Mirk kinase inhibition blocks the in vivo growth of pancreatic cancer cells

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ABSTRACT

The Mirk/dyrk1B gene is upregulated and sometimes amplified in pancreatic ductal carcinomas. In poor microenvironmental conditions Mirk mediates cell survival by maintaining cancer cells in a largely quiescent, noncycling state and by decreasing toxic ROS levels through maintaining expression of a series of antioxidant genes. Premature entry into cycle, increased ROS levels, DNA damage, and apoptosis follow Mirk kinase depletion or inhibition. Mirk kinase inhibitor EHT5372 treated Panc1 spheroids lost quiescence markers coincident with an increase in cyclin A showing entry into cycle, and exhibited DNA damage, apoptosis and smaller size. EHT5372 treatment in vivo led to an increased fraction of Ki67 positive, cycling cells in Panc1 xenografts whose size was reduced. Pdx-1-cre LSL/KrasG12D/Ink4a/Arf null B6 mice always develop pancreatic cancer, allowing only 30% survival by 8 weeks, while each of the Mirk kinase inhibitor treated mice survived 8 weeks. Mirk inhibition led to a roughly four-fold increase in tumor αSMA-positive fibroblasts and large stromal collagen-rich infiltrates in the pancreas that can restrain tumor growth. The mTOR inhibitor RAD001 alone, or together with EHT5372, reduced pancreatic cancer size 30-fold, while the drug combination reduced the number of microscopic tumor foci 2-fold compared to RAD001 alone.

INTRODUCTION

Pancreatic ductal adenocarcinoma is a highly lethal disease with few treatment options, and causes the death of over 40,000 people annually. About 70% of cases are initiated by mutation in the K-ras gene. Oncogenic K-ras is required for the formation of benign pancreatic tumors, their progression, and the maintenance of invasive cancers [1],[2],[3]. Mutant K-ras has been shown to activate the serine/threonine kinase Mirk/dyrk1B through the Rac1/MKK3 signaling pathway [4] so Mirk should be an active kinase during pancreatic cancer development. Metabolic stress, oncogenes such as mutant ras, and the rapid growth of tumor cells raise reactive oxygen species (ROS) levels, making ROS levels generally higher in tumor cells than in normal cells. Tumor cells with mutant ras survive the toxic stress of high ROS by becoming dependent on non-oncogenes such as antioxidant genes [5]. Mirk lowers ROS levels by increasing expression of antioxidant genes [6]. Mirk is an active kinase in pancreatic, ovarian and colon cancer cells and is an active kinase in a murine model of pancreatic cancer where Mirk restricts Hedgehog initiated Gli1 activity to the stromal compartment [7]. Significantly, Mirk maintains the viability of the most aggressive subset of pancreatic cancer cells, those that can undergo clonal growth [4], which should include the tumor stem cells. The Mirk gene is within the 660 kb core region of the 19q13 amplicon found in 12% of all primary pancreatic cancers, but in 33% of the more advanced T3 and pT4 tumors, lymph node metastases and distant metastases [8]. Mirk protein is found in 90% of resected pancreatic cancers [9]. Pharmacologic inhibition of the kinase Mirk/Dyrk1B by the small molecule inhibitor RO5454948 enabled escape from arrest in G0 quiescence, and increased ROS levels, DNA damage, and apoptosis in pancreatic cancer cells that had entered cycle [10], and escape from quiescence led to more apoptosis in cancer cells with mutant p53 in addition to low expression of G1 CDK inhibitors [11]. Similar results were seen with Mirk depletion. Mirk expression is much lower in most...
normal diploid cells [12] than in cancer cells. In addition, direct measurement of Mirk kinase activity showed that kinase activity was much more elevated in ovarian cancer cells than in normal diploid ovarian epithelial cells [11]. The viability of normal diploid fibroblasts and epithelial cells was not affected by Mirk depletion [13] or by Mirk kinase pharmacologic inhibition [11]. The current study tested whether Mirk kinase could be targeted in vivo in xenografts of Panc1 cells with an amplified Mirk gene and in a genetic model of pancreatic cancer with no known Mirk amplification.

RESULTS AND DISCUSSION

Mirk kinase depletion or inhibition leads to DNA damage, increased ROS levels, and apoptosis

We speculated that inhibition of Mirk kinase might be effective in murine models of pancreatic cancer in which the Mirk gene was amplified, as it is in 12% of all primary pancreatic cancers, and in 33% of metastases...
Mirk was effectively depleted from Panc1 cells made quiescent by serum-starvation (Fig.1A). DNA damage was shown in the serum-starved cells by increased levels of H2AX phosphorylated at S349 (γH2AX), with slightly more in the Mirk-depleted cells. Histone protein H2AX molecules become phosphorylated on serine-139 near their carboxyl terminus when they are within the chromatin at a double-stranded DNA break site, and create a focal site for DNA repair within a short time of DNA damage [17]. The Mirk-depleted cells were then released from quiescence by replating at lower density in growth medium. Half of the cultures were treated with toxic levels of the chemotherapeutic drug gemcitabine to induce DNA damage. Although gemcitabine killed many cells, the remaining cells exhibited more DNA damage if they were Mirk-depleted as well (Fig.1A). Thus Mirk depletion in Panc1 cells stressed by poor culture conditions or a chemotherapy drug correlates with more DNA damage. Similarly, Panc1 cells treated with a range of concentrations of either of two Mirk kinase inhibitors, RO5454948 or EHT5372, showed increased DNA damage detected by antibody to phosphorylated H2AX as well as increased amounts of the apoptosis marker cleaved PARP (Fig.1B). RO5454948 is not stable in rodents, so the Mirk kinase inhibitor EHT5372, with greater stability (Methods) was tested. ROS species are known to increase DNA damage, and Mirk lowers ROS levels by increasing expression of a series of at least 9 antioxidant genes including superoxide dismutase 2 and ferroxidase [6], probably through its transcriptional co-activator activities [18], [19]. Both EHT5372 and RO5454948 increased ROS levels in a dose-dependent manner (Fig.1E), as the Mirk inhibitor RO5454948 did in an earlier study [10]. The concentrations that induced the highest ROS levels also induced more DNA damage and more apoptosis, with 5 and 10µM EHT5372 and 0.5 and 1µM RO5454948 being optimal.

EHT5372 showed selective inhibitory effect on Mirk protein in a screen of over 300 protein kinases [20]. However, how much of the growth inhibitory effect on Panc1 cells was due to targeting Mirk, and not off-target kinases? We compared the growth of Panc1 cells with MIA cells, which have been reported not to express Mirk [9]. The EHT5372 EC50 was 1.2µM on Panc1 cells, but could not be determined on MIA cells, as levels from 1 to 10µM inhibited MIA growth about 10%. (Fig.1C). Thus EHT5372 targeted other kinases than Mirk within MIA cells, but this off-target effect only led to a minor effect on growth. A second test of the specificity of the EHT5372 was to determine whether the inhibitor reduced expression of Plac8, placenta specific antigen. This protein has been shown to suppress pancreatic cancer formation by blocking autophagy [21]. In prior studies Mirk depletion had been shown to reduce Plac8 expression about 5-fold [19]. Panc1 cells exhibit low levels of Plac8 [21]. However, treatment of these cells with either EHT5372 or RO5454948 markedly decreased Plac8 expression (Fig.1D). These two studies indicate that EHT5372 phenocopies Mirk depletion, and that growth inhibition by this inhibitor is primarily due to inhibition of Mirk kinase.

Inhibition of Mirk kinase by EHT5372 has a role in inducing apoptosis of spheroid cells

Tumor cells grown attached to typical tissue culture plates have a limited ability to model tumor cells in vivo. Tumor cells in spheroids, non-adherent multicellular aggregates, are highly resistant to chemotherapy, compared with tumor cells grown attached, and may better reflect drug sensitivity in vivo [22], [23], [24], and thus enable selection of a dosage that might be effective in animal models. Panc1 cells formed into spheroids when cultured in ultra-low attachment plates (Figs. 2A&B). Mirk protein was expressed in Panc1 spheroid cells (Fig.2B). Treatment with 10µM of the Mirk kinase inhibitor EHT5372 led to a 13-fold decrease in Panc1 spheroid size, as measured from photomicrographs, and increased the number of single cells (Fig.2A), with a dose-dependent decrease in spheroid size seen at 1µM and above. AsPc1 spheroids, with no Mirk gene amplification, required much higher levels of the inhibitor to reduce spheroid volume (Fig.2A). Panc1 spheroids were treated for 7 days with the Mirk kinase inhibitor EHT5372 alone, or together with the mTOR inhibitor RAD001. In Panc1 spheroids cells Mirk inhibition led to 10-fold more DNA damage as shown by increased levels of γH2AX, and about 6-fold more apoptosis as assayed by cleavage of PARP (Fig.2B). In earlier studies RAD001 increased the toxicity of EHT5372 towards ovarian ascites cancer cells from a series of patients, as well as ovarian cancer cell lines [25]. However, addition of RAD001 had little effect on Panc1 spheroids (Fig.2B). Thus the Mirk kinase inhibitor reduced the number of Panc1 cells in the spheroids, consistent with the cells in the outside layers dying of apoptosis and reducing spheroid volume. Interestingly, others have reported that...
stable short hairpin RNAs against Mirk/dyrk1B prevented the A498 renal carcinoma cell line from forming spheroid cultures [26]. Disruption of spheroids to single cells is a step in their dissolution.

In contrast to the results with Panc1 cells, in BxPc3 pancreatic cancer cells with no Mirk gene amplification, the combination of 5µM RAD001 and 10µM EHT5372 killed almost all cells (Fig.2C), while cell growth was inhibited only about 50% by 5µM RAD001 alone (data not shown). Possibly RAD001 increased the amount of DNA damaging ROS released by Mirk kinase inhibition [27], or increased expression of Mirk in BxPc3 cells [27], or both.

**Mirk kinase inhibition allows Panc1 spheroid cells cultured in vitro as spheroids or Panc1 cells grown as xenografts in vivo to inappropriately enter cycle**

Earlier studies showed that spheroids formed from ovarian cancer cells are largely in a quiescent, dormant state, expressing elevated levels of the quiescence markers p130/Rb2 and the CDK1 p27 compared to cycling adherent cells, and about 85% of the spheroid cells were in G0/G1 by flow cytometry [28]. Likewise, Panc1 spheroids exhibited the quiescence marker p130/Rb2 and the CDK inhibitor p27, which serves to block cell cycling (Fig.3A). Blocking the Mirk contribution to cell quiescence allows some cancer cells to enter cycle although remaining in poor culture conditions [10], [29]. In prior studies, Mirk kinase inhibition by RO5454948 forced more SW620 colon cancer cells to enter cycle as assayed by an increase in BrdU-incorporating cells [10], and Mirk kinase depletion enabled serum-starved HD6 colon cancer cells to leave quiescence and traverse G1 to the G0/G1 boundary with S phase [13]. Likewise, inhibition of Mirk kinase reduced the quiescence marker p130/Rb2 about 5-fold in Panc1 spheroid cells (Fig.2B, Fig.3A). The DREAM complex component p130/Rb2 sequesters transcription factors necessary for cell cycling [30], [31]. Significantly, EHT5372 at 5 and 10µM strongly reduced p27 levels, while at the same time increased levels of cyclin A, a
marker of cells in S phase (Fig. 3A), showing movement into cycle. Thus EHT5372 inhibition of Mirk kinase enabled more Panc1 cells grown in three-dimensional culture as spheroids to leave the quiescent state and enter cycle although they remained in serum-limited culture conditions. The loss of quiescence proteins and increase in cyclin A by EHT5372 at 5 and 10µM paralleled an increase in DNA damage as shown by increased levels of γH2AX, and the apoptosis markers cleaved caspase3 and cleaved PARP (Fig. 3A), showing that inappropriate entry into cycle correlated with DNA damage, apoptosis and the resulting cell loss in these Panc1 spheroids.

Likewise, inhibition of Mirk/dyrk1B in vivo led to inappropriate entry into cycle and cancer cell loss. Athymic mice bearing palpable tumors 3 weeks after injection of 1 million Panc1 cells were subjected to twice weekly 0.1ml intraperitoneal injection with Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 0.8mg/kg, 2mg/kg, or 4mg/kg, equivalent to 2, 5 or 10µM, or with diluent over a 2-week period. Tumors from each group were dissected free of other tissues, weighed and then fixed and stained for the proliferation antigen Ki67. Many pancreatic cancer cells in vivo are quiescent in G0 [32]. G0 cells do not express the proliferation related antigen Ki67. Only about 22% of Panc1 xenograft cancer cells were Ki67 positive and thus in cycle (Fig. 3B, 0 lane). EHT5372 treatment increased the fraction of Ki67 Panc1 tumor cells at every concentration tested, 2, 5 or 10µM (Fig. 3B). However, comparison of the fractions of Ki67 positive cells by unpaired two-tailed t-test showed only the 10µM EHT5372 value was statistically greater than control, p=0.0082 (Fig. 3B). Thus Mirk kinase inhibition increased quiescent pancreatic cancer cell entry into cycle in vivo.

The Mirk kinase inhibitor EHT5372 reduces the growth of Panc1 pancreatic cancer xenografts in a dose-dependent manner

In the xenograft experiment shown in Fig. 3B, tumors from each group of mice were weighed, and

![Fig. 3: Mirk kinase inhibitor treatment in vitro or in vivo leads to increased entry into cell cycling, and DNA damage and apoptosis.](image)

A. Panc1 spheroids were treated 3 or 7 days with the Mirk kinase inhibitor 0.5-10µM EHT5372 or 10µM RAD001 before analysis by western blotting of quiescence proteins p130/Rb2 and p27, cyclin A as a measure of entry into cycle, histone H2AX phosphorylation as a measure of DNA breaks, the apoptotic marker cleaved PARP and cleaved caspase 3, and for blotting controls actin and a cross-reacting band to confirm equal loading. B. 4 week old J:NU athymic mice (Jackson Labs) were injected subcutaneously under the backskin with 1 million viable Panc1 cells, 5 mice per group. After 3 weeks, palpable tumors were detected, and mice were subjected to twice weekly 0.1ml intraperitoneal injection with Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 2, 5 or 10µM (4mg/kg), or diluent over a two week period. Tumors from each group of mice were fixed, and stained for the proliferation marker Ki67, a DNA polymerase subunit. Sections containing all of the tumors in each group were photographed with the same contrast, and printed. Each print was “gridded”, and all of the cells within 4 alternate grid boxes were counted to eliminate counter bias. 454+/-21 cells were counted per treatment. Comparison of Ki67 percents by unpaired two-tailed t-tests for control vs. 10µM EHT5372 was statistically significant, p=0.0082.
only 10µM EHT5372 statistically reduced tumor size (Fig.4A). This experiment was repeated with a 10-fold increase in the number of Panc1 cells injected and addition of two other drugs (Fig.4B). Pharmacological inhibition of Mirk kinase allows quiescent tumor cells with low or absent expression of CDKN2A (p16) to escape quiescence even under suboptimal culture conditions and enter cycle prematurely with unrepaired DNA damage, where some undergo apoptosis [11]. We speculated that an increase in apoptosis might occur if additional checkpoints were inactivated. In an initial survey with the Chk1 inhibitor LY2603618, a combination of this agent and the Mirk kinase inhibitor EHT5372 led to complete dispersal of the Panc1 spheroids (data not shown). Athymic mice bearing palpable Panc1 pancreatic cancer xenografts were subjected for 2 weeks to twice weekly 0.1ml intraperitoneal injections to give respectively, 4 mg/kg Mirk/dyrk1B inhibitor EHT5372, 25 mg/kg gemcitabine, 4.4 mg/kg Chk1 inhibitor LY2603618, PBS diluent or combinations as indicated. Gemcitabine had no effect. However, the short duration of treatment with the Mirk kinase inhibitor was sufficient to reduce tumor growth 3 fold (Fig.4B). Comparison of tumor weights by unpaired two-tailed t-tests for control vs EHT5372 showed that the differences were statistically significant, p=0.0277. Unexpectedly, the Chk1 inhibitor LY2603618 increased tumor growth about 60% over control values, as the tumors appeared more vascularized, but addition of the Mirk kinase inhibitor reduced tumor size about two-fold with a statistical difference, p=0.0388 for LY2603618 vs. LY2603618 and EHT5372. The Chk1 inhibitor was expected to decrease tumor growth, not increase it, so the finding that the Mirk inhibitor reduced tumor growth even in the presence of LY2603618 strengthens the observation that Mirk kinase inhibition by itself is enough to decrease the in vivo growth of pancreatic cancer cells known to contain a highly amplified Mirk gene and elevated levels of active Mirk kinase [9]. Thus the Mirk kinase inhibitor blocked the xenograft growth of pancreatic cancer cells with an amplified Mirk gene under two different experimental conditions.

Mirk expression levels are very low in most normal cell types except for skeletal muscle [12] where it blocks ROS generated by muscle contraction [33], suggesting that this kinase has a non-critical function in most normal cells. Supporting this interpretation, the weights of mice treated with EHT5372 were unchanged, with similar weights seen in mice treated with 2, 5, or 10µM drug or diluent only (Fig.4C). The treated mice were lively, and showed no apparent distress, or differences in behavior compared to control mice. No other assays for viability were performed.

Mirk kinase inhibition maintained mouse viability in a genetic model of pancreatic cancer

A genetically defined murine model of pancreatic cancer pairs deletion of the CDK inhibitor p16 gene with cre-lox activation of a mutant Kras gene localized to the
pancreas by the Pdx-1 transcription factor [34], and such
tumors express Mirk protein (Supplementary Fig.1).
Pdx-1-cre LSL/KrasG12D/Ink4a/Arf null mice were
genotyped at weaning, then either treated with
EHT5372 alone or left untreated for eight weeks when all
mice should show tumor growth [34], or until death or
sacrifice because of ill health. More mice were added to
the untreated group, 10 in all, because only three control
mice lived eight weeks, while four others either died on
day 52 or 53, or had to be sacrificed at 53 days because of
ill health (Fig.5A). In Fig. 5B tumors from mice that died
before 8 weeks or were sacrificed due to poor health are
indicated with arrowheads. The remaining three control
mice died at 45 days or earlier. These control mice were
not undersized compared to their littermates, as Pdx-1-cre
LSL/KrasG12D/Ink4a/Arf null mice that were undersized
and ill-appearing from an early age were not included in
any of the groups. In marked contrast, all 10 of the mice
 treated with the Mirk kinase inhibitor (MKI) EHT5372
lived for the entire 8 weeks (Fig.5A).

When the pancreases were excised, those from the
untreated group were enlarged and felt hard, compared to
the pancreas from a genetically more normal littermate
(Pdx-1-cre/p16null). The sectioned pancreases were
stained with hematoxylin & eosin to reveal structure.
The width and length of all tumors in each pancreas
was measured from H&E slides using a 10x magnifying
loop, and the total volume calculated. Surprisingly from
the viability data (Fig.5A), a similar mean tumor size
was seen in the pancreases excised from mice treated
with the Mirk kinase inhibitor and the untreated controls
(Fig.5B). The two largest tumors from the untreated mice
and from the EHT5372-treated mice were compared
by microscopy (Fig.6). The pancreases from untreated
mice were often almost completely taken over by tumor
with some stromal component (Fig.6A&B). However,
each pancreas from the Mirk inhibitor treated mice had
some tumor with a large solid core of stroma (Fig.6B)
surrounded by normal-appearing tissue (Fig.6A), possibly
enough normal pancreas to enable these mice to live 8
weeks. The stoma was collagen-rich and more extensive
than that found in the tumors from the untreated mice
(Fig.6B). Sonic hedgehog signaling induces stromal growth
in the pancreas, but is blocked in the pancreatic
epithelial cells by Mirk/dyrk1B [7], [35]. Possibly
inhibiting Mirk kinase activity in the pancreatic cancer
cells enabled more sonic hedgehog signaling and thus
more stromal growth. Also, inhibition of Mirk might push
the p16 null fibroblasts into cycle. About 10% of normal
diploid fibroblasts, when accumulated in G0 quiescence
by serum-starvation, responded to Mirk kinase inhibition
by entering cycle and moving to G2 [11]. In this murine
model, the stromal fibroblasts were p16 null, so might
respond more strongly to Mirk kinase inhibition, so there
might be a larger population to respond to signals from
the pancreatic cancer cells to make stroma. To test this

Fig.5: Treatment with Mirk inhibitor EHT5372 maintains
the viability of Pdx-1-cre LSL/KrasG12D/Ink4a/Arf null B6 mice for 8 weeks, but leads to
extensive stromal growth in the p16 null background and thus large tumors, while the addition of the mTOR
inhibitor RAD001 or RAD001 alone reduces size of pancreatic cancers. A. Pdx-1-cre LSL/KrasG12D/Ink4a/Arf
null B6 mice were genotyped at weaning, then injected twice weekly with 4 mg/kg Mirk/dyrk1B inhibitor (MKI) EHT5372
(4 mice), EHT5372 plus 5 mg/kg RAD001 (6 mice), 5 mg/kg RAD001 (5 mice), or left untreated (10 mice) until sacrifice at
8 weeks. The days mice survived are plotted. B. The volume of the pancreatic cancers arising in each mouse was determined
from width2 x length/2 of all tumors detected in an H&E section, and the total volume per mouse was added together. Only the
tumors in untreated mice that lived to 52-56 days are included. Mean+/SE shown. The control untreated values are statistically
different from the EHT5372+RAD001 values, p=0.0166, and different from the RAD001 only values, p=0.0483, both 2 tailed
unpaired t tests. The mean +/-SE sizes of the tumors in cubic mm were 257+/-82, 258+/-109, 4.8+/-1, 6.6+/-4 for untreated, MKI,
RAD, MKI+RAD, respectively. C. The total numbers of tumor foci in the H&E sections from the Mirk kinase inhibitor (MKI)
plus RAD001 and the only RAD001 treated Pdx-1-cre LSL/ KrasG12D/Ink4a/Arf null B6 mice after 8 weeks were counted.
The number of foci includes the tumors of 1mm of greater, as well as the foci, which could only be seen using a microscope.
hypothesis, the fibroblast content in tumors was measured by immunohistochemistry for the fibroblast marker alpha smooth muscle actin (SMA). The Mirk inhibitor treated tumors had many more fibroblasts than the untreated tumors (Fig.6C). Comparison of an equal number of sections by densitometry showed that the EHT5372-treated tumors had roughly four times the number of SMA+ fibroblasts as the controls, consistent with the increased stroma. Desmoplasia in pancreatic cancer was thought to increase aggressiveness. However, recent studies employing myofibroblast depletion have shown a strong protective role by stroma in this cancer [36], [37], which might explain the enhanced viability of the Mirk kinase inhibitor treated mice.

Mirk kinase inhibition plus mTOR inhibition strongly reduced pancreatic cancer size in the genetic model

In our earlier studies, the combination mTOR inhibitor RAD001 and EHT5372 killed over 90% of ovarian cancer ascites taken directly from patients and maintained as spheroids in culture [25]. In the current study RAD001 did not increase the toxicity of the Mirk kinase inhibitor EHT5372 towards Panc1 spheroid cells (Fig.2B), but RAD001 did increase its toxicity towards BxPC3 cells, which have no Mirk gene amplification (Fig.2C). Possibly RAD001 would enhance the toxicity of EHT5372 in the genetic mouse model with no known Mirk amplification.

Pdx-1-cre LSL/KrasG12D/Ink4a/Arf null mice were bred, genotyped at weaning, then either treated with RAD001 alone or with RAD001 and EHT5372 for eight weeks, or until death. All of the mice treated with EHT5372 and RAD001 were viable for 8 weeks, as were all but one of RAD001- treated mice (Fig.5A). All of the mice treated with RAD001 or with both the Mirk kinase inhibitor and the mTOR inhibitor RAD001 had detectable pancreatic cancer at 8 weeks, but only as small nodules. When the total tumor volumes were calculated both groups had tumors that were over 30 times smaller than tumor volumes in the control, untreated mice (Fig.5B).
Interestingly, there was no enhanced stromal growth when the mTOR inhibitor was added with the Mirk kinase inhibitor (Fig.6B). Examination of the slides from the RAD001-only treated mice showed that these pancreases had a large number of microscopic tumor foci that were too small to be measured for tumor volume. These were about twice as many tumor foci in the pancreases from mice treated with only RAD001 compared with the pancreases from mice treated with RAD001 and the Mirk kinase inhibitor (Fig.5C), suggesting that at a time later than 8 weeks, the RAD001-treated mice would have more pancreatic tumors than the mice treated with both inhibitors. Thus the Mirk kinase inhibitor reduced the number of minute tumors when added together with the mTOR inhibitor, and this drug combination strongly reduced pancreatic cancer growth and maintained mouse viability. Although the mice in all groups were similar in size within standard deviation when sacrificed, the mice treated with both inhibitors were slightly larger in general than the untreated controls, 21.3+/−2.9g vs. 20.1+/−4.6g, while those treated with the Mirk inhibitor or RAD001 alone averaged 20.6+/−3.1g and 20.8+/−2.8g, respectively.

As compiled in the Cancer Genome Atlas, the PI3K/Akt/mTOR/p70S6K signaling pathway is frequently deregulated in solid tumors, including pancreatic cancers. Ras proteins directly bind and activate PI3 kinases. Mutant K-ras-driven lung adenocarcinomas undergo stasis and partial regression if the binding between K-ras and the PI3K subunit p110alpha is blocked [38], showing that the PI3K signaling pathway is essential for tumor maintenance initiated by mutant K-ras, at least in a lung adenocarcinoma model. The mTOR inhibitor RAD001 (everolimus) has been approved by the FDA for renal cell carcinoma, giant cell astrocytoma in tuberous sclerosis patients, hormone receptor positive/HER2 negative breast cancer and in neuroendocrine pancreatic cancer [39]. This is a relatively limited range of cancers possibly because mTOR inhibitors upregulate several survival pathways including growth factor receptors. One of the survival proteins upregulated by mTOR inhibition is the Mirk/dyrk1B kinase, through CREB binding sites in the Mirk promoter [27]. RAD001 upregulates Mirk expression, leading to Mirk protein levels elevated up to 10-fold in various pancreatic cancer and ovarian cancer cell lines [27]. We were surprised that RAD001 had such strong effects in the genetic mouse model, as it does not have such effects in patients as a single agent, but the genetic model has not always been predictive.

CONCLUSIONS

Recent studies have shown that sonic-hedgehog-dependent pancreatic cancer stroma suppresses or restrains pancreatic cancer development, and that Shh-deficient tumors had reduced stromal content, but were more aggressive [36], [37]. Thus the Mirk inhibitor effects of increasing fibroblast growth and subsequent stromal collagen content in the genetic model (Fig.6) may explain why these tumors were not as lethal to their hosts as the untreated tumors (Fig.5A). Thus, the combination of RAD001 with a Mirk kinase inhibitor may warrant further exploration as a possible therapeutic modality.

MATERIALS & METHODS

Cell lines, media, western blotting, MTT assays, ROS measurements, and transfections were as described [6]. Western blotting was quantitated from autoradiographs using the UN-SCAN-IT gel 5.3 program. All cell lines were purchased from the ATCC in 2009, and their identities confirmed by STR profiles in June, 2014 by Promega through the ATCC. Seventeen short tandem repeat (STR) loci plus the gender determining locus, Amelogenin, were amplified using the commercially available PowerPlex® 18D Kit from Promega. Each cell line sample was processed using the ABI Prism® 3500xl Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems). Appropriate positive and negative controls were run and confirmed for each sample submitted. The submitted profiles were an exact match for the Panc1 ATCC human cell line in the ATCC STR database (14 of 14 loci) and the BxPC3 human cell line (12 of 12 loci). In May 2012, short tandem repeat profiling of 15 loci was used to authenticate the TOV21G cell line.

Mirk/dyrk1B inhibitors

EHT5372 was the gift of Diaxonhit (Paris, France). EHT5372 at 1µM was a stable compound in a human liver microsome study, with 77.4% of the drug remaining after 60 min, with a half-life of over 120 min as assayed by LC-MS [20]. EHT5372 had an IC50 of 0.28nM on the synthetic peptide substrate Dyrktide, and was highly selective in a screen of 339 kinases, including members of the dyrk family [20]. The Chk1 inhibitor LY2603618 and the mTOR inhibitor RAD001 were purchased from Selleck Chemicals. All other reagents were from Sigma.

Spheroid culture preparation

1 million cells are plated in a 100mm tissue culture dish in 10ml spheroid media and allowed to attach overnight. Cells are then trypsinized, washed twice, then suspended in 10 ml spheroid media (DMEM supplemented with 0.5% FBS) and placed in an ultra-low attachment dish (Fisher), and in 2-3 days spheroid formation occurred. Viable cell numbers in spheroids were determined by metabolism of MTT. All biochemical experiments were repeated at least twice.
Mouse studies

J:NU athymic mice (Jackson Labs) were injected subcutaneously under the backskin with either 1 million (experiment 1, 4 week old mice) or 10 million viable Panc1 cells (experiment 2, 7 week old mice). When 1 million cells were injected, after 3 weeks palpable tumors were detected, and mice were then subjected to twice weekly 0.1ml intraperitoneal injection with Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 2, 5 or 10µM, or diluent over a 2-week period. When 10 million cells were injected, after 12 days palpable tumors were detected, and mice were subjected to twice weekly 0.1ml intraperitoneal injection with either Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 10µM (4mg/kg), gemcitabine to give a final concentration of 25mg/kg, the Chk1 inhibitor LY2603618 to give a final concentration of 4.4 mg/kg, or combinations as indicated, or diluent over a 2 week period. Tumors were excised, weighed and fixed for routine histology and stained for Ki67 expression.

Pdx-1-cre LSL/KrasG12D/Ink4a/Arf null B6 mice were bred from founder mice Pdx-1-cre (B6.FVB-Tg(Ipf1-cre)1Tuv, LSL Kras G12D (B6.129-Kras tm4Tj), Ink4a/Arf null (B6) (B6.129-Cdkn2a tm1Rdp) from the NCI, genotyped at weaning by PCR of tail snips, and then, until 8 weeks of age or death or sacrifice due to illness, subjected to twice weekly 0.1ml intraperitoneal injection with either 4mg/kg Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 10µM, 5mg/kg RAD001 to give a final concentration of 5.2µM, the combination, or left untreated. Mice were sacrificed by carbon dioxide inhalation and weighed. Their pancreases were excised, subjected to twice weekly 0.1ml intraperitoneal injection with Mirk/dyrk1B inhibitor EHT5372 to give a final concentration of 10µM, 5mg/kg RAD001 to give a final concentration of 5.2µM, the combination, or left untreated. Mice were sacrificed by carbon dioxide inhalation and weighed. Their pancreases were excised, fixed for routine histology and stained for Ki67 expression.

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