide docking score is a log scale, so this predicted a 100-fold improvement in affinity). Based on the structure of compound **G**, a number of covalent inhibitors with electrophile warheads at different positions were designed and evaluated using Schrödinger's Covalent Docking program²⁵. This showed that having a 2-chloroacetamide electrophile at the *meta*-position (compound **YZ0468**) had the best-predicted affinity score *in silico* (docking structure showed in Fig. 3A). On the basis of this indazole scaffold, a customized compound library employing amide coupling, reductive aminations, and cross coupling reactions was created (Fig. 3C) and evaluated using

Schrödinger's Glide docking and Covalent Docking program. The top-scoring compounds were synthesized and tested in the fluorescence polarization assay to further assess inhibition activity.

Synthesis of covalent KRAS* inhibitors

A set of promising covalent small molecule inhibitors was thus synthesized (Table S3). As an example, the synthetic route developed for compound **YZ0711** is illustrated in Fig. 3D. The indazole scaffold was synthesized by heating 2-fluoro-5-nitrobenzonitrile and hydrazine monohydrate in refluxing ethanol followed by the selective BOC-protection of the *N*-1 position of the indazole and subsequent palladium-catalyzed hydrogenation²⁶. The resulting diaminoindazole could be acylated selectively at the *C*-5 amine using an acid chloride. In the next step, the *C*-3 amine was acylated in pyridine to yield compound 4. Catalytic hydrogenation of the nitro group in compound 4 followed by acylation of the resulting aniline with chloroacetyl chloride and selective deprotection of the BOC-group provided the desired product.

Biophysical measurements

We developed an *in vitro* fluorescence polarization assay to test the ability of small molecules to displace BODIPY-GTP, a fluorescent analog of GTP. In this assay, test compounds that were able to competitively replace BODIPY-GTP bound to KRAS* resulted in a decrease in the polarization of BODIPY-GTP (Fig. 4A).

The designed covalent inhibitors and non-covalent inhibitors were able to compete with fluorescent GTP binding for the engineered mutant, but not for KRAS^{G12V} (Fig. 4, Fig. S5), thus satisfying the selectivity criterion. In addition, inhibitor binding was catalyzed by EDTA but not SOS1, a guanine nucleotide exchange factor that promotes guanine nucleotide exchange (Fig. S5). Compared with compounds bearing acrylamide and vinyl sulfonate warheads, compounds bearing a chloroacetyl warhead had a better ability to bind to KRAS* (Fig. 4B). This might result from the relatively small pocket in KRAS*. Indeed, an extra chloro group on the benzyl moiety of YZ0571-1 decreased potency, consistent with a small available space in the binding site. In addition, a non-covalent analog (YZ0571-4) was not able to displace BODIPY-GTP. Based on this result, a series of covalent inhibitors incorporating chloroacetyl warheads were synthesized. Compound YZ0714 and YZ0719 had comparable binding affinity to YZ0571-1. Compound YZ0719 has low activity in a cell viability assay, which might result from its low cell membrane permeability (Table S2). Compound YZ0711 had the best selectivity at inhibiting KRAS signaling among these electrophiles (data not shown) and was selected for further analysis.

WaterMap (Schrödinger), which calculates thermodynamics associated with water hydration sites in protein active sites^{27–28}, showed that compound YZ0711 displaced nearly all high-energy hydration sites in the nucleotide binding site of KRAS* (Fig. 4D, Fig. S6). This result indicated possible favorable binding between compound YZ0711 and KRAS* protein.

Mass-spectrometry analysis of covalent binding

To determine whether compound YZ0711 could covalently bind to KRAS*, purified KRAS* (20 μ M) was incubated with 200 μ M YZ0711 and 1 mM EDTA overnight at 4°C. The protein was then labeled with iodoacetamide and digested with trypsin. The resulting peptides were analyzed by nano LC/MS. LC/MS analysis revealed a modification of peptide (103–117) with YZ0711 at cysteine (C114) or lysine (K117) residues (Fig. 5A). A peptide (103–117) lacking cysteine but containing lysine in KRAS^{G12V} was not modified with YZ0711 (Fig. 5B), indicating that YZ0711 modification was exclusive to C114 in KRAS*. C114 modification with YZ0711 was estimated to be 94% in KRAS* (Table MS1).

Cell-based assay

The KRAS* MEFs and KRAS^{G12V} MEFs described above were used in cellular assays. We investigated whether the test compounds were able to penetrate cell membranes and covalently label KRAS*. Using LC-MS, we found accumulation of compound YZ0711 in KRAS* MEFs was 1.4 fold compared to the compound concentration in the medium (Table S2). Desthiobiotin-GTP, which can covalently modify conserved lysine residues in the nucleotide-binding site, was used to pull down KRAS in cell lysates. KRAS* MEFs treated with 50 μ M YZ0711 has a decreased KRAS enrichment (Fig. 6A), suggesting that YZ0711 is able to penetrate the cell membrane and target the GTP/GDP binding site.

In a cell growth inhibition assay (Fig. 6B and Fig. S7), KRAS* MEFs and KRAS^{G12V} MEFs were treated with DMSO, 25 μ M YZ0711, or 25 μ M YZ0756 (a structurally similar compound as YZ0711) for 4 days and cell numbers were counted on a Vi-Cell XR Cell Viability Analyzer. Compound YZ0711 showed 80% inhibition of KRAS* MEF number and 50% inhibition to KRAS^{G12V} MEFs, indicating that YZ0711 exhibits some off-target effect, but it is more selective to KRAS*. The cellular inhibition test using BRAF^{V600E}-CAAX RASless MEFs also indicated that compound YZ0711 selectively inhibit KRAS* MEFs' growth (Fig. S7). Analog YZ0756 exhibited 25% inhibition in KRAS* MEFs and 15% inhibition to KRAS^{G12V} MEFs, which is less potent than compound YZ0711.

Inhibition of RAS signaling pathway

To test whether compound YZ0711 can disrupt KRAS*-effector binding, RAF RBD protein, which can selectively pull down GTP loaded KRAS*, was used. The GTP-loaded KRAS* protein was incubated with DMSO or YZ0711 (40 μ M) for 15 min and the reaction was stopped by adding MgCl₂ (65mM), then incubated with RAF RBD protein. Minimal KRAS* was observed for YZ0711-bound KRAS* compared with GTP-loaded KRAS* (DMSO control group) (Fig. 6C). To further investigate whether compound YZ0711 can inhibit the RAS-RAF-MEK-ERK and RAS-PI3K-AKT signaling pathways in cells, the phosphorylation levels of ERK and AKT were analyzed by western blot (Fig. 6D). Treatment of KRAS* MEFs with YZ0711 resulted in decreased pERK and pAKT levels. KRAS^{G12V} MEFs were more resistant to this effect. Treatment of KRAS* MEFs with YZ0711 also resulted in decreased RAS-bound BRAF, suggesting YZ0711 can disrupt RAS signaling (Fig. 6E).

DISCUSSION

Before embarking on a small molecule drug discovery project directed against a specific target protein, it is critical to first validate the therapeutic effectiveness of the target in a specific disease, as well as the therapeutic index associated with target inhibition²⁹. “Chemical” and “genetic” methods are two main approaches used in this target validation where small molecules or genetic tools are used to modulate the target function³⁰. Genetic methods including gene knockout and RNAi may have problems of generating null mutants for essential genes and lacking alignment between RNAi and inhibitor studies³⁰. Small molecule approaches can overcome above difficulties and provide additional information about druggability, toxicity, and safety of inhibiting a target.

KRAS is an essential gene and its gene product interacts with multiple upstream and downstream effectors. However, there is a lack of suitable chemical tools that can directly target *KRAS*. We developed a scalable system for testing the *in vitro* consequences of pharmacological inhibition of *KRAS*. An enlarged binding pocket was engineered into GTP binding site of *KRAS* through mutation of two conserved residues to alanine. The engineered *KRAS* is functionally distinguishable from *KRAS*^{G12V} in cell. We have designed and synthesized a small-molecule probe that covalently binds to an engineered *KRAS* mutant with high affinity and displaces GTP from the binding pocket. The probe allows selective binding to the engineered protein, but not the wild-type protein. The probe is also cell membrane permeable and can selectively inhibit RAS signaling in cells. Covalent inhibitors are known to have off-target activity by forming a covalent bond with other proteins. We employed chloroacetyl warhead, which has low intrinsic reactivity, to minimize this off-target effect²⁴. The probe we designed is selective to the engineered *KRAS* but also has some extent of off-target effect in cells. The better selectivity and higher potency could be achieved by getting co-crystal structure of ligand-protein complex, and further optimization of ligand structure and activity. The crystallization research is ongoing in our lab and we'll report it in future after we have progress.

Small GTPases contribute to multiple cellular processes and different stages of cancer development and progression^{31–36}. They have conserved GTP/GDP binding pockets and share similar activation/deactivation mechanisms. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP by GTP, while GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of small GTPases. In the active state (GTP-bound state), small GTPases associate with a variety of effectors and promote downstream signaling. The system we designed here can be expanded for the design of allele selective small molecule inhibitors of other proteins in small GTPase family, thus providing tools to validate small GTPases. Considering the high affinity of small GTPases for GTP (nanomolar range) and the high concentration of GTP in cells (~0.5 mM)²², a covalent inhibitor is generally necessary to compete with nucleotide binding. To achieve high selectivity and minimize off-target effect, a targeted covalent inhibitor need to be designed, which requires available protein crystal structures and structure-and-activity studies. Besides, choosing a protein which is easy to be crystalized will be highly beneficial for the study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Design of engineered KRAS allele

(A) The engineered KRAS* harbors an enlarged pocket and a cysteine residue, which enables the design of a covalent molecule selectively target KRAS* over KRAS^{G12V}. (B) Sequence alignment of RAS and small GTPases superfamily (conserved residues are in BOLD). (C) Representation of KRAS G12V (PDB code: 4TQ9) and engineered KRAS* (structure was minimized using Schrödinger Prime).

Figure 2. Establishment of engineered KRAS knock in cell lines

(A) RAF-RBD can selectively pull down GTP-loaded KRAS* but not GDP-loaded KRAS*. (B) Amount of exchanged GDP and GTP in KRAS^{G12V} as a function of time. (C) Amount of exchanged GDP and GTP in KRAS* as a function of time. (D) Western blot indicated the overexpression of RAS in KRAS^{G12V} MEFs and KRAS* MEFs. RAS downstream signaling was activated upon EGF treatment. The *endogenous* KRAS MEFs are MEFs transfected with empty pBabe vector but without hydroxygamosifen selection. The transfected pBabe puro KRAS G12V vector has a FLAG tag (*N*-terminal on insert) causing it to migrate slower through the gel compared with the endogenous KRAS and KRAS* (KRAS* w/o FLAG tag was inserted into the pBabe vector). (E) qPCR shows over expression of KRAS in the transfected cell lines.

Figure 3. Design and synthesis of covalent inhibitors

(A) Design of a first series of small molecules and representation of the proposed binding mode of compound 6 in KRAS*. The benzyl moiety is designed to target the engineered hydrophobic pocket. Electrophile warheads are designed to covalently react with C114. Covalent docking of compound 6 in KRAS* showed favorable formation of covalent bond between C114 and the vinyl sulfone moiety (The docking pose is shown in the figure). The ligand is predicted to form hydrogen bond interaction with A146, C114, S17, Y32 residues; and Pi-cation interaction with K117, and K147 residues (docking was conducted by Schrödinger Covalent Docking). (B) Design of a second series of small molecule and representation of the proposed binding mode of compound YZ0468 in KRAS*. Covalent docking of compound YZ0468 in KRAS* showed favorable formation of covalent bond between C114 and the chloroacetyl moiety (The docking pose is shown in the figure). The ligand is predicted to form hydrogen bond interaction with D30, D119, T144, S145 residues; Pi-cation interaction with K117; and Pi-Pi interaction with F28 (C) Combinatorial library design (created by MOE) (libraries of carboxylic acids, aldehydes, acid chlorides, and amines are collected from Sigma-Aldrich). (D) Synthesis route of compound YZ0711

Figure 4. Fluorescent polarization assay shows the competitive displacement of GDP in KRAS* protein

(A) Fluoro-GTP binds in GTP-binding site to yield a high fluorescence polarization (FP) value. With the displacement of small molecule inhibitors, fluoro-GTP shows a small FP value. (B) The structure and activity of selected covalent inhibitors. Covalent inhibitors selectively occupy GTP binding pocket of KRAS*, but not KRAS^{G12V} protein. Compound YZ0571-1, bearing a 2-chloroacetyl chloride moiety, has optimal activity in the fluorescence polarization assay and cell viability assays. (C) The structure and activity of selected covalent inhibitors. Covalent inhibitors selectively displace GDP from KRAS* protein, but not KRAS^{G12V} protein. (D) Compound YZ011 is covalently docked in KRAS* (The docking pose is shown in figure). WaterMap shows the thermodynamic-properties of hydration site around the protein active site. Stable hydration sites ($G < 3$ kcal/mol) are shown in green, significantly unstable hydration sites ($G > 5$ kcal/mol) are shown in red, and moderately unstable sites ($3 \text{ kcal/mol} < G < 5 \text{ kcal/mol}$) are shown in brown. Compound YZ0711 displaces nearly all high-energy waters in the binding site.

Figure 5. YZ0711 covalently labels KRAS* but not KRAS G12V
Liquid chromatography/mass spectrometry of peptide (residues 103–117) in KRAS* (**A**) and KRAS^{G12V} (**B**) Peptide 103–117 in KRAS* was detected to be labeled by YZ0711 at either Lysine or Cysteine residue. No labeling was detected in peptide 103–117 in KRAS^{G12V}.

Figure 6. Compound YZ0711 activity in cell

(A) Desthiobiotin-GTP pull down: treatment of *KRAS** MEFs with 100 μ M YZ0711 for 6hs prior to probing with desthiobiotin-GTP decreases the amount of KRAS that can be pulled down with streptavidin compared to control group. (B) Vi-cell assay: MEFs were treated with 25 μ M YZ0711 or 25 μ M YZ0756, the cell density was measured every 12 hs for 4 days. YZ0711 is more lethal to *KRAS** MEFs than *KRAS G12V* MEFs. (C) *KRAS** was loaded with GTP first, then incubated with DMSO or compound YZ0711 for 15min at room temperature and the reaction was stopped by adding $MgCl_2$ (65 mM). Compound YZ0711 disrupted the binding of *KRAS** with RAF RBD. (D) MEFs were treated with DMSO, 1 μ M, 5 μ M, and 25 μ M YZ0711 for 24hs. The pERK and pAKT levels were decreased in *KRAS** MEFs but not *KRAS G12V* MEFs with the treatment of YZ0711. (E) Co-Immunoprecipitation (IP) of B-RAF with RAS from *KRAS** MEFs cell line after treatment with compound YZ0711 (100 μ M).