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BRIEF REPORT

Targeting oncogenic protein kinase Cι for treatment of mutant *KRAS* LADC

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ABSTRACT
Lung cancer is the leading cause of cancer death in the US with ~124,000 new cases annually, and a 5 y survival rate of ~16%. Mutant *KRAS*-driven lung adenocarcinoma (*KRAS* LADC) is a particularly prevalent and deadly form of lung cancer. Protein kinase Cι (PKCι) is an oncogenic effector of *KRAS* that activates multiple signaling pathways that stimulate transformed growth and invasion, and maintain a *KRAS* LADC tumor-initiating cell (TIC) phenotype. PKCι inhibitors used alone and in strategic combination show promise as new therapeutic approaches to treatment of *KRAS* LADC. These novel drug combinations may improve clinical management of *KRAS* LADC.

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KRAS mutation is an oncogenic driver required for LADC tumor initiation and maintenance

Activating *KRAS* mutations are detected in approximately 33% of LADC. *KRAS* mutations are also present in preneoplastic lesions of the lung (atypical alveolar hyperplasias, AAH), suggesting early acquisition during lung neoplasia.[1] Genomic sequencing of lung adenomas in situ revealed both regional histological and genomic heterogeneity; however, *KRAS* mutations were observed uniformly throughout individual lesions, indicating that mutant *KRAS* drives early tumor cell transformation, selection and evolution.[2] Expression of mutant *KrasG12D* in the mouse lung is sufficient to drive LADC initiation, providing in vivo evidence that *KRAS* is a key oncogenic driver of LADC initiation.[3,4] Interestingly, systemic delivery of *KRAS* siRNA significantly inhibited tumor growth and metastasis of mutant *KRAS* LADC tumors, demonstrating a continued dependency on mutant *KRAS* signaling for tumor maintenance.[5]

Therapeutic targeting of *KRAS* LADC

Small molecule inhibitors designed to directly target *KRAS* have focused on several approaches: agents designed to decrease exchange of GDP for GTP, increase the hydrolytic activity of mutant *KRAS*, or inhibit effector binding and/or activation (recently reviewed by Marcus and Mattos).[6] Inhibitors of *KRAS* processing/membrane association, and disruption of interaction with chaperones that transfer *KRAS* to and from the cell membrane have also been investigated.[7] However, despite these efforts, agents directly targeting *KRAS* have been of limited clinical utility. As a consequence, extensive efforts have more recently focused on defining key oncogenic *KRAS* signaling pathways, and targeting more druggable downstream signaling components of these *KRAS*-dependent pathways.

PKCι is required for *KRAS* LADC

Studies from our lab and others demonstrate that atypical PKCι promotes tumorigenesis in numerous human tumor types in vitro and in vivo.[8,9] PKCι is frequently targeted for tumor-specific genetic alteration and/or overexpression in many human tumor types, including myelogenous leukemias,[10] glioma,[11] triple negative breast cancer[12] and cancers of the lung,[13] colon,[14] pancreas[15] and ovary.[16-18] PKCι was the first PKC isozyme to be identified as a bonafide oncogene in human cancer; first demonstrated by our group in non-small cell lung cancer (NSCLC),[13] and subsequently in ovarian and other cancers.[18,19] Atypical PKCs directly interact with oncogenic *RAS* in vitro and in vivo, implicating them as mediators of oncogenic *RAS* signaling.[20] PKCι is required for *Kras* LADC tumorigenesis in mice, and for the transformed growth of *KRAS* LADC cell lines, revealing PKCι as a critical effector in *KRAS* LADC.[21,22]

PKCι promotes the transformed growth and invasion of *KRAS* LADC cells by activating an oncogenic

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RAC1-MEK-ERK proliferative signaling pathway.[22,23] In *KRAS* LADC cells, PKC*ι* forms an oncogenic complex with the polarity protein PAR6 through PB1-PB1 domain interactions.[23] The RHO family GTPase guanine nucleotide exchange factor ECT2 binds this complex and serves to activate the RHO family GTPase RAC1, which in turn drives a RAC1-PAK-MEK-ERK signaling cascade.[23-25] PKC*ι* directly phosphorylates ECT2 at T328, an event that promotes ECT2 binding to the PKC*ι*-PAR6 complex, RAC1 activation, and transformed growth and invasion in NSCLC cells.[23,24] PKCι-RAC1-MEK-ERK signaling drives proliferation and invasion, at least in part, by stimulating expression of matrix metalloproteinase 10 (MMP10).[23,26,27] Taken together, these data demonstrate that PKC*ι* drives a critical proliferative signaling pathway required for the transformed growth of *KRAS* LADC.

A critical factor regulating NSCLC susceptibility to chemotherapy-induced apoptosis is splice site selection within the *BCL-X* gene, yielding either pro-apopotic Bcl-x(s) or anti-apoptotic Bcl-x(L).[28,29] Downregulation of PKC*ι* in *KRAS* LADC A549 cells significantly decreased cell survival and reduced expression of SAP155, an RNA trans-acting factor that promotes Bcl-x pre-mRNA splice site selection to generate the pro-survival Bcl-x(L).[30] Re-expression of Bcl-x(L) in PKC*ι*-depleted cells rescued cell survival, demonstrating that PKC*ι* promotes survival of *KRAS* NSCLC cells by regulating alternative splicing of the *BCL-X* pre-mRNA.[30]

PKC*ι* drives a *KRAS* LADC tumor-initiating cell (TIC) phenotype

KRAS LADC tumors are comprised of a heterogeneous population of cells that exhibit a hierarchy of tumorigenic potential. Cells at the top of this hierarchy, tumor-initiating cells (TICs), exhibit a high level of tumor-initiating activity and an enhanced capacity to recapitulate *KRAS* LADC in vivo.[31-33] TICs exhibit the unique capacity to both self-renew, and form a more differentiated, but highly proliferative tumor cell population, driving both tumor initiation and maintenance.[34,35] Tumors harboring mutant *KRAS* exhibit enhanced chemoresistance,[36] radiation resistance,[37] and poor survival;[38] all properties associated with the TIC phenotype, suggesting that *KRAS* is an important driver of the LADC TIC phenotype.[39,40]

We have identified PKCι as a key mediator of the *KRAS* LADC TIC phenotype.[21,41] PKCι is highly expressed in mutant *Kras* transformed bronchioalveolar cells, a putative stem cell population and cell-of-origin of LADC in the mouse lung.[21] PKC*ι* deficiency dramatically reduces *Kras*-mediated transformation and expansion of the bronchioalveolar population, thereby inhibiting *Kras*-driven tumor formation.[21] MMP10, a critical target of PKCι proliferative signaling, is also required for *Kras*-mediated bronchioalveolar stem cell expansion and LADC tumorigenesis.[42] MMP10 is elevated in human *KRAS* LADC TICs, and both PKCι and MMP10 are required for LADC TIC behavior and tumor formation.[26,41] These data demonstrate that PKCι is required for maintenance of the *KRAS* LADC TIC phenotype, and indicate that MMP10 is an important mediator of PKCι-driven growth of *KRAS* LADC TICs.

Notch3 is also required for *KRAS* LADC TICs self-renewal, tumor initiation and maintenance.[32,33] We recently demonstrated that PKCι regulates Notch3 expression in *KRAS* LADC TICs by phosphorylating the ELF3 transcription factor, thereby promoting its occupancy of the *NOTCH3* promoter. *KRAS* LADCs require expression of PKCι, ELF3 and Notch3 for TIC cell growth, clonal expansion and tumorigenesis.[41] A critical feature of TICs is the ability to undergo symmetric cell division to self-renew, as well as asymmetric cell division to generate a more differentiated, highly proliferative population of tumor cells.[34] PKCι-ELF3-Notch3 controls the TIC phenotype by regulating asymmetric cell division, a process required for tumor initiation and expansion.[41] Interestingly, the PKCι-ELF3-Notch3 signaling axis is specific for mutant *KRAS* LADC TICs, since this cascade is not observed in LADC TICs harboring wild-type *KRAS* or in lung squamous cell carcinoma (LSCC). Our previous work demonstrated that PKCι drives a LSCC TIC phenotype by activating a distinct PKC*ι*-SOX2-Hedgehog (Hh) signaling axis that drives Hh-dependent TIC growth.[41,43]

PKC*ι* inhibitors as anti-tumor agents

Given the potential for PKC*ι* as a therapeutic target, we and others have sought to identify PKC isotype-selective inhibitors of PKC*ι*. Recently, an isotype selective, ATP-competitive inhibitor of PKC*ι* activity (CRT0066854) was identified and characterized to suppress transformed growth of mutant Ras cancer cell lines.[44] A separate screen for molecules that could bind a unique sequence in the PKC*ι* catalytic domain and inhibit its kinase activity identified ICA-1, capable of blocking PKC*ι* substrate phosphorylation and transformed growth in neuroblastoma cells.[45] While both of these inhibitors hold promise as PKC*ι*-targeted agents, evidence of their effectiveness as therapeutic agents awaits pre-clinical evaluation. To identify inhibitors of PKC*ι* that could be rapidly translated to clinical application, we conducted a high-throughput screen of FDA-approved drugs for compounds that inhibit oncogenic PKC*ι* signaling. We reasoned that compounds that can disrupt the PB1-PB1 domain interaction between PKC*ι* and its oncogenic

partner PAR6 would exhibit anti-tumor activity.[46] Our screen identified the anti-rheumatoid agent aurothiomalate (ATM) as a selective inhibitor of the PKCι-PAR6 interaction.[46,47] ATM, and the structurally related anti-rheumatoid drug, auranofin (ANF), selectively bind the PB1 domain of PKCι and block PAR6 binding, inhibit PKCι-mediated oncogenic signaling (i.e. the RAC1-MEK-ERK signaling axis) and block transformed growth and invasion of NSCLC cells in vitro and in vivo.[27,46,47] The anti-tumor activity of these agents is dependent upon their ability to bind PKCι and inhibit PKCι-PAR6 signaling since a PB1 domain PKCι mutant that no longer binds ATM but retains binding to PAR6, can support transformed growth and confer resistance to the growth inhibitory effects of ATM.[47] ANF is currently being evaluated clinically as an anti-tumor agent in lung and ovarian cancer patients.[48,49] These compounds are well tolerated in the oncology setting and exhibit promising therapeutic potential.[49]

Though ATM and ANF exhibit efficacy as single agents in pre-clinical models, it is likely they will find optimal clinical use in strategic combination with other agents. In this regard, we have demonstrated synergistic pre-clinical efficacy of ANF in combination with a SMO inhibition in LSCC.[43] In mutant *KRAS* LADC, combining ANF and a gamma-secretase inhibitor (GSI; inhibitor of Notch signaling) exhibited synergistic activity in *KRAS* LADC in vitro and in vivo, demonstrating effective "vertical blockade" of this critical signaling pathway (Fig. 1).[41]

We have also assessed ATM in combination with inhibitors of signaling pathways frequently activated in *KRAS* LADC, including rapamycin (mTOR), Erlotinib (EGFR) and the multikinase inhibitor, Sorafenib (Fig. 2A). Whereas each of these drug combinations showed synergistic growth inhibitory effects in one or more cell line, the combination of ATM and rapamycin showed highly significant synergistic activity in 3 out of 3 mutant *KRAS* LADC cell lines tested (Fig. 2A). Although mTOR inhibitors have shown clinical activity in lung cancer patients, a significant impediment to their use has been either intrinsic or acquired resistance. Combined ATM and rapamycin exhibits enhanced anti-tumor activity against *KRAS* LADC tumor growth in vivo (Fig. 2B). Taken together these data indicate that combined PKCι and mTOR inhibition may be an effective "horizontal blockade" strategy to treat mutant *KRAS* LADC, including those tumors that exhibit intrinsic or acquired resistance to mTOR inhibition (summarized in Fig. 1). A phase I trial is currently ongoing to assess this combination clinically.

Summary

Preclinical studies demonstrate that PKCι inhibition represents a viable therapeutic approach to treatment of *KRAS* LADC, particularly when used in strategic combination with agents targeting other KRAS effectors. Beyond *KRAS* LADC, PKCι promotes the oncogenic phenotype of many other cancers; therefore clinical development of PKCι-targeted therapies may provide effective therapeutic options for many other oncology patients.

Materials and methods

Materials: *KRAS* mutant LADC cell lines, A549, A427 and H460, were purchased from American Type Culture

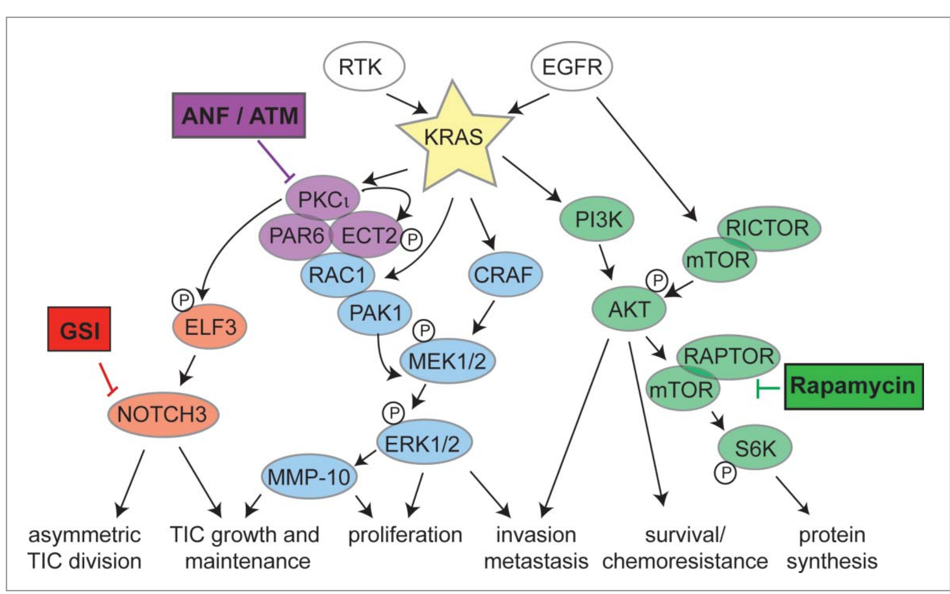

Figure 1. Clinically relevant PKCι-targeted combination therapies for treatment of *KRAS* LADC. "Vertical Blockade" of the PKCι-ELF3-Notch3 signaling axis and "Horizonal Blockade" of PKCι and mTOR signaling pathways are denoted.

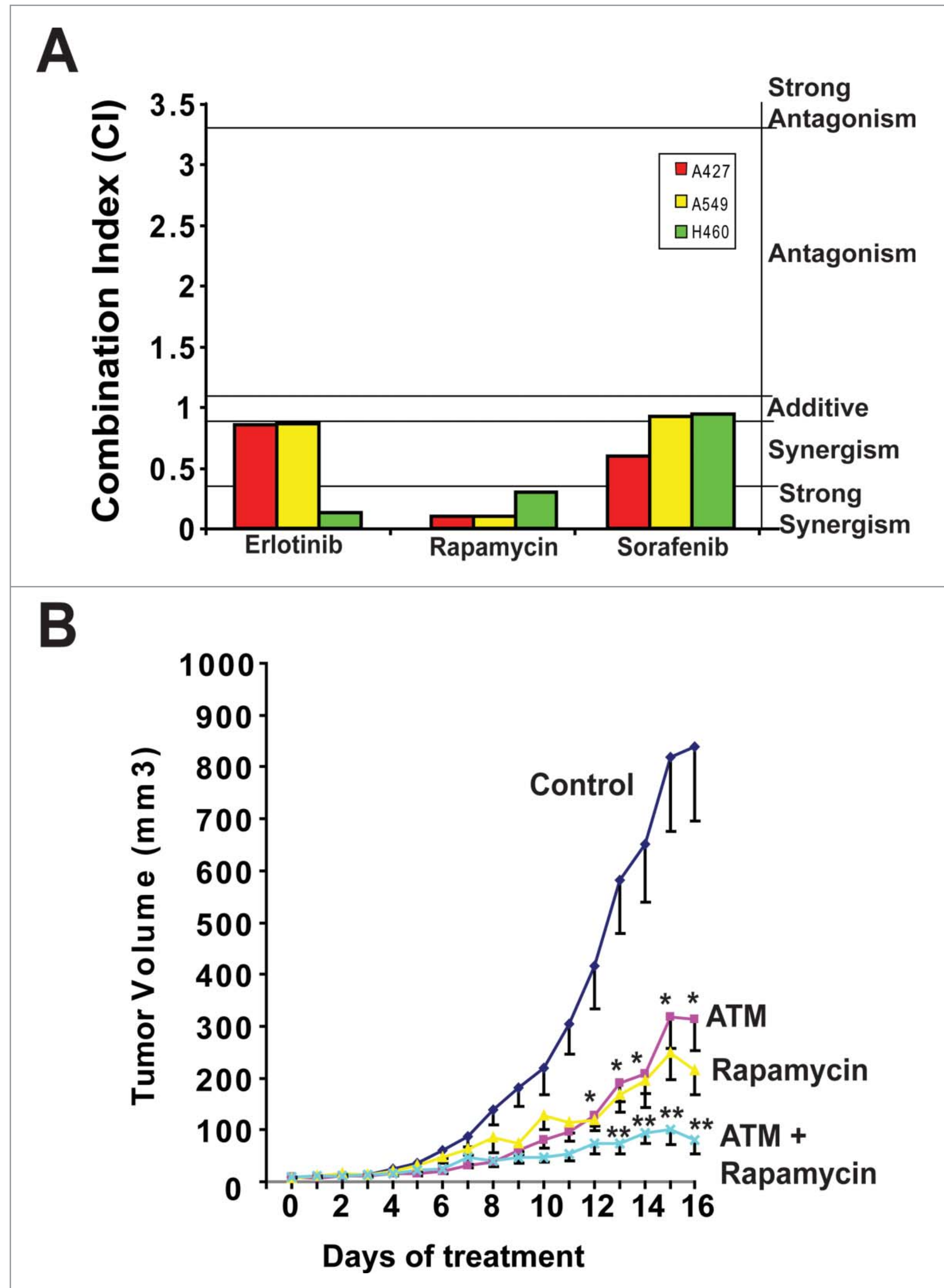

Figure 2. ATM and rapamycin exhibit synergistic growth inhibitory activity against *KRAS* LADC. (A) Combination Index (CI) analysis of ATM combined with Erlotinib, rapamycin and sorafenib for inhibition of anchorage independent growth of *KRAS* LADC cells. (B) Combined ATM and rapamycin significantly inhibits growth of H460 *KRAS* LADC xenograft tumors. Mice were treated with ATM (60 mg/kg/day) and/or rapamycin (5 mg/kg/day) or diluent alone (control). Mean +/− SE is plotted. n = 8–9/group. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ vs. rapamycin or ATM alone.

Collection and maintained in culture conditions as recommended.

In vitro studies: Anchorage-independent growth in soft agar was assessed as previously described.[27] Drug interactions were analyzed using the median effect combination index of Chou and Talalay, calculated using CalcuSyn software.[50] n = 5/dose group and results are representative of 2 independent experiments.

In vivo studies. 5×10^6 H460 cells were injected into the flank of 4–8 week old immunocompromised nude mice (Jackson Labs). Tumor cells were allowed to engraft and tumor size was monitored 3×/week by caliper measurements. Tumor volume was calculated as length x width x height x 0.5236. Mice were randomly distributed into treatment group when tumor average size = 10 mm^3. Mice were administered ATM (60 mg/kg) and/

or rapamycin (1 mg/kg) daily by intraperitoneal injection and humanely harvested after 16 d treatment. Control mice were injected daily with equivalent volumes of diluent only.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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