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An inhibitor of the pleckstrin homology domain of CNK1 selectively blocks the growth of mutant KRAS cells and tumors

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Abstract

Cnk1 (connector enhancer of kinase suppressor of Ras 1) is a pleckstrin homology (PH) domain-containing scaffold protein that increases the efficiency of Ras signaling pathways, imparting efficiency and specificity to the response of cell proliferation, survival, and migration. Mutated KRAS (mut-KRAS) is the most common proto-oncogenic event, occurring in approximately 25% of human cancers and has no effective treatment. In this study, we show that selective inhibition of Cnk1 blocks growth and Raf/Mek/Erk, Rho and RalA/B signaling in mut-KRAS lung and colon cancer cells with little effect on wild type (wt)-KRAS cells. Cnk1 inhibition decreased anchorage-independent mut-KRas cell growth more so than growth on plastic, without the partial “addiction” to mut-KRAS seen on plastic. The PH domain of Cnk1 bound with greater affinity to PtdIns(4,5)P₂ than PtdIns(3,4,5)P₃, and Cnk1 localized to areas of the plasma membranes rich in PtdIns, suggesting a role for the PH domain in the biological activity of Cnk1. Through molecular modeling and structural modification, we identified a compound PHT-7.3 that bound selectively to the PH domain of Cnk1, preventing plasma membrane co-localization with mut-KRas. PHT-7.3 inhibited mut-KRas, but not wild type KRas cancer cell and tumor growth and signaling. Thus, the PH domain of Cnk1 is a druggable target whose inhibition selectively blocks mutant KRas activation, making Cnk1 an attractive therapeutic target in patients with mut-KRAS-driven cancer.

Keywords

mutant KRAS; CNKSR1; PH domain; inhibitor

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Introduction

Cnksr1 (connector enhancer of kinase suppressor of Ras 1) hereafter called Cnk1, is a multidomain scaffold protein important for cell proliferation, survival, and migration (1,2). Protein scaffolds assemble components of signaling pathways in a way that increases their interaction and prevents their inactivation, thus imparting efficiency, sensitivity and specificity to the ultimate signaling response (2–4). Cnk1 has been reported to act as a scaffold for a number of Ras and Rho GTPase family members, while translocating its binding partners to cell membranes where signaling is initiated (4–7). Cnk1 is a scaffold for the Ras/Raf/Mek/Erk signaling cascade (3,8), possibly as part of a cell membrane Ras signaling nanocluster (9,10). Inhibiting *Drosophila* Cnk has been reported to block Ras1 signaling by disrupting a complex between Ras1 and Raf (11).

Point mutation of the *KRAS* gene (mut-*KRAS*) is the most common proto-oncogenic event in human cancer, and is found in approximately 25% of human cancers with highest levels in pancreatic, colon cancer, and lung adenocarcinoma (12). Mut-KRas activates downstream signaling that ultimately leads to the mut-KRas phenotype of altered proliferation, anchorage independent growth, invasion and tumorigenesis (13). Mut-KRas is a particularly insidious oncogene because it not only drives cancer growth but also overrides the effects of molecularly targeted therapies (14). The difficulty of inhibiting mut-KRas has led to attempts to target mut-KRas downstream effector pathways but such agents have displayed a narrow therapeutic window impeding adequate inhibition of pro-oncogenic signals (15). Direct inhibitors of mut-KRas are in development (16,17) but currently there no effective therapy for mut-KRas tumors.

We were interested to find whether inhibiting Cnk1 would block KRas in mammalian cells. Cnk1 has a phosphoinositide (PtdIns) lipid binding pleckstrin homology (PH) domain, and is found localized to areas of the plasma membranes rich in PtdIns (18) suggesting a role for the PH domain in the biological activity of Cnk1. We have previously shown that the PH domains of signaling proteins can be selectively inhibited with small molecules (19), and we therefore explored whether inhibiting the PH domain of Cnk1 might be a way to inhibit mut-KRas activity. Through molecular modeling and structural modification we have identified a small molecule probe compound that binds selectively to the PH domain of Cnk1 preventing plasma membrane co-localization with mut-KRas, and having the ability to inhibit mut-KRas, but not wild type KRas cancer cell and tumor growth.

Materials and Methods

Tissue culture

Mut-KRas MiaPaCa-2 pancreatic cancer cells, M27 MiaPaCa-2 with both mut-*KRAS* mutant alleles deleted (20), mut-KRas HCT-116 colon cancer cells, and HKK2 HCT-116 with its single mut-*KRAS* allele deleted (21), were provided by Dr. Natalia Ignatenko, University of Arizona, Tucson, AZ. NSCLC cell lines were obtained from Dr. John Minna UT South Western, Dallas, TX (Table S1). All cell lines were routinely tested to be mycoplasma free and the identity of each line authenticated before study, and 2 month intervals while in culture, by the Genomics Shared Resource at SBP.

Cell transfection

Studies were conducted using SmartPool siRNA (Dharmacon, Lafayette, CO). A validation study (Figure S1) was conducted using CNK1 siRNAs from a second manufacturer (Qiagen, Valencia, California). Total siRNA concentration was kept at 40 nM for single or multiple siRNA combinations. Knockdown efficiency was determined by Western blotting of cell lysates 72 hours post transfection.

Western blotting

Cells for Western blotting were grown in RPMI medium with 10% FBS for 24 hr. Primary rabbit monoclonal antibodies used for Western blotting were anti: Erk, Egfr, Mek1/2, c-Raf, phospho-Akt Ser473, phospho-ErkThr202,Tyr220, phospho-EgfrTyr1068, and phospho-Mek1/2Ser217/221 (Cell Signaling Technology, Danvers, MA), Cnk1 (Abcam, Cambridge, MA), RalA, RalB, and phospho-c-RafSer338/Tyr340 (EMD Millipore, Billerica, MA), and KRas mouse antibody (Novus Biologicals, Littleton, CO). RalA, RalB, Rho and Ras family GTP activation kits were from EMD Millipore (Billerica, MA) and were used according to manufacturer instructions.

Cell proliferation assays

To measure 2D growth on plastic cells were treated for 24 hr with non-targeting siRNA or si*CNK1*, replated and 72 hr later, or after incubation with small molecule inhibitors for 72 hr, growth measured using the XTT Cell Viability Assay (Biotium, Hayward, CA). For 3D spheroid growth 20,000 HCT-116 colon cancer cells or wt-KRas HCT-116 HKH2 cells were treated for 24 hr with non-targeting siRNA or si*CNK1*, and allowed to form spheroid clusters for 48 hr under non-adherent conditions in a hanging drop of media. The spheroids were then transferred to a non-adherent 3D-nanoculture plate (Scivax, Sunnyvale, CA) and volume calculated by the modified ellipsoidal formula (22). Anchorage independent growth in soft agarose employed a modification of a previously published 96 well method (23).

Reverse Phase Protein Assay

Reverse phase protein array (RPPA) was carried out on cell lysates by MD Anderson Cancer Center Functional Proteomics RPPA Core Facility using a 208 protein/phosphoprotein cell signaling and cell cycle antibody panel as previously described (24).

Molecular modeling

Comparative modeling was used to construct a homology model of the PH-domain of Cnk1 using the published structures of 4 human PH-domains with high homology to Cnk1 PDB codes: 1U5D, 1U5F, 1U5G and 1UPQ. Using proprietary modeling algorithms and state of-the-art commercial drug discovery software and ligand-based approaches we screened an *in silico* library of over 3 million compounds and identified lead compounds. For further details see Supplemental Methods S1. Pharmacological properties of the modeled agents used for selection included Log P, metabolic and mutagenic features, oral absorption, hERG and Caco-2 scores.

Surface plasmon resonance spectroscopy for PH domain binding

The PH domain of Cnk1 and other signaling protein PH domains were expressed as fusion proteins with glutathione S-transferase (Gst) at the N-terminus. Analysis of small molecule binding used surface plasmon resonance (SPR) spectroscopy on a Biacore T200 (GE Healthcare) with a CM5 sensor chip and Gst capture kit. For further experimental details of the SPR method see Supplemental Methods 2.

Compound synthesis

For chemical structures see Table 1 and Table S2, and for synthetic methods see Table S2 Schemes S1 and SII.

Confocal laser scanning microscopy

Confocal laser scanning microscopy and fluorescence lifetime imaging microscopy (FLIM) were carried out as described in Supplemental Methods 3.

Approximately 10^7 A549, H441 and H1975 NSCLC cells in log cell growth were suspended each in 0.2 mL PBS and injected subcutaneously into the flank of female *NOD-SCID* mice with 10 mice per group. When the tumors reached 50 to 150 mm³ daily dosing was begun with PHT-7.3 (PHusis Therapeutics, La Jolla, CA) at 200 mg/kg ip, or erlotinib at 75 mg/kg po (25), or trametinib at 0.3 mg/kg po (26). Tumor volumes based on twice a week caliper measurements were calculated as previously described (22). Mouse body weight was measured weekly. All animal experiments were approved by SBP IACUC.

Results

CNK1 inhibition selectively inhibits mut-KRas dependent growth and signaling in a panel of cell lines

We first examined the growth of wt-KRas and mut-KRas cell lines after KRas and Cnk1 siRNA knockdown. We used a panel of non-small cell lung cancer (NSCLC) cell lines grown in 2D on plastic, and showed that treatment with siRNA to KRAS gave only a small inhibition of the growth of wt-KRas cells, and inhibited the growth of some but not all mut-KRas cells (Figure 1A, Figure S1). That not all mut-KRas cells are growth inhibited by si*KRAS* (i.e. addicted to KRAS) has been previously reported for 2D culture (27,28). When we used si*CNK1* we saw no growth inhibition of wt-KRas NSCLC cells but marked inhibition of mut-KRas cells although without the pattern of addiction seen with si*KRAS*. siRNA knockdown of KRas and Cnk1 protein of >80% maximal by 72 hr was confirmed for a number of cell lines (Figure S2). We saw no difference in the effects of Cnk1 inhibition in cells with various KRas or p53 mutations (Table S1). We next studied the effect of KRas and Cnk1 knockdown on KRas downstream signaling, and found minimal effects of KRas knockdown in wild type NSCLC and HKH2 colon cancer cells, while in mut-KRas cells there was upregulation of p-Akt, p-Egfr, p-Erk, probably involving negative regulatory loops (29) but otherwise only modest cell line dependent effects on KRas signaling (Figures 1B and 1C). Others have also found that KRas knockdown has relatively small and cell line dependent effects on KRas signaling (13,27,30). Levels of RalA/B, pan-Rho-GTP and pan-

Ras-GTP were decreased in mut-KRas HCT-116 colon cancer cells, but pan-Rho-GTP was increased and only Ral/B decreased in isogenic wt-KRas HKH2 cells (Figures 1C and S3).

It has previously been demonstrated that the effects of mut-KRas inhibition are strongly enhanced in anchorage independent culture (31). To determine if Cnk1 knockdown phenocopied that effect of mut-KRas knockdown we tested the anchorage independent growth of HCT-116 colon cancer cells by hanging drop spheroid formation (Figure 1D). HKH2 cells lacking the mutant KRAS allele showed no growth inhibition in standard 2D culture, but had a >90% growth arrest in spheroid culture, as previously reported for anchorage independent culture (21). The growth of HCT-116 cells treated with siCNK1 was also markedly inhibited in spheroid culture. Thus, CNK1 inhibition leads to selective growth inhibition of mut-KRas NSCLC cells with little effect on wt-KRas cell growth, and without the pattern of KRAS addiction seen with inhibition of mut-KRas. Inhibition of KRas downstream signaling by siRNA to KRas and Cnk1 was modest, and variable depending on the cell line, while inhibition of RalA/B-GTP, pan-Rho-GTP and pan-Ras-GTP was also seen when Cnk1 was inhibited.

We next studied the effect of treatment with si*KRAS* or si*CNK1* on the cell cycle. There was no change in the cell cycle distribution of HKH2 cells lacking the mutant KRAS allele, while parental HCT-116 cells retaining mut-KRas showed an increase in cells in the G1 phase to levels identical to the HKH2 cells (Figure 2A). Notably, G1 cell cycle arrest is the reported phenotype in MEF's lacking any Ras isoform (32). Noteworthy is that we found that CRISPR knockout of CNK1 in mut-KRas cells was lethal and prevented clonal outgrowth. We also studied the effects on cell growth of expression of wild type full length Cnk1, or Cnk1-EEALAN with a mutated PH domain making it unable to bind PtdIns (Figure 2B, Figures S4A and S4B). While no effect was observed on the growth of wt-KRas cells, expression of full length Cnk1 resulted in inhibition of the growth of mut-KRas cells, almost to levels seen with Cnk1 knockdown. This is a predicted effect for a scaffold protein where its expression at concentrations in excess of its binding partners will sequester these partners in incomplete complexes leading to combinatorial inhibition of signaling activity (33). The expression of Cnk1-EEALAN unable to bind PtdIns similarly inhibited cell growth, as did expression of just the Cnk1 PH domain, presumably by displacing membrane bound Cnk1. A similar effect has been previously reported for the Cnk1 PH domain on RhoA signaling (6). The use of a myristoylated Cnk1, often used to enhance nonspecific membrane attachment of PH domain proteins (34) (Figure S4C), also showed inhibition of cell growth consistent with the need for Cnk1 to be attached to areas of membrane with high PtdIns. Thus, the level of Cnk1 in cells could be critical for cell growth, with either too much or too little inhibiting cell growth, which would be consistent with it acting as a scaffold protein, while PH domain competition or non-selective membrane attachment through myristoylation is also inhibitory.

When we used fluorescent protein tagged human Cnk1-GFP and KRas-RFP in HEK293 cells, Cnk1 and KRas were seen in the cytoplasm and at the plasma membrane, but co-localized only at the plasma membrane (Figure 2C). When transfected with mut-KRas(G12D)-RFP the cells displayed an altered morphology with many plasma membrane extensions consistent with KRas induced transformation, and co-localization of Cnk1 and

mut-KRas at discrete areas of the plasma membrane at the ends of the membrane protrusions. FLIM spectroscopy showed no change in fluorescence lifetime of cells co-transfected with Cnk1-GFP and wt-KRas-RFP compared to Cnk1-GFP transfection alone (Figure 2D). However, a reduction in the fluorescence lifetime was observed in cells co-transfected with Cnk1-GFP and mut-KRas-RFP. These results suggest there is a close proximity (<10 nm), or physical binding of Cnk1 with mut-KRas, but not wt-KRas. Thus, Cnk1 localizes in close proximity with mut-KRas, and Cnk1 knockdown copies the growth inhibitory effects of mut-KRas deletion independent of the type of KRas mutation, with more pronounced inhibition in 3D culture, while without a significant effect on wt-KRas cell growth.

To determine if Cnk1 knockdown replicated the selective deletion of the mut-KRAS allele we used RPPA with an array of 208 signaling and other proteins, in 16 mut-KRas NSCLC cell lines with various activating mutations in KRas treated with either a non-targeting siRNA control or siRNA to *CNK1* (Figure 2E). We found only three proteins that were significantly down regulated across these lines after Cnk1 knockdown, phosphorylated Rb1 serine 807, Cyclin B1 and CDK1 (Figure 2F). The data suggests that Cnk1 inhibition abrogates the ability of mut-KRas to drive Rb1 phosphorylation allowing Rb1 to engage E2F, a transcriptional promoter of cell cycle progression, and repress its activity. The inability of these cells to progress from the G1 phase to the S phase and eventually into mitosis is evident by reduced levels of Cyclin B1.

Cnk1 PH domain binding compounds disrupt co-localization of Cnk1 and mut-KRas

Cnk1 serves as a scaffold protein and has no catalytic activity to disrupt but does have a PH domain that may be responsible for appropriate localization to the plasma membrane through PtdIns binding in the Cnk family of proteins (35). Mut-KRas is also reported to interact with PtdIns through a positively charged lysine region in its hypervariable region (36). We first measured the affinity of the Cnk1 PH domain for PtdIns(3,4)P₂, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, and found that Cnk1 bound PtdIns(4,5)P₂ with the highest affinity (K_d = 0.18 μM) (Figure 3A). As no crystal structure exists for the Cnk1 PH domain, we constructed a homology model of the PH-domain of Cnk1 and used *in silico* screening of a library of commercially available compounds to identify possible binding ligands (Figure 3B). One of the compounds, PHT-7.0, was found to bind to the PH-domain of Cnk1 with K_d = 10.9 μM (Table 1). Using PHT-7.0, a series of analogues with modifications of the ester side arm were designed, modeled and synthesized to screen for compounds with increased potency and desirable pharmacokinetic properties (Table S1 and Table S2 Schemes SI and SII). From a number of compounds generated and screened, PHT-7.3, a dioxane analog, was found to have the highest binding affinity to the Cnk1 PH domain K_d = 4.7 μM (Figure 3C). Additionally, PHT-7.3 showed selective binding to the PH domain of Cnk1 compared to the PH domains of Pdk1, Btk, Akt1, and Plekha7 (Table S3), and displaced PtdIns(3,4,5)P₃ from binding to the PH domain of Cnk1 (Figure 3D and Table S4). To study if PH domain binding compounds blocked co-localization of Cnk1 and mut-KRas in cells, confocal microscopy was performed in cells treated with PHT-7.0 or PHT-7.3. Both PHT-7.0 and PHT-7.3 blocked the co-localization of Cnk1 and mut-KRas at the cell plasma membrane (Figure 3E) and FLIM lifetime plots confirmed that both compounds inhibited the binding of

Cnk1 to mut-KRAS (Figure 3F). Thus, a series of small molecule probe compounds have been identified that bind to the PtdIns(3,4,5)P₃ binding pocket of the PH domain of CNK1 and prevent the interaction between Cnk1 and mut-KRas, but not wt-KRas at the plasma membrane.

The Cnk1 PH domain binding compound PHT 7.3 inhibits the proliferation of mut-KRas cells *in vitro* and elicits signaling changes similar to CNK1 inhibition

PHT 7.0 and analogs 7.3 and 7.10 (Table 1) were tested in a small group of mut-KRas cells and found to inhibit cell proliferation (Figures S5). PHT-7.3 was the most consistently active (Figure S6), and was chosen for further study. PHT-7.3 did not inhibit the growth (IC₅₀ > 100 μM) of normal mouse or human normal fibroblasts, pancreatic duct, lung, colon or myoblast cells (Table S5). When tested against a panel of NSCLC cell lines, wt-KRas cell line 2D growth was not inhibited except for two lines H1437 and H2023. Five mut-KRas cell lines also showed no inhibition of growth in 2D culture, while 7 were inhibited (Figure 4A). Growth inhibition was not related to levels of Cnk1 protein in cells (Figure S7). However, using 3D anchorage independent growth all the mut-KRas lines were sensitive to PHT-7.3 with IC₅₀s averaging 4.4 μM, and as low as 0.3 μM (Figure 4B). Wt-KRas cell growth was still not inhibited by PHT-7.3, except for H1437, which has activated KRas signaling due to a MAP2K1 mutation (37), and H2023. Cell migration was also inhibited (Figure S8). Analysis of downstream K-Ras signaling in a panel of mut-KRAS cells recapitulated the increase in p-Akt, p-Egfr, and pErk seen with deletion and siRNA knockdown of Cnk1, and with decreased RalB and Rho activation (Figures 4C and D, and S9). Thus, treatment with PHT-7.3 mimics the growth and signaling effects of deletion of mut-KRas and Cnk1 knockdown, and displays a preferential growth inhibition of mut-KRas cell lines over wt-KRAS lines that is enhanced in anchorage independent conditions.

Antitumor Activity

PHT-7.3 demonstrated the desirable qualities of binding to the PH domain of Cnk1, selective inhibition of mut-KRas NSCLC cell growth and signaling, good *in vivo* pharmacokinetic properties, and was selected as a probe compound for further *in vivo* evaluation. We tested PHT-7.3 for antitumor activity, and when dosed daily at 200 mg/kg ip for up to 20 days, PHT-7.3 exhibited cytostatic antitumor activity in the mut-KRas(G12S) A549 xenograft and mut-KRasG12V H441 xenograft (Figures 5A and 5B) but not in the wt-KRas H1975 NSCLC xenograft (Figure 5C). Measuring the activity of downstream pathways revealed increased levels of p-Egfr (Figure 5D) and down-regulation of activated RalB and Rho signaling (Figure 5E). PHT-7.3 administered in combination with daily erlotinib 75 mg/kg or trametinib 0.3 mg/kg gave additive antitumor effects with both agents, and with erlotinib there was a tumor regression for the duration of treatment (Figure 6A and 6B). There was no loss of body weight with PHT-7.3 treatment (Figure S10). Thus, *in vivo* antitumor studies with PHT-7.3, alone and in combination with erlotinib or trametinib, show inhibition of mut-KRas tumor growth but little effect on wt-KRas tumor growth.

Discussion

Our studies show that the scaffold protein Cnk1 closely colocalizes with mut-KRas at the plasma membrane, and a small molecule inhibitor of the PH domain of Cnk1, PHT 7.3, prevents the colocalization, decreases Raf/Mek/Erk signaling, and causes arrested mut-KRas but not wt-KRas, cell and tumor growth. Cnk1 has also been reported to regulate Rho activity by binding to constitutively active RhoA(G14V) and RhoH, and the Rho GEFs MLK2/3 and Mkk7, leading to activation of the JNK MAP kinase pathway (5–7). Rac which also acts through Jnk signaling is similarly regulated by Cnk1 (7), as is RalGDS a GEF for RalA/B (6,8), and IPECF1 a GEF for Arf (30). Consistent with these reports we found the inhibition of Cnk1 decreased levels of active pan-Rho-GTP and pan-Ras-GTP and RalA/B-GTP, but we did not study other pathways that might be affected. There are also reports that Cnk1 regulates Akt activity driving cell proliferation through inhibition of FoxO (38), and cell invasion through activation of Nf- κ b (39). It is not clear at this time the role these other Cnk1 dependent pathways play in regulating KRas signaling and cell growth.

An important consideration is why the growth of wt-KRas cancer cells is not sensitive to Cnk1 inhibition? It may simply be that wt-KRas is not important in cells with other cancer drivers, and indeed we saw minimal change in Raf/Mek/Erk and Akt signaling, or cell growth in wt-KRas cells when KRas was inhibited. Although we saw that Cnk1 was localized at the plasma membrane in proximity to wt-KRas, FLIM studies showed it did not directly engage wt-KRas, as it did mut-KRas. It is also possible that the short life time of active wt-KRas-GTP precludes assembly of a Cnk1 scaffold signaling complex, unlike with constitutively activated mut-KRas-GTP.

The Cnk1 inhibitor we developed, PHT-7.3, blocked the growth of some but not all mut-KRas cells in 2D culture, but was much more potent at inhibiting anchorage independent growth in all mut-KRas cell lines tested. A secondary finding of these studies is that the phenomenon of Ras oncogene “addiction” where some mut-KRas cells are dependent on the oncogene for their growth, while others are not (25,26), was only seen under 2D growth conditions, and not seen with anchorage independent growth of mut-KRas cells. Anchorage independent growth is a hallmark phenotype of cancer cell growth but is often neglected when studying mut-KRas (30).

Cnk1 possesses a PH domain that we found preferentially binds PtdIns(4,5)P₂ (K_d = 0.18 μ M). It has been reported that Cnk1 localizes to plasma membranes rich in PtdIns (35) suggesting to us a role for the PH domain in directing the loaded Cnk1 scaffold to the same plasma membrane vicinity as mut-KRas, which is also found in regions of high membrane PtdIns (36). The PH domain of Cnk1 is known to be important for ability to interact with the active form of RhoA to stimulate its transcriptional rather than cytoskeleton effects (5,6). Using our experience of small molecule PH domain inhibitors for signaling kinases (19,40,41), we developed PHT-7.3 a small molecule probe compound that can displace bound PtdIns from the PH-domain of Cnk1 and block the membrane association of Cnk1 with mut-KRas. PHT-7.3 also inhibits mut-KRas and Rho signaling, and selectively blocks mut-KRas cell and tumor growth, but not that of wt-KRas cancer cells, nor normal human and rat fibroblasts, myoblasts and lung and colon epithelial cells. An unanswered question is

why PHT-7.3, which binds the PH domain of Cnk1 with micromolar affinity, can inhibit mut-KRas cell growth at sub-micromolar concentrations. Discrepancies between biochemical and cell growth inhibitor concentrations are well documented for kinase inhibitors and other drugs, and have been attributed to clustering and stacking of binding proteins at specific areas of the cell surface membrane (42–44). This could happen for PHT-7.3, or being a lipophilic molecule it could concentrate in, or close to the cell membrane aqueous interface, which is a very different environment compared to aqueous phase biochemical measurements of compound binding.

In summary, our studies have found that the scaffold protein Cnk1 is found in close proximity to mut-KRas at the cell plasma membrane through binding of Cnk1's PH domain, and Cnk1 inhibition prevents mut-KRas cell growth, particularly anchorage independent growth, exceeding the effects of KRas inhibition. In addition we found that although Cnk1 colocalizes with wt-KRas at the plasma membrane, it is not tightly associated, and Cnk1 inhibition does not inhibit wt-KRas cell growth or downstream signaling. Through molecular modeling and structural modifications we identified a compound PHT-7.3 that binds selectively to the PH domain of Cnk1 preventing plasma membrane binding with mut-KRas, and with the ability to inhibit mut-KRas, cancer cell and tumor growth and signaling, but not that of wt-KRas. Thus, the PH domain of Cnk1 is a druggable target whose inhibition selectively blocks mutant KRas activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Findings identify a therapeutic strategy to selectively block oncogenic KRas activity by inhibiting the PH domain of Cnk1, which reduces its binding to cell membranes and decreases the efficiency of Ras signaling and tumor growth.

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Figure 1. CNK1 silencing causes growth and signaling inhibition of mut-KRas but not wt-KRas cells.

A. Effect of siKRAS (left panel) and siCNK1 (right panel) on 2D growth on plastic of NSCLC cell lines. Wt-KRas cells are shown by shaded bars, and mut-KRas cells by filled bars. Values are cell growth expressed relative to non-targeting (siScr) treated control cells. Values are mean of 3 determinations and bars are S.E. **B.** Western blots showing the effects of 72 hr siRNA knockdown of KRas or Cnk1, compared to non-targeting (siScr) control cells, on downstream signaling in wt-KRas and mut-KRas NSCLC cells. **C.** Western blot of 72 hr siRNA knockdown of KRas or Cnk1, compared to siScr on downstream signaling in isogenic mut-KRas HCT-116 colon cancer cells and wt-KRas HKH2 cells. **D.** Isogenic mut-KRas HCT-116 colon cancer cells and wt-KRas HKH2 cells treated for 24 hr with siScr or siCNK1, and allowed to form spheroids for 48 hr in a hanging drop of media. Lower panel, typical photomicrographs showing HCT-116 parental cells, or HKH2 wt-KRas cells treated

with siScr or siCNK1. Upper panel shows quantitation of the data. Values are mean of 10 measurements and bars are S.E.

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Figure 2. Effects of Cnk1 inhibition on cell cycle, mutant effects Cnk1s on growth, and membrane colocalization of Cnk1 and KRas.

A, Cell cycle analysis of wt-KRas HKH2 and mut-KRas HCT-116 cells treated with siKRAS or siCNK1 for 72 hr. siScr is a non-targeting control. **B**, Effects on wt-KRas and mut-KRas NSCLC cell growth: 1 empty vector; 2 full length Cnk1; 3 Cnk1 PH domain; 4 full length Cnk1 with a non-PtdIns binding PH domain (mut Cnk1-EEALA); and 4 myristoylated Cnk1. Values are mean of 3 determinations with S.E. **C**, Confocal microscopy of HEK293T cells expressing Cnk1-GFP and either wt-KRas or mut(G12D)-KRas-RFP after incubation in serum free medium overnight. **D**, FLIM showing the average fluorescence lifetime distribution for Cnk1-GFP alone (top), Cnk1-GFP in the presence of

wt-KRas-RFP (middle) or mut-KRas-RFP (bottom). The lifetime measurements were taken from the co-localization images. Cnk1-GFP alone and Cnk1-GFP co-transfected with wt-KRas-RFP showed comparable fluorescence lifetime that centered around 2.2 ns. In contrast, cells co-transfected with CNK1-GFP and mut-KRas-RFP the average lifetime decreased to 2.1 ns. The reduction in average fluorescence lifetime is significant and due to FRET, a strong indicator of a direct interaction. **E**, RPPA of the top most down regulated and upregulated cell cycle and signaling proteins (cut-off + and – 0.4 arbitrary units from a total of 208 proteins studied) between mut-KRas HCT-116 and wt-KRAS HKH2 colon cancer cells (KRas, open bars), and between HCT-116 24 hr non-targeting siRNA and si*CNK1* treated cells (CNK1, filled bars). Values are arbitrary units. **F**, Changes in cyclin B1, Cdk1, and pRb (ser807) as the only proteins changed in a large panel of mut-KRas (solid lines) but not wt-KRas (dashed lines) cell lines treated with non-targeting siRNA (left of each panel) or *siCNK1* (right of each panel).

Figure 3. Small molecule inhibitors of the CNK1 PH domain prevent phosphoinositide binding and displace membrane mut-KRas.

A, Isothermal titration calorimetry of the Cnk1 PH domain with different PtdIns phosphates. 30 mM of Cnk1 PH domain was titrated with 0 to 300 mM of the indicated diC8 PtdIns phosphate. **B**, Docking of compound PHT-7.3 into a structural model of the Cnk1 PH domain bound to Ins(1,3,4,5)P₄. The structural model of the PH domain of Cnk1 was built using 1U27 as a template and PHT-7.3 docked using SwissDock. Ins(1,3,4,5)P₄ is shown in yellow, and PHT-7.3 in cyan can be seen burying into a pocket occupied by phosphate at position 4 on the inositol ring and so inhibit binding to inositol phosphate head groups. **C**, SPR sensorgram showing binding of PHT-7.0, PHT-7.3 and PHT-7.10, to the Gst tagged PH domain of Cnk1. **D**, SPR sensorgram showing displacement of PtdIns(3,4,5)P₃ binding to

the PH domain of Cnk1 by 50 μ M PHT-7.3 **E**, Confocal microscopy of HEK293 cells expressing Cnk1-GFP and mut(G12D)-KRas-RFP incubated for 1 hr with 50 μ M PHT-7.0 or PHT-7.3. Both compounds blocked the colocalization of Cnk1 and mut-KRas shown in the merged images compared to non-treated control. **F**, FLIM lifetime plots showing that compounds PHT-7.0 and PHT-7.3 reversed the shortened fluorescence lifetime (shown by dashed line) of the compound-treated cells indicating they inhibited Cnk1 interaction with mut-KRAS interaction.

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Figure 4.

A, Waterfall plot of the IC_{50} s of PHT-7.3 for inhibition of 2D growth on plastic of a panel of NSCLC cell lines with wt-KRas cells shown by shaded bars, and mut-KRas cells by filled bars. An IC_{50} greater than 100 μ M is shown by >. Values are mean of 3 determinations and bars are S.E. **B**, Plot of the IC_{50} s of PHT-7.3 for inhibition of 3D growth in soft agarose of the same a panel of NSCLC cell lines. Inset is a semilogarithmic waterfall plot of the inhibition of mut-KRas NSCLC growth. **C**, Western blot showing inhibition of downstream signaling and RalA/B, Rho and total Ras GTP levels in A549 mut-KRas NSCLC cells exposed to different concentrations of PHT-7.3 for 24 hr, and **D**, in other mut-KRas NSCLC cells exposed to 25 μ M PHT-7.3 for 24 hr.

Figure 5. *In vivo* antitumor activity and inhibition of KRas signaling by a CNK1 PH domain inhibitor.

A, Antitumor activity of PHT-7.3 administered at 200 mg/kg ip daily for 20 days to mice with mut-KRas A549 NSCLC xenografts. In all plots the horizontal bar shows the duration of dosing. There were 10 mice per group. Bars are S.E. and P value at end of dosing are ** < 0.01, * < 0.05. **B**, Antitumor activity of PHT-7.3 administered at 200 mg/kg ip daily for 20 days to mice with mut-KRas H441 NSCLC xenografts. **C**, Antitumor activity of PHT-7.3 administered at 200 mg/kg ip daily for 21 days to mice with wt-KRas H1975 NSCLC xenografts. **D**, Western blot showing inhibition of downstream KRas signaling in A549 NSCLC tumor xenografts at 4 and 24 hr after a single dose of PHT-7.3 at 200 mg/kg ip, and after 6 days dosing with PHT-7.3 at 200 mg/kg ip at 4 hr after the last dose. There were 4 mice per time point. **E**, Western blot of RalA/B, Rho and total Ras GTP in the same tumor samples. There were 4 mice per time point.

Figure 6. Antitumor combination studies of PHT-7.3.

A, Antitumor activity of PHT-7.3 administered at 200 mg/kg ip, with and without the Egfr inhibitor erlotinib at 75 mg/kg po, daily for 8 days to mice with mut-KRas A549 NSCLC xenografts. In all plots the horizontal bar shows the duration of dosing. There were 10 mice per group. Bars are S.E. Comparisons of groups at the end of dosing are shown by vertical lines on the right with P values ** < 0.01, * < 0.05. **B**, Antitumor activity of PHT-7.3 administered at 200 mg/kg ip, with and without the Mek inhibitor trametinib at 0.3 mg/kg po, daily for 20 days to mice with A549 mut-KRas, wt-EGFR NSCLC xenografts. There were 10 mice per groups, bars are S.E.

Table 1

Compounds identified as CNK1 PH domain inhibitors

Compound	Structure	Cnk1 binding Kd μ M	Growth inhibition IC ₅₀ μ M		Pharmaco kinetics	
			wt-KRas	mut-Ras	t _{1/2} β min	Cl ml.min/kg
PHT-7.0		10.9	67	20	3	ND
PHT-7.3		4.7	69	19	260	133
PHT-7.10		15.2	55	22	28	34

ND = not detectable

Binding of the compounds to the PH domain of Cnk1 was measured by SPR spectroscopy. Growth inhibition is the mean IC₅₀ for 3D growth inhibition of a set of wt-KRas NSCLC cells H266, H1299, H1975, H3255, H1437, H2023 and mut-KRas NSCLC cells H2122, Calu1, H2009, H441, H358, H1573, H23, H1373, A549, H1944, H647. Pharmacokinetic parameters were determined in mice following intraperitoneal administration of the compounds at 10 mg/kg.