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Farnesyltransferase-mediated Delivery of a Covalent Inhibitor Overcomes Alternative Prenylation to Mislocalize K-Ras

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Abstract

Mutationally activated Ras is one of the most common oncogenic drivers found across all malignancies, and its selective inhibition has long been a goal in both pharma and academia. One of the oldest and most validated methods to inhibit overactive Ras signaling is by interfering with its post-translational processing and subsequent cellular localization. Previous attempts to target Ras processing led to the development of farnesyltransferase inhibitors, which can inhibit H-Ras localization but not K-Ras due to its ability to bypass farnesyltransterase inhibition though alternative prenylation by geranylgeranyltransferase. Here we present the creation of a neo-substrate for FTase that prevents the alternative prenlation by geranylgeranyltransferase and mislocalizes oncogenic K-Ras in cells.

Brief Synopsis

K-Ras is the most common oncogene found across all cancers. We describe a chemical work-around of K-Ras's ability to bypass farnesyltransferase inhibitors by creating a neo-substrate of farnesyl transferase to inhibit K-Ras localization.

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Supporting Information. Experimental methods, supplementary figures, Synthetic procedures and characterization.

Author Contributions

C.J.N., G.H., and K.M.S.-designed research, G.H. designed, synthesized and characterized the *in vitro* activity of the molecules C.J.N. performed or supervised the cellular assays. All authors analyzed data and contributed to the manuscript.

Introduction

The ability to associate with membranes is a critical feature for the cellular localization and activity of many proteins[1,2]. Early studies of the Ras oncogene highlighted these features when deletions localized to the C-terminal tail were found to prevent its membrane localization, which in turn prevented the ability of oncogenic Ras mutants from transforming cells[3]. Further work identified the cysteine residue 4 amino acids from the C-terminus that is conserved in all Ras proteins as necessary for their membrane localization[4]. This cysteine along with the 3 terminal amino acids define the CaaX motif, which was found to be sufficient to signal for the covalent attachment of either a geranylgeranyl lipid group by geranylgeranyltransferase (GGTase), or in the case of Ras, a farnesyl group catalyzed by farnesyltransferase (FTase) to the targeted cysteine[2,5–6]. This alkylation is then followed by the cleavage of the 3 terminal amino acids and methylation of the new C-terminal carboxyl group to create a hydrophobic C-terminus suitable for interacting with membranes (Fig. 1 path a)[7–8].

The finding that this post-translational modification was necessary for Ras signaling drove the development of numerous potent peptidomimetic farnesyltransferase inhibitors (FTI) in order to target the roughly 16% of cancers containing activating Ras mutations[9]. These agents showed exceptional activity in H-Ras driven pre-clinical models with robust inhibition of Ras's transforming ability and no obvious dose limiting toxicities[10,11]. However, in clinical trials dominated by patients harboring the more common K-Ras mutations, the inhibitors failed to show any significant benefit over the control arms[12–13].

This un-anticipated failure was largely attributed to several previously unappreciated differences in the processing of the various Ras isoforms. K-Ras4b, the predominant isoform of K-Ras in cells (hereafter referred to as K-Ras), was found to bind to FTase more strongly than H-Ras, which would necessitate a higher drug concentration to effectively block K-Ras farnesylation[14]. Additionally, while H-Ras is solely a substrate of FTase, in the presence of an FTI, K-Ras can be alternatively prenylated by GGTase, providing a cellular bypass mechanism that allows for its proper downstream processing, membrane localization, and signaling (Fig. 1 path b)[14–15]. Several attempts were made to develop either selective GGTase inhibitors that could be used in combination with an FTI or dual inhibitors of FTase and GGTase. Among these were high doses of statins, which prevent the cellular biosynthesis of pre-cursors to farnesyl pyrophosphate and geranylgeranylpyrophosphate, thereby allowing them to act as dual inhibitors. This attempt, along with other dual inhibitor strategies have encountered potential toxicity issues associated with GGTase inhibition that have limited their clinical advancement[1,16].

The failure of farnesyl transferase inhibitors to cause mislocalization of K-Ras led us to wonder if an alternative chemical strategy based on farnesyl transferase could be employed. We envisioned developing an alternative substrate for farnesyl transferase, which could covalently modify the K-Ras C-terminal cysteine. Such a strategy would prevent subsequent processing steps while simultaneously blocking alternative prenylation resulting in the mislocalization of K-Ras to the cytoplasm. Here we describe the design of such neo-substrates for FTase and demonstrate their ability to covalently modify the CaaX motif of K-

Ras in an FTase dependent manner *in vitro*. Cell permeable pro-drugs were shown to mislocalize multiple different oncogenic mutants of K-Ras into the cytoplasm and when combined with statins inhibit oncogenic K-Ras signaling. These results suggest this neo-substrate strategy for blocking K-Ras prenylation may prove to be a viable approach towards inhibiting a broad spectrum of Ras oncogenes.

Results

Design and activity of electrophilic farnesyl transferase inhibitors of K-Ras

In designing a pharmacological strategy for K-Ras inhibition we sought a solution that would circumvent both the issue of K-Ras's high affinity for FTase and its ability to bypass inhibition through GGTase. We hypothesized that if we could irreversibly label the normally farnesylated cysteine of K-Ras with a small molecule it would prevent all forms of prenylation and prevent the subsequent processing and translocation steps, thereby compromising the ability of K-Ras to signal (Fig. 1 path c). Since the C-terminal tail of K-Ras is relatively unstructured with no obvious nearby pockets for a small molecule inhibitor to derive binding affinity, we envisioned harnessing the active site of FTase to deliver the inhibitor to K-Ras.

As a starting point for the design of a neo-substrate for FTase, we analyzed the crystal structure of the farnesyl-competitive FTI **1** bound to FTase and the C-terminal peptide of K-Ras (Fig. 2a)[17]. FTI **1** features a hydroxamate ester and a phosphonate, which together mimic the pyrophosphate moiety of farnesyl pyrophosphate. The most useful observation from the structure was that the hydroxamate nitrogen is only about 6 Å from the target K-Ras cysteine and is oriented in such a way that attaching an electrophile at this position would likely direct it towards the cysteine. However, direct incorporation of an amide-linked electrophile at this nitrogen would result in an unstable N-alkoxyimide structure; thus we made some simplifying adjustments to improve the chemical stability and facilitate the synthesis of multiple analogs, arriving at the general structure **2** (Fig. 2b).

The vinylsulfonamide **3** was the first FTase neo-substrate synthesized to show covalent labeling of recombinant full-length K-Ras in an FTase-dependent reaction, as judged by intact protein liquid chromatography-mass spectrometry (LC/MS). While only labeling 6% of the total K-Ras, it was encouraging that no reaction was observed in the absence of FTase, indicating that the enzyme is in fact promoting the non-natural reaction. The low intrinsic reactivity of **3** in the absence of FTase could potentially be attributed to the unfavorable repulsive interaction between the negatively charged phosphonate and an approaching negatively charged nucleophile. Such interactions would be alleviated by the positively charged active site residues of FTase effectively neutralizing the negatively charged phosphonate.

Switching the electrophile from the vinyl sulfonamide to a propynamide (**4**) did not by itself improve the extent of K-Ras labeling. However, the combination of the propynamide and adding one methylene spacer between the electrophile and phosphonate improved the conjugation to 45% (**5**). While the activity of **5** was encouraging, we were concerned about the nonspecific toxicity of the reactive propynamide electrophile[18]. We considered that we

might fare better with an electrophilic warhead whose reactivity could be enhanced by the active site of FTase. In this regard, epoxides stood out as a group susceptible to Lewis and Brønsted-acid catalysis that could potentially take advantage of the positively charged residues and zinc cofactor present in the active site. In fact, epoxy amide **6** proved to be just such an electrophile with lower uncatalyzed reactivity than **5** and improved FTase-dependent K-Ras labeling (54%) as determined in this assay. A representative LC/MS chromatogram of K-Ras following the reaction with **6** in the presence of FTase is shown in Fig. 2c. Importantly, we were unable to detect covalent labeling of K-Ras in the analogous experiment without FTase using this method. We also explored dicarboxylates as an alternative phosphomimetic group (compound **7**); however, this resulted in compounds with reduced activity compared to **6**. Finally, we prepared as a negative control a desoxy analog of **6** that lacks the epoxide electrophile. As expected, we were unable to detect any labeling of K-Ras with the resulting compound (**8**) using this assay.

One outstanding question was whether neo-substrate **6** could undergo transfer to other protein substrates of FTase in addition to K-Ras. From a therapeutic standpoint, it would presumably be desirable if the neo-substrate showed at least some specificity for K-Ras over other farnesylated proteins. To investigate this, we treated a purified sample of the closely related protein H-Ras under identical conditions using compound **6** and FTase. However, no covalent labeling was detected in this assay (*SI*, Fig. S1), whereas the positive control farnesylation reaction using farnesyl pyrophosphate proceeded as expected. We considered two explanations for the apparent selectivity. First, K-Ras is known to be among the tightest binding and overall most efficient substrates of FTase [19]. It is reasonable to think that this higher efficiency might also carry over to our unnatural neo-substrate. Additionally, previous work examining FTase-catalyzed prenylation reactions of various peptides has shown that certain prenyl donors can show selectivity for one peptide sequence, while others can have inverted selectivity[20–25]. Thus it is possible that our use of K-Ras labeling as a primary screen led us to a neo-substrate that is in fact partially optimized for K-Ras over other farnesylated proteins athough it is unlikely that this degree of specificity would apply to all of the prenylated substrates within a cell.

Naturally, we had some concern surrounding the fact that our neo-substrates retain a farnesyl-like tail, and thus could conceivably still target K-Ras for proper membrane localization. However, we noted that the K-Ras-**6** adduct has an exposed negatively charged phosphonate as well as an α-hydroxy amide resulting from epoxide opening. Upon deeper consideration, it seemed unlikely to us that the K-Ras-**6** adduct would be similar enough to farnesylated K-Ras to undergo the remaining steps of post-translational processing (enzymatic proteolysis and methylation) as well as carrier-promoted trafficking to the membrane.

Neo-substrate 6 Mislocalizes m-Cerulean K-Ras (G12C)

In order to evaluate the cellular activity of the phosphonate containing compounds, we masked the phosphonate moiety of compound **6** and the negative control **8** as a di-pivalate ester pro-drug (referred to as **6*** and **8***). We then evaluated the ability of the cell permeable **6*** and **8*** to mis-localize a dox-inducible mCerulean K-Ras containing the oncogenic

mutation G12C (mCerulean K-Ras (G12C)). A 9h induction of m-Cerulean K-Ras showed that the labeled K-Ras was properly processed and trafficked to the periplasmic membrane (Fig. 3a). Treatment of cells with high doses of the HMG Co-A reductase inhibitor lovastatin, which prevents the cellular synthesis of both the farnesyl-pyrophosphate and geranylgeranyl pyrophosphate substrates thus acting as a dual FTase and GGTase inhibitor, prevented this accumulation at the membrane as did **6***. In contrast, the non-electrophilic inhibitor **8*** had no effect on the localization of K-Ras (Fig. 3a).

In order to quantify the degree of K-Ras mislocalization, we measured the fluorescence intensity of the labeled K-Ras across a linescan spanning from a cell's cytoplasm towards the extracellular space. We then applied a sigmoidal fit to the fluorescent signal that was used to align all scans at the sigmoidal midpoint i.e. the membranes edge. A representative example for cells treated either with DMSO or **6*** is shown in Fig. 3b with the linescan shown as an arrow and the resulting trace highlighted in red. Linescans were generated from 50 cells per condition each shown in gray. The ratio of membrane localized K-Ras to cytoplasmic K-Ras was calculated (representative example in *SI* Fig. S2) demonstrated that **6*** but not the non-electrophilic **8*** is capable of mis-localizing oncogenic K-Ras into the cytoplasm as effectively as the positive control compound lovastatin.

6 Prevents K-Ras bypass of FTase inhibitors and mislocalizes K-Ras

We next evaluated the ability of our inhibitors in the PSN-1 cell line, which contains a G12R K-Ras mutation and has previously been shown to bypass FTase inhibition[26]. Cellular fractionation validated that K-Ras could escape both a peptide competitive, L744,832 (L744), or a farnesyl pyrophosphate competitive, FPT inhibitor III (FPT3), FTI and maintain its proper membrane localization (Fig. 4a). However, targeting both FTase and GGTase using either lovastatin (Lova) or the GGTase inhibitor, GGTI 298 (GG), in addition to the peptide competitive FTase inhibitor (L744+GG) resulted in robust mislocalization of K-Ras to the cytoplasm (Fig. 4a). Similar to the dual inhibitor treatment and consistent with the fluorescently-tagged Ras system, **6*** but not **8*** was able to successfully alter the localization of K-Ras to the cytoplasm (Fig. 4a). This activity was both time and dose dependent (Fig. 4b, c) and was also seen in SW-620 cells, which have a G12V K-Ras mutation (*SI*,Fig. S3). To verify that the cellular activity of neo-substrate **6*** was dependent on FTase as it was *in vitro* and not an artifact of the electrophile, we treated cells with either **6*** alone or in combination with increasing doses of the peptide competitive FTase inhibitor. We hypothesized that the peptide competitive FTase inhibitor would prevent K-Ras from coming into contact with the **6** + FTase complex and allow it to bypass the neo-substrate through GGTase. As shown in figure 4d, this co-treatment strategy was effective in preventing the mislocalization of K-Ras into the cytoplasm, indicating that the cellular ability of **6*** to mislocalize K-Ras is dependent on FTase.

To evaluate the effect of mislocalizing K-Ras from the membrane on MAPK signaling, we probed p-ERK levels in PSN-1 cells after a 24h treatment with a dose response of **6***. While these concentrations were sufficient to mislocalize a detectable amount of K-Ras to the cytoplasm by western blot at this time point, p-ERK levels remained high. This lack of activity could potentially be due to the increased expression of K-Ras that kept the level of

membrane bound K-Ras consistent despite a portion now residing in the cytoplasm (Fig 4b, c, 5a).

We reasoned that the potency of neo-substrate **6*** could be enhanced by reduced competition with the natural substrate, farnesyl pyrophosphate. Statins, which decrease the cellular concentration of farnesyl pyrophosphate, may therefore increase the potency of **6*** by allowing it to more effectively compete for the FTase active site. The co-treatment of cells with 1 μM of Lovastatin plus **6*** was sufficient to inhibit p-ERK signaling (Fig. 5a). The reverse combination experiment also showed that the addition of 10 μM **6*** to a dose response of lovastatin also increased its ability to inhibit p-ERK (Fig. 5b). This increased activity was specific to the electrophilic compound **6*** and not **8*** or the peptide competitive FTase inhibitor (Fig. 5c) and also resulted in an increase in the anti-proliferative ability of **6*** but not **8*** in PSN-1 cells (Fig. 5d).

Discussion

The term “undruggable target” serves as a placeholder until strategies are developed to overcome the unique challenges associated with the molecular recognition of proteins by small molecules that alter their biological output. While considerable effort has focused on how to drug structurally defined protein domains that lack obvious small molecule binding pockets, a less studied category of “undruggable” domains are those with little or no stable tertiary structure. The most notorious example of an undruggable protein in oncology, oncogenic K-Ras, possesses two structural domains that typify each category: the structured G-domain responsible for engaging with effectors and initiating downstream signaling and the flexible C-terminal tail responsible for membrane localization. Recently, the G-domain has been successfully targeted by exploiting the unique chemical reactivity of the oncogenic G12C mutation[27–28]. Here, we developed a chemical strategy to target the flexible C-terminal tail of K-Ras by taking advantage of its known biology to identify an interacting partner that offered a small molecule binding pocket that we could use to deliver our inhibitor in a targeted manner.

Previous generations of inhibitors targeting K-Ras localization focused on inhibiting the function of FTase itself leaving the translated K-Ras CaaX motif available for prenylation by GGTase, which is sufficient for downstream processing and K-Ras localization. By directly targeting the cysteine side chain with a neo-substrate strategy, we were able to overcome this innate resistance mechanism and mislocalize K-Ras to the cytoplasm with a single agent. Other groups have previously tried to mislocalize K-Ras using un-natural farnesyl derivatives, but the resulting agents were either non-cell permeable, or required high concentrations of an alcohol precursor that relied on subsequent cellular processing to form the necessary un-natural farnesyl pyrophosphate[29–31]. An alternative strategy to alter the localization of K-Ras by inhibiting the interaction between fully processed K-Ras and PDE-δ has also been described and has been shown to successfully alter oncogenic K-Ras signaling[32,33].

While neo-substrate **6*** was capable of inhibiting the proper cellular localization of K-Ras in cell lines containing oncogenic variants it is likely that it would affect other FTase substrates

within the cell including wt K-Ras. This lack of mutant K-Ras vs. wt K-Ras specificity may limit the ultimate therapeutic index (TI) of the neo-substrate strategy. One interesting feature of mutant K-Ras is the preferential use of splice variant −4B compared to WT K-Ras which commonly contains splice variant −4A. The two splice variants result in differential C-terminal tail sequences where farnesylation occurs. Splice variant −4B has been correlated with downregulation of non-canonical Wnt/Ca2+ signaling and more aggressive metastatic potential (cite: 1. Wang, M.-.T. *et al.* K-Ras Promotes Tumorigenicity through Suppression of Non-canonical Wnt Signaling. *Cell* **163,** 1237–1251 (2015).). The K-Ras-4A sequence is closely related to the H-Ras C-terminal tail 9 (CITATION: Stephen, A. G., Esposito, D., Bagni, R. K. & McCormick, F. Dragging Ras Back in the Ring. *Cancer Cell* **25,** 272–281 (2014).). Since neo-substrate **6** distinguishes between K-Ras-4B and H-Ras, there may be some degree of selectivity between oncogenic K-Ras and WT K-Ras based on differential splice variants.

The clinical failure of FTI's led to an abandonment of the therapeutic area. However, there is renewed interest in FTI's that although lacking in efficacy against K-Ras have been verified to be potent inhibitors of H-Ras and are currently being tested in clinical trials of patients harboring tumors with oncogenic H-Ras mutations[34]. These may soon lead to the long awaited breakthrough of Ras inhibitors, which our data suggests could eventually be expanded to a broader patient population harboring K-Ras mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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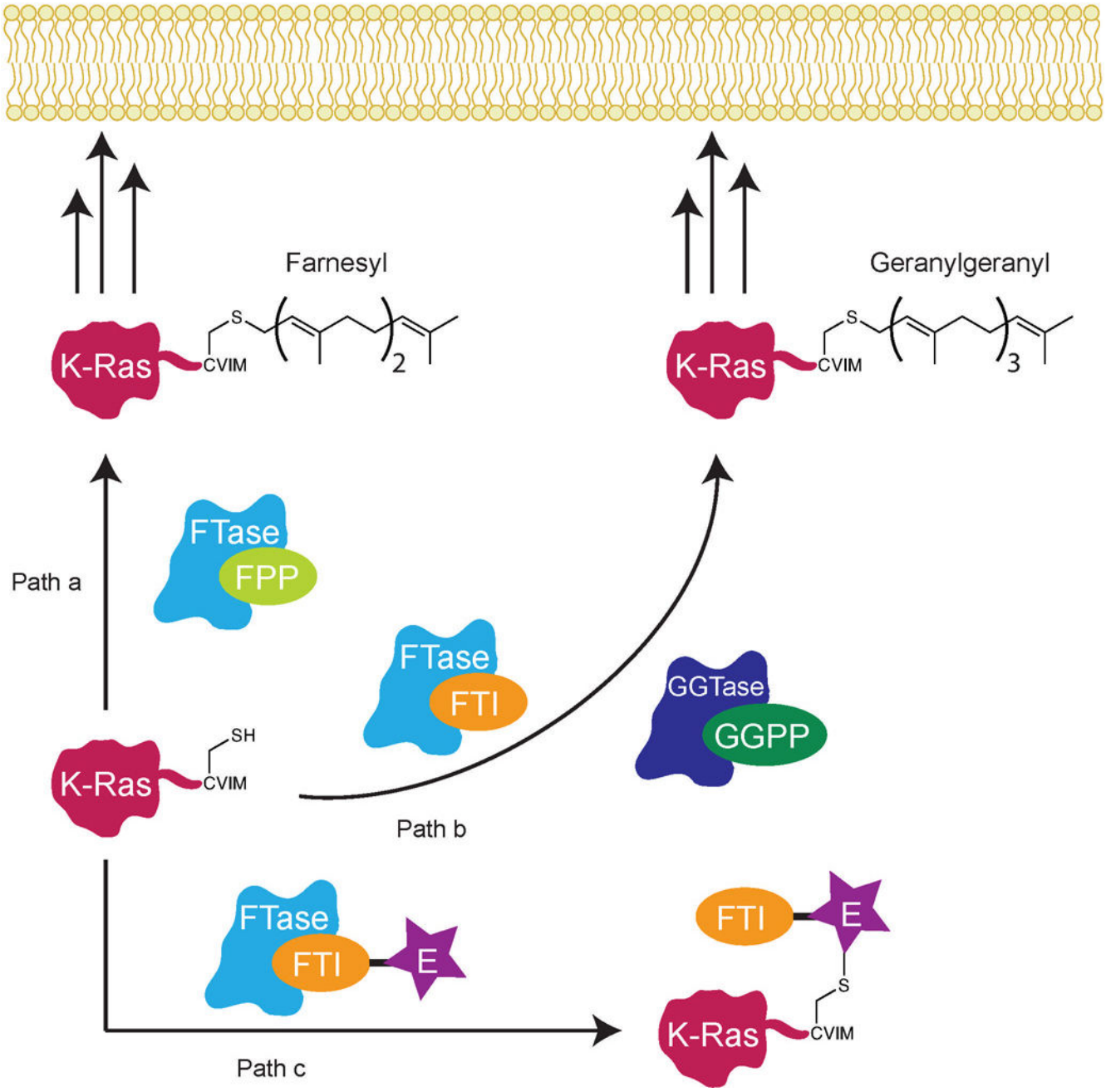

Figure 1.

Proposed method to overcome innate resistance to inhibition of K-Ras post translational modifications. **Path a** depicts the normal course of K-Ras post translational modification by FTase, which leads to membrane localization after several subsequent steps. **Path b** shows the scenario when the active site of FTase is blocked with an FTI: a second prenylation enzyme, GGTase, can catalyze geranylgeranylation of K-Ras, which still leads to properly localized and functional K-Ras. **Path c** represents our proposed approach: proper incorporation of a cysteine-reactive chemical electrophile (E) into an FTI could allow FTase

to irreversibly transfer a covalent inhibitor to the normally prenylated cysteine, thereby blocking any other modification at the site and causing mislocalization of K-Ras.

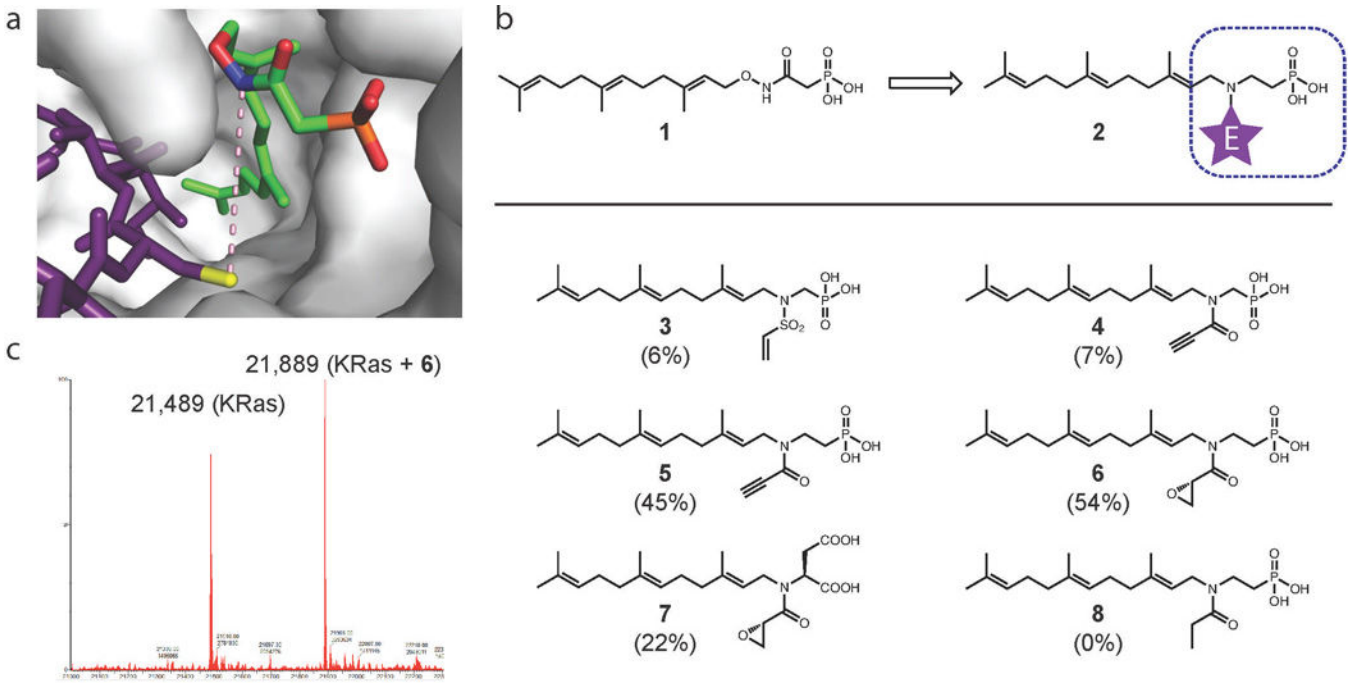

Figure 2.

Design and *in vitro* activity of electrophilic FTI. **a.** The ternary crystal structure of a known reversible inhibitor **1** (green) complexed with FTase (gray surface) and the c-terminal tail of K-Ras (purple) (PDB:1D8D) suggests a possible site of attachment for an electrophile on a nitrogen in the inhibitor (blue) towards the target K-Ras cysteine (yellow). **b.** The known reversible inhibitor **1** was adapted into the general scaffold **2** to facilitate incorporation of an electrophile (E) and variation of the electrophile-charged phosphonate "warhead" (dashed box). The structures of electrophilic FTI **3**–**7** and non-electrophilic control **8** are shown along with their percent modification of K-Ras in an *in vitro* LC/MS assay (20 μM inhibitor, 10 μM K-Ras, 10 μM FTase, 4 hrs). **c.** Representative LC/MS chromatogram of the reaction between K-Ras and inhibitor **6**. Peaks have been re-labeled for clarity.

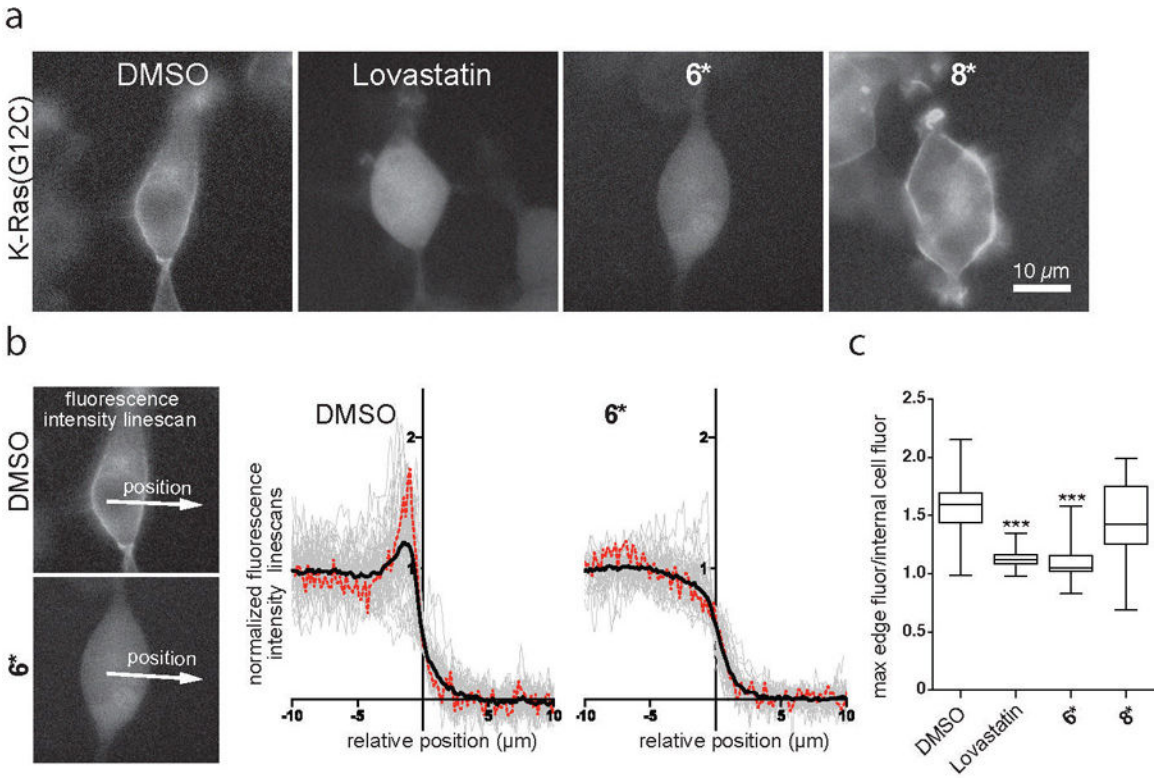

Figure 3.

6* displaces K-Ras(G12C) from the plasma membrane. **a**. Representative images showing the localization of mCerulean-K-Ras (G12C) in 293 cells after 9 hours of treatment with DMSO, Lovastatin, **6*** or **8*** (25μM). Bar=10μm. **b**. Fluorescence intensity linescans were measured across the edge of cells as depicted by the arrows overlaid on the fluorescence images. Aligned and normalized intensity linescans (see Materials and Methods) are plotted for cells treated with DMSO or **6*** (n=50, gray lines). The average linescan for each population (black line) and linescans corresponding to the cells shown on the fluorescence images (dashed red line) are highlighted in the graphs. **c**. The ratio of the maximum edge fluorescence to internal cellular fluorescence was calculated for each cell (see Materials and Methods) and summarized in box and whiskers plots for each population of cells (n=50). Whiskers correspond to min/max values. Statistically significant differences between DMSO- and drug-treated cells were calculated using the Mann-Whitney test (***p<0.0001).

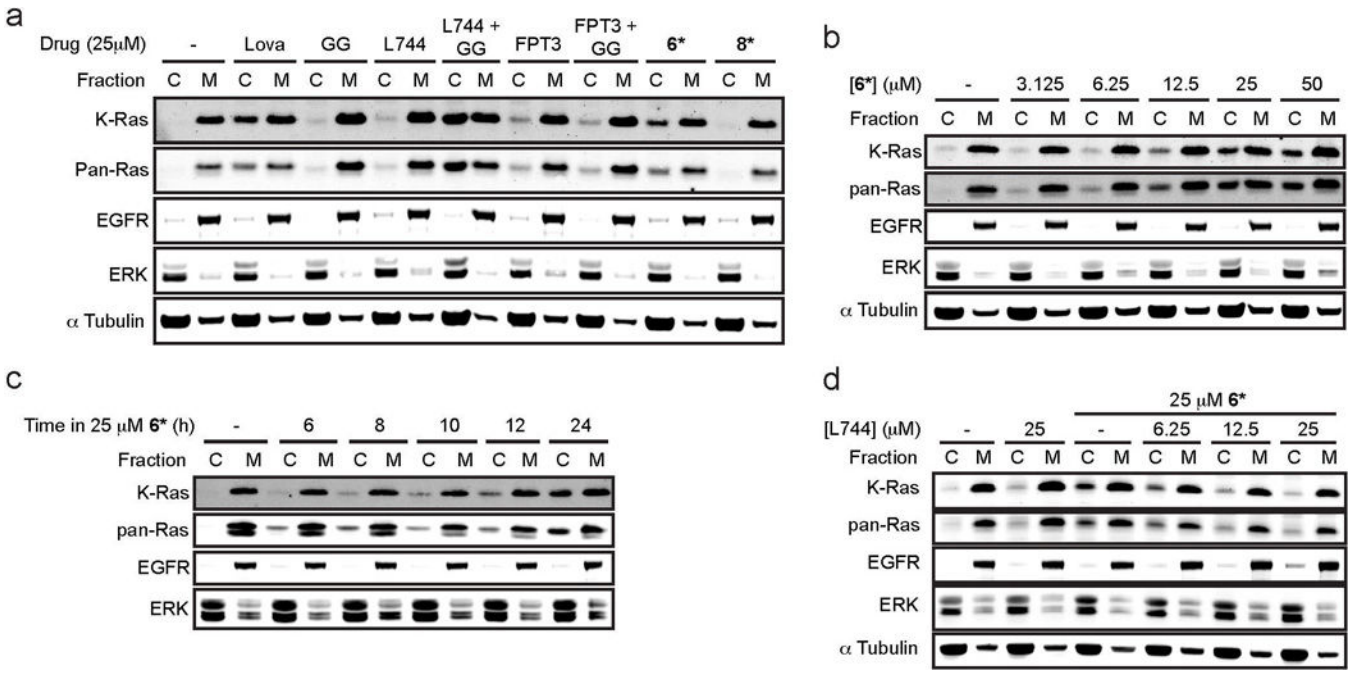

Figure 4.

6* mislocalizes oncogenic K-Ras into the cytoplasm. **a**. PSN-1 cells (K-Ras G12R) were treated with the indicated inhibitors for 24 h and the lysates were separated into cytoplasmic (C) and membrane (M) fractions. K-Ras can bypass both the peptide competitive and farnesyl pyrophosphate competitive FTase inhibitors through GGTase in order to maintain its proper localization. In contrast, **6*** induces the mislocalization of K-Ras to the cytoplasm. Lova = Lovastatin, inhibits FTase and GGTase by blocking cellular synthesis of their activated prenylation substrates, GG = GGTI 298, peptide competitive GGTase inhibitor, L744 = L744,832 peptide competitive FTase inhibitor, FPT3 = FPT III, farnesyl pyrophosphate competitive inhibitor, the pro-drug version of **1. b. 6*** inhibits the membrane localization of K-Ras in a dose dependent manner at 24h. **c**. The mislocalization of K-Ras by **6*** is time dependent. **d**. Cellular fractionation of PSN-1 cells treated with either L744, **6***, or **6*** in the presence of increasing concentrations of L744 show the cellular activity of **6*** is dependent on K-Ras binding to FTase.

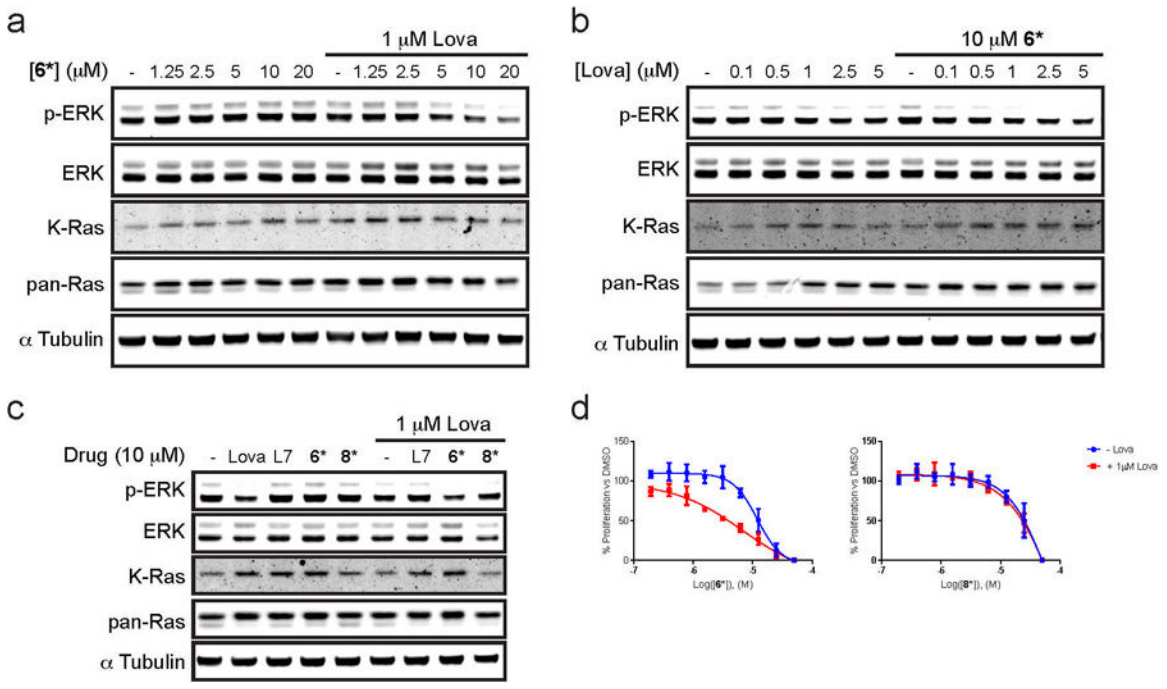

Figure 5.

6* in combination with statins can inhibit K-Ras signaling and proliferation. **a-c**. PSN-1 cells were treated with the indicated inhibitors for 24h and whole cell lysates were analyzed by the specified antibodies. **d**. 72h proliferation of PSN-1 cells against a dose response of either **6*** or **8*** +/− 1 μM Lova (mean ± S.D., n=2).