Targeting pancreatic cancer metastasis by inhibition of Vav1, a driver of tumor cell invasion

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Azathioprine inhibits Vav1-dependent invasion

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Abstract:

Pancreatic cancer, one of the most lethal forms of human cancer, is largely resistant to many conventional chemotherapeutic agents. While many therapeutic approaches focus on tumor growth, metastasis is a primary factor contributing to lethality. Therefore, novel therapies to target metastatic invasion could prevent tumor spread and recurrence resulting from local and distant metastasis. The protein Vav1 is aberrantly expressed in over half of pancreatic cancers. Its expression promotes activation of Rac and Cdc42 and leads to enhanced invasion and migration, as well as increased tumor cell survival and proliferation, suggesting that Vav1 could be a potent therapeutic target for pancreatic cancer. The purine analogue azathioprine, well-known for its function as an anti-inflammatory compound, was recently shown to function by inhibiting Vav1 signaling in immune cells. We therefore hypothesized that azathioprine could also inhibit Vav1 in pancreatic tumor cells to reduce its pro-invasive functions. Indeed, we have found that treatment of cultured pancreatic tumor cells with azathioprine inhibited Vav1-dependent invasive cell migration and matrix degradation, through inhibition of Rac and Cdc42 signaling. Further, azathioprine treatment decreased metastasis in both xenograft and genetic mouse models of pancreatic cancer. Strikingly, metastasis was dramatically reduced in Vav1-expressing tumors arising from p48Cre+/+, KRasG12D/+, p53<sup>F/+</sup> mice. These inhibitory effects were mediated through Vav1, as Vav1-negative cell lines and tumors were largely resistant to azathioprine treatment. These findings demonstrate that azathioprine and related compounds could be potent anti-metastatic agents for Vav1-positive pancreatic tumors.
Introduction

Pancreatic cancer is one of the most lethal forms of human cancer, with a 5-year survival rate of approximately 5% (1). This poor prognosis is due in large part to late detection, metastatic invasion, and resistance to conventional chemotherapeutics. Indeed, even with treatment with standard chemotherapeutic agents, the average survival is just 6-11 months (2, 3). Therefore, new therapeutic targets and approaches are necessary to treat this aggressive tumor type.

Metastasis is a major cause of cancer lethality, accounting for as many as 90% of cancer deaths. Strikingly, recent data suggest that metastasis occurs very early in the development of pancreatic cancer, possibly even prior to the formation of a primary tumor (4, 5). While a goal of cytotoxic therapies is to minimize metastatic tumor growth, therapies specifically designed to inhibit the mechanisms of invasion that drive metastasis are not currently utilized as part of cancer treatment. Therefore, targeting invasion and metastasis of pancreatic tumors should be considered as an arm of therapeutic plans for treating pancreatic cancer, even in the absence of detectable metastatic disease (6).

Factors that specifically promote metastasis by pancreatic tumors could provide valuable therapeutic targets for minimizing the spread of pancreatic cancer. The protein Vav1 is aberrantly expressed in over half of pancreatic cancers, and its expression correlates with a poor prognosis in patients (7). In cultured tumor cells and in mouse models, ectopic Vav1 expression increases invasive cell migration (8, 9), as well as oncogenic transformation, proliferation, and survival (7, 10). In fact, Vav1-positive cell lines have become dependent upon Vav1 expression, as siRNA-mediated depletion of Vav1 results in decreased cell viability and an inhibition of
invasive migration. Vav1 expression is normally restricted to hematopoietic cells, where it is an important regulator of immune cell development and activation and is implicated in hematological malignancies. Vav1 is an activator of the small GTPases Rac1 and Cdc42, which are potent regulators of actin cytoskeletal dynamics, through its function as a guanine nucleotide exchange factor (GEF). Rac1- and Cdc42-dependent actin cytoskeletal remodeling is a driving factor in cell migration and invasion, and is a part of the machinery that induces metastatic dissemination. Further, Vav1 is a dominant signaling molecule downstream of both oncogenic K-Ras and Src (7, 9). Its ectopic expression, potent pro-invasive role, and dominance to known oncogenes indicate that Vav1 could be a potent therapeutic target for pancreatic cancer.

Azathioprine is a well-established, well-tolerated drug used as an anti-inflammatory agent and immunosuppressant for treatment of inflammatory bowel disease or following transplantation. Though azathioprine has been used clinically for decades, it was only recently discovered that a metabolite of azathioprine is a potent inhibitor of Vav1/Rac (11-13). Azathioprine is metabolized to 6-thio-GTP, which can be loaded onto Rac1 in place of GTP. 6-thio-GTP can be hydrolyzed to 6-thio-GDP, but its release is unable to be catalyzed by Vav exchange factors (11). In primary T cells, this converted the CD28 stimulatory signal to a pro-apoptotic signal. While the specificity for the Vav1 GEF was not defined, the immune-suppressive effects of azathioprine are consistent with the restricted expression of Vav1 to the immune system.

As azathioprine inhibits Vav1 function in immune cells in human patients, we hypothesized that azathioprine could attenuate Vav1’s pro-invasive function in pancreatic cancer cells. The overall strategy is to test if azathioprine could be an anti-metastatic agent for patients with Vav1-positive tumors. Here, we demonstrate that inhibition of Vav1-mediated invasion by
azathioprine significantly disrupts metastatic potential. These findings reveal a new function for a well-established class of pharmacological agents in the treatment of pancreatic cancer.

Methods

Cell culture, transfections, and azathioprine treatment

DanG, PANC1, and CFPAC human PDAC cells were maintained in DMEM with 10% FBS; BxPC3, Panc04.03, and L3.6 human PDAC cells were maintained in RPMI with 10% FBS. The cell lines were obtained from the ATCC or from Dr. Daniel Billadeau or Martin Fernandez-Zapico (Mayo Clinic). The genetic and phenotypic heterogeneity of these cell lines reflect the genetic diversity of human tumors (14, 15). CA1D breast cancer cells (Dr. Ruth Lupu, Mayo Clinic) were maintained in DMEM/F12 50/50 with 5% horse serum. All cells were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in a 37°C incubator with 5% CO₂.

RNAi targeting human Vav1, Rac1, Cdc42, or a nontargeting control (Dharmacon) were transfected using RNAiMax (Invitrogen) according to the manufacturer’s instructions. Vav1: 5’-CGUCGAGGUCAAGCACAUU-3’, Rac1: 5’-GAGGAAGAGAAAAUGCCUG-3’, Cdc42: 5’-TTCAGCAATGCAGACAATTAA-3’. For cultured cells, azathioprine (Sigma) was diluted in 0.1 N NaOH (stock concentration of 5 mM, final concentration of 5μM or 10 μM). Plasmids encoding myc-tagged active Cdc42 Q61L (Addgene plasmid 12974) or active Rac Q61L (Addgene plasmid 12983) were kindly provided by Gary Bokoch. mCherry Vav1 was described previously (8). Mouse Vav1 cDNA was provided by Dr. Daniel Billadeau (Mayo Clinic).

Migration and Invasion Assays
For transwell invasion, PVP-free filters with 10 μM pores (Neuroprobe) were coated with 0.1% gelatin. The lower chamber of a blind-well chamber (Neuroprobe) contained medium with 10% FBS and azathioprine (0 or 5 μM). 2x10^5 cells were plated in the upper chamber in medium containing 0.1% FBS and azathioprine (0 or 5 μM), and incubated for 20 hours to allow migration across the filter. The nuclei on the top and bottom of the filter were stained using DAPI and scored to calculate the percent of cells that invaded across the filter.

Cell migration was measured using a wound healing assay as described (8). Cells were grown to confluence on gridded coverslips, and incubated with azathioprine (0 or 5μM) before (30h) and after wounding. Cells were imaged at t=0 and t=24 hours (t=7 hours for Panc04.03 cells).

**Matrix Degradation**

Coverslips were coated with Oregon Green-conjugated gelatin (Invitrogen) as described (16). Cells were pretreated with azathioprine (0 or 5μM) for 48 hours, then replated on gelatin-coated coverslips for 7 hours (DanG) or 24 hours (BxPC3, CFPAC, CA1D) in the presence or absence of azathioprine. Coverslips were fixed, permeabilized, and stained for actin using TRITC-Phalloidin (Sigma). Fluorescence micrographs were acquired using a Zeiss Axiovert 35 epifluorescence microscope (Carl Zeiss) using a 63x objective and a Hamamatsu OrcaII camera (Hamamatsu Photonics) with iVision software. Images were processed using Adobe Photoshop software (Adobe). Adjustments were applied uniformly to the entire image. The percentage of cells degrading the matrix was scored. The area of degradation was quantified by the segmentation function using iVision software.
Immunoblotting

Rac and Cdc42 assays were described previously (8, 9). To verify knockdowns, cells were lysed in NP-40 lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 2 mM Na$_3$VO$_4$, 15 mM NaF, Complete protease inhibitors (Roche)). Mouse tumor samples were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 mM Na$_3$VO$_4$, 15 mM NaF, Complete protease inhibitors (Roche)) with sonication. Protein concentrations of the soluble lysates were determined by BCA assay (Pierce), and proteins were resolved by SDS-PAGE and immunoblotted using the following primary antibodies: Vav1 (Novus 3A11), Rac1 (Millipore 23A8), Cdc42 (BD Biosciences), Actin (Sigma A2066). Secondary antibodies conjugated to horseradish peroxidase were from Biosource International. Chemiluminescent signals were detected using Supersignal West Pico or Femto Chemiluminescence substrates (Thermo), and exposure to autoradiography film (HyBlot CL).

Quantitative RT-PCR

Metastatic tumor samples (from liver) from human PDAC patients were provided by the Mayo Clinic SPORE in Pancreatic Cancer. VAV1 transcript expression in human PDAC samples was determined by real time quantitative RT-PCR using PerfeCTa SYBR Green SuperMix (Quanta). RNA was extracted using TRizol reagent (Invitrogen) following the manufacture’s protocol and then purified using the RNeasy Mini kit (QIAGEN). Five hundred ng of total RNA was reverse-transcribed using a High-Capacity cDNA kit (Applied Biosystems). A portion of the total cDNA was amplified by real-time PCR. Expression was determined using a C1000 Thermal Cycler (Bio-Rad). 18s rRNA was used as the endogenous control gene. All
reactions including controls were performed in triplicate. The relative target gene expression was normalized to the endogenous reference gene (18s rRNA) and determined using the DC_T method. Primer sequences: VAV1 sense: ATGACAGCAGAAGGACTGTAC, anti-sense: TGCCAAGGCAACCAGGGCTCAGCAGTA. 18S sense: AACCCGTTGAACCCCATTCGTGAT, anti-sense: AGTCAAGTTCGACCGTCTTCTCAG.

**Proliferation and Survival Assays**

Cells were seeded at low density, then treated with azathioprine (0, 5, or 10 μM) for 4 days. The culture medium was then reserved, and the cells were trypsinized and resuspended in the reserved medium to retain any dead cells. Cell number and death were determined by counting on a hemocytometer in the presence of trypan blue. Alternatively, the cells were treated as described above and analyzed by MTS assay (CellTiter96 Aq Non-Radioactive Cell Proliferation Assay, Promega).

**Animal Studies**

All animal experiments were conducted with the approval of the Mayo Clinic Institutional Animal Care and Use Committee and in accordance with the requirements of the National Institutes of Health. All mice were monitored daily for health and well-being. Female athymic nude mice used for xenograft models were purchased from Harlan. Mice to generate the KPC mouse model (p48^Cre^/+; KRas^{G12D}; p53^{Flox/+}) were provided by Martin Fernandez-Zapico (Mayo Clinic, Rochester, MN) and were described previously (17-19). Mice to generate the PDX-Cre; KRas^{G12D}, p53^{Flox/Flox} and PDX-Cre; KRas^{G12D}, p53^{Flox/R172H} mouse models were provided by Baoan Ji (Mayo Clinic, Rochester, MN). For animal experiments, azathioprine was
solubilized in 0.1 N NaOH, and then diluted in sterile D-PBS and sterile filtered. Mice were treated with 0, 5, or 10 mg/kg body weight azathioprine three times per week via IP injection in a volume of 10 μl/g. For the 0 mg/kg group, mice were injected with D-PBS containing 2mM NaOH, the same final concentration as the azathioprine solution. No adverse events were detected due to azathioprine treatment. Mice were sacrificed upon a weight loss of >10% body weight, or when moribund, by CO2 inhalation or pentobarbital injection (Fatal Plus).

**Xenograft models.** For the subcutaneous xenograft model, 5-6 week old female athymic nu/nu mice were anesthetized by IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and 10x10^6 DanG or BxPC3 tumor cells in resuspended in100 μl growth medium were injected into the left flank. Tumors were allowed to develop over 5 days (DanG) or 25 days (BxPC3) until reaching a volume of approximately 100 mm^3 prior to commencing azathioprine treatment. Mice were matched for tumor size for assignment into treatment groups. Tumor volume was measured at the time of azathioprine treatment. For orthotopic xenograft implantations, 5-6 week old female athymic nu/nu mice were anesthetized by IP injection of ketamine and xylazine, followed by IP injection of Buprenex, and 1x10^6 DanG or L3.6 cells resuspended in 100 μl D-PBS were injected into the head of the pancreas. Azathioprine treatment commenced one week after implantation. Upon necropsy, macroscopic metastases were scored. The target sample size was based on prior studies (20).

**Genetic models.** Male and female p48Cre^+/; LSL-KRasG12D^+/; p53^Flox/+ mice on a mixed genetic background were treated with azathioprine or vehicle 3 times per week beginning at 5 weeks of age until sacrifice. Mice were randomly assigned to treatment groups, balancing male and female mice in each group. When possible, littermates were assigned to different treatment groups. An initial sample size of 19 was selected to provide 80% power to detect differences in
the number of metastases between drug-treated mice and control mice, based on our preliminary results. Mice that did not form primary pancreatic tumors (<10%) were not included in the analysis. In both the orthotopic and genetic mouse model, upon necropsy, the intestinal mesentery, liver, spleen, diaphragm, and abdominal cavity were all inspected for detectable metastatic tumors (≥1 mm diameter), which were counted and measured using calipers.

**Statistical Analysis**

Statistical analyses were conducted using Microsoft Excel or GraphPad Prizm. Data are displayed as the mean +/- SEM. Statistical significance was determined using a Student’s t test. For Kaplan-Meier survival curves, a log-rank test was used. A p value of 0.05 represented a statistically significant difference.

**Results**

**Azathioprine inhibits Vav1 invasion in vitro**

The exchange factor Vav1 is aberrantly expressed in over half of human pancreatic cancers, and markedly amplifies the invasive and migratory properties of tumor cells (7-9). Therefore, we propose that Vav1 is important in promoting the metastasis of pancreatic cancer. Strikingly, in addition to expression in primary tumors, we have found that Vav1 is also expressed in metastatic human tumors. Liver metastases from human PDAC patients were analyzed by quantitative RT-PCR. Aberrant Vav1 expression was found in nearly half of the tumors tested, with a subset having extremely high expression of Vav1 (Figure 1A). These
findings demonstrate Vav1 expression in metastatic tumors, and are consistent with our findings that Vav1 may promote tumor cell invasion. Indeed, depletion of Vav1 from pancreatic tumor cells completely inhibits invasive transwell migration, an \textit{in vitro} model for metastatic invasion (Figure 1B, (8, 9)). Based on these findings and our previous studies, we tested if inhibition of Vav1 could reduce the invasive potential of tumors.

Azathioprine is used clinically as an inhibitor of Vav1 in the immune system (11). Therefore, we hypothesized that azathioprine could also be used to inhibit Vav1 function during invasion and migration in pancreatic cancers. To test this, we first assessed invasion \textit{in vitro} using a transwell invasion assay. To determine if azathioprine was specific for Vav1, we took advantage of multiple pancreatic cancer cell lines, some of which express Vav1 (DanG, CFPAC, Panc04.03), and some of which do not (PANC1, BxPC3, L3.6) (Supplemental Figure 1, (7-9)). The cells were pre-treated with or without azathioprine for two days at 5 \( \mu \text{M} \), a dose that is reported to be physiologically relevant and comparable to that in patients under azathioprine treatment (12). Azathioprine dramatically reduced transwell invasion by DanG, CFPAC, and Panc04.03 cells (Figure 1C), similar to siRNA-mediated depletion of Vav1 (9). In contrast, azathioprine had no effect on transwell invasion by cell types that do not express Vav1 (PANC1, BxPC3, or L3.6, Figure 1D). These findings indicate that azathioprine potently inhibits tumor cell invasion \textit{in vitro}, and suggest that this may occur in a Vav1-dependent manner.

While these findings correlate azathioprine’s anti-invasive effects with Vav1 status, tumor cell lines contain multiple mutations that may account for a differential sensitivity to azathioprine. Therefore, to directly test if azathioprine regulated Vav1-dependent invasion, we manipulated Vav1 expression in one of the isolated cell lines. PANC1 cells, which do not express Vav1, were transfected with either mCherry-tagged Vav1 or mCherry vector, and treated
with azathioprine for two days prior to seeding in a transwell migration assay. Overexpression of Vav1 significantly increased the invasion of the tumor cells. Importantly, this Vav1-dependent invasion was completely blocked by azathioprine treatment (Figure 1E). These data provide strong and direct support of the premise that azathioprine can inhibit Vav1-mediated invasive migration of pancreatic tumor cells.

Metastatic invasion requires the remodeling and degradation of the extracellular matrix in order for tumor cells to escape from the primary tumor and invade into a secondary environment. We have shown that Vav1 contributes to tumor cell invasion through the formation of matrix-degrading invadopodia (9). Therefore, we determined if azathioprine could also inhibit Vav1-dependent matrix degradation. To test this, cells were plated on a fluorescent gelatin substrate, and degradation was measured by the loss of fluorescence from the matrix. Azathioprine treatment significantly reduced the percentage of Vav1-positive cells capable of degrading the matrix, and dramatically reduced the amount of matrix degradation per cell (Figure 2A-C). Importantly, we only observed this inhibition in the Vav1-expressing cells (DanG, CFPAC), but not in cells that do not express Vav1 (BxPC3, or the breast cancer cell line CA1D). Taken together, these data indicate that azathioprine treatment inhibits Vav1-dependent invasive properties in vitro.

To directly test if azathioprine inhibits matrix degradation through Vav1, Vav1 expression was attenuated in DanG cells using siRNA, and the Vav1-knockdown cells were treated with azathioprine and evaluated for matrix degradation. Treatment of the cells with either azathioprine or Vav1 siRNA significantly reduced the percent of cells able to degrade the matrix (Figure 2D-E). However, azathioprine had no further inhibitory effect in the Vav1-depleted cells.
These data indicate that Vav1 is required for the inhibitory effects of azathioprine on matrix degradation, and are consistent with azathioprine inhibiting Vav1-dependent invasion (11).

As an additional component of invasion, we also tested if azathioprine could inhibit cell migration by using a wound healing assay. Azathioprine treatment did inhibit migration by DanG cells, but had no effect on any of the other cell lines tested (Vav1-positive or Vav1-negative), suggesting that inhibition of migration is not the primary mechanism by which azathioprine inhibits tumor cell invasion (Supplemental Figure 2).

Azathioprine reduces Rac and Cdc42 activation in Vav1-expressing pancreatic tumor cells

This study was based on the premise that azathioprine can inhibit Vav1-dependent Rac activation in primary T cells (11, 12). Therefore, we tested if azathioprine affected Rac activation in pancreatic tumor cells. DanG cells were treated with 5 μM azathioprine for 48 hours, and Rac activation was analyzed using a p21-binding protein pulldown assay for active Rac/Cdc42. Indeed, azathioprine treatment caused approximately a 30-40% reduction in Rac activation, consistent with previous reports (Figure 3A, (11, 12)). However, a reduction in Rac activation was not observed in PANC1 cells, which do not express Vav1 (Figure 3B). These data are consistent with the inhibition of Vav1-mediated Rac activation by azathioprine.

Vav1 regulates invadopodia formation and matrix degradation through activation of Cdc42, a GTPase related to Rac but with distinct functional effects (9). However, while 6-thio-GTP binds to Cdc42, it was not previously found to inhibit Cdc42 activation in T cells (11). We therefore tested if azathioprine inhibited matrix degradation through a reduction in the activity of Cdc42. DanG cells were incubated with 5 μM azathioprine for 48 hours, and then active Cdc42 was assessed by biochemical pulldown. Similar to Rac, we observed a 30-40% reduction in the
amount of active Cdc42 in pancreatic cancer cells (Figure 3C). However, in Panc1 cells, which do not express Vav1, azathioprine did not decrease Cdc42 activation (Figure 3D). These data suggest that, in addition to regulating Rac activation, azathioprine can also inhibit Vav1-mediated activation of Cdc42. This likely reflects the role of Vav1 in activating both Rac and Cdc42 in pancreatic cancer cells, whereas Vav1 may be more specific for Rac in T cells.

As this effect on Cdc42 was somewhat surprising (11), we used additional approaches to test the requirement for Cdc42 in azathioprine-mediated inhibition of matrix degradation. First, Cdc42 or Rac were depleted in DanG cells by siRNA-mediated knockdown, followed by incubation with azathioprine for 48 hours prior to assessing matrix degradation on a fluorescent gelatin substrate. Knockdown of Cdc42 reduced the percent of cells degrading the matrix, similar to azathioprine treatment. However, azathioprine had no further inhibitory effect on the Cdc42-depleted cells, suggesting that Cdc42 is required for azathioprine’s inhibitory effects (Figure 3E). In contrast, while knockdown of Rac also reduced the percent of cells degrading matrix, azathioprine treatment further inhibited matrix degradation by 50%, suggesting that azathioprine’s inhibitory effects on matrix degradation are independent of Rac.

Next, we tested if the inhibitory effects of azathioprine could be overcome by constitutive activation of Cdc42 or Rac. DanG cells were transfected with an empty vector, active Cdc42 Q61L, or active Rac1Q61L, and treated with 5 μM azathioprine for 48 hours prior to plating on the fluorescent gelatin substrate. Azathioprine reduced the percent of cells degrading the matrix, and this could be completely reversed by the expression of active Cdc42 Q61L, but not active Rac (Figure 3F). Taken together, these data suggest that azathioprine inhibits both Rac and Cdc42 in pancreatic cancer cells, and that its inhibitory effects on matrix degradation are mediated through a reduction in Cdc42 activity.
Azathioprine reduces Vav1-dependent metastasis in vivo

These promising results using cultured PDAC cells suggested that azathioprine inhibits Vav1-dependent matrix degradation and invasion in vitro. We next extended these findings to in vivo models of pancreatic cancer metastasis. First, an orthotopic xenograft model was utilized using either Vav1 positive (DanG) or Vav1-negative (L3.6) cell lines. The pancreatic tumor cells were injected into the head of the pancreas in athymic nude mice, and the mice were treated with azathioprine or vehicle control (D-PBS) by IP injection for 3 (DanG) or 4 (L3.6) weeks. Upon necropsy, the number of macroscopic metastatic lesions was quantified, with metastases forming primarily in the intestinal mesentery, but also on the liver and in the abdominal cavity. Azathioprine treatment (5 mg/kg) significantly reduced the number of metastasis by 50% compared to the vehicle-treated control (Figure 4A). In contrast, azathioprine had no effect on the metastasis of the Vav1-negative cell line L3.6 (Figure 4B). Together with the in vitro data described above, these findings suggest that azathioprine treatment inhibits Vav1-dependent metastasis of pancreatic tumor cells.

As Vav1 is used clinically to target the immune system, it was important to evaluate its effects on metastasis in an immunocompetent genetic mouse model of pancreatic cancer. As all of the experiments to this point have utilized human Vav1, we tested if mouse Vav1 was similarly sensitive to azathioprine’s anti-invasive effects. PANC1 tumor cells, which do not express Vav1, were transfected with either a mouse Vav1 cDNA or empty vector, treated with azathioprine for two days, then seeded in a transwell invasion assay. Consistent with human Vav1, overexpression of mouse Vav1 significantly increased invasive transwell migration by the tumor cells. Importantly, this Vav1-dependent invasion was completely blocked by azathioprine.
treatment (Supplemental Figure 3A). Therefore, mouse Vav1 can similarly promote the invasive capability of PDAC cells, and also appears as sensitive to azathioprine as the human Vav1 protein (Figure 1E).

We utilized the genetic KPC mouse model (p48Cre+/; LSL-KRasG12D+/; LSL-p53Flox/+) (17, 19), and treated these mice with 0, 5, or 10 mg/kg azathioprine three times weekly beginning at 5 weeks of age, until the animals became moribund. Upon necropsy, the number of metastatic lesions was scored. Nearly 90% of the control mice formed macroscopic metastases, again, primarily to the intestinal mesentery, and occasionally to the liver (30% of mice) and elsewhere in the peritoneal cavity, indicating that the tumors have invasive capability. Strikingly, treatment with azathioprine at 5 mg/kg caused a significant 50-70% reduction in the number of metastases formed (Figure 4C-D), and the number of mice developing metastases was reduced by 40-70%. This resulted in improved survival for the azathioprine-treated mice by 2 weeks, particularly at the 10 mg/kg dose (Figure 4G).

The central hypothesis of this study is that azathioprine could be an anti-metastatic agent for patients with Vav1-positive tumors, consistent with our data that azathioprine selectively inhibits invasion by Vav1-expressing cells. Therefore, we tested the Vav1 expression of the primary and metastatic tumors isolated from the KPC mice by immunoblotting lysates from the primary tumor. Vav1 was robustly expressed in nearly 80% of the tumors formed, consistent with the ectopic expression of Vav1 in pancreatic cancers (Supplemental Figure 3). We next stratified the metastasis data based on the Vav1 expression of the tumors. Strikingly, when Vav1-positive tumors are considered, azathioprine treatment caused an even more significant reduction of pancreatic cancer metastasis (Figure 4E). The number of metastases per mouse was reduced by 70-80%, for both the 5mg/kg and 10mg/kg treatment groups. In addition, the azathioprine-
mediated improvement in survival was enhanced among the mice with Vav1-positive tumors. Treatment with azathioprine increased survival time by 4-5 weeks compared to the control mice, particularly at the 10 mg/kg dose (Figure 4H). Taken together, these data support the model that azathioprine inhibits Vav1 function in pancreatic tumor cells in culture and in mice to inhibit pancreatic cancer cell invasion and metastasis and improve survival.

While azathioprine treatment strongly reduced metastasis, it did not reduce primary tumor size in the genetic mouse model of PDAC (Figure 4F), in the orthotopic mouse model, or in a flank injection model using DanG (Vav1-positive) or BxPC3 cells (Vav1-negative, Supplemental Figure 4A-C). Similar experiments were conducted using more aggressive mouse models of pancreatic cancer (PDX-Cre, LSL-KRas\textsuperscript{G12D/+}, LSL-p53\textsuperscript{Flox/Flox}, or PDX-Cre, LSL-KRas\textsuperscript{G12D/+}, LSL-p53\textsuperscript{Flox/R172H}). However, these mice formed tumors very rapidly and had a mean survival time of only 8 weeks, and importantly, did not develop metastases. Again, azathioprine treatment had no effect on tumor size, and in this aggressive, rapid model, did not improve survival time (Supplemental Figure 4D-E).

Azathioprine treatment did reduce tumor cell proliferation \textit{in vitro}, as measured by a reduction in cell number, without a significant increase in cell death. However, this inhibition was independent of Vav1 status, as both Vav1-positive and Vav1-negative cells responded similarly (Supplemental Figure 5A-C), suggesting this is a Vav1-independent effect of azathioprine. And importantly, this inhibition did not translate to a reduction in tumor burden \textit{in vivo} (Supplemental Figure 4), possibly due to limitations in penetration of the desmoplastic tumor stroma, or due to signals from the supportive tumor microenvironment. Therefore, these data suggest that the effects of azathioprine on Vav1-dependent metastasis and survival are due to its anti-invasive effects, and not due to an effect on cell proliferation or viability.
Discussion

Vav1 expression drives multiple steps in the tumorigenic and metastatic process, indicating that it could be a key node for therapeutic intervention (7-9). Here we extend our basic cell biological findings into preclinical models of pancreatic cancer, and demonstrate that treatment with the drug azathioprine significantly reduced Vav1-dependent tumor cell invasion and metastasis. While reported as a Vav1/Rac inhibitor, the primary known mechanism and function of azathioprine has been as an anti-metabolite that suppresses the immune response and inflammation. This study reveals a surprising role for azathioprine as an inhibitor of metastasis, and illustrates the need for basic research into mechanisms of drug action and into the cell biological pathways regulating disease states.

Azathioprine has been utilized successfully in the clinical setting for over 50 years to treat inflammatory disorders, to treat leukemia, and to suppress the immune system following transplantation. However, it was unclear how azathioprine could induce immune-specific effects. Further, levels of 6-thioguanine incorporation into the DNA did not appear to be sufficient to explain its cytotoxic effects (11, 12, 21). The recent finding that azathioprine inhibits Vav/Rac signaling was quite surprising, but is consistent with both the hematopoietic-specific effects of azathioprine and the hematopoietic expression pattern of Vav1. Our data are supportive of a model where Vav1 is required for the inhibitory effects of azathioprine. Other GEFs are still able to activate Rac and Cdc42 in the presence of azathioprine, consistent with the remaining Rac/Cdc42 activation following azathioprine treatment or Vav1 knockdown (Figure 3 and (8,
The exchange action of other GEFs could certainly be inhibited by azathioprine; however, ectopically expressed Vav1 may be the primary GEF driving matrix degradation, invasion, and metastasis, thereby making sensitivity to azathioprine most obvious in the context of the invading tumor cell.

When used as an immune suppressant or anti-inflammatory agent, azathioprine is taken daily by patients for years, and is reasonably well-tolerated. While side effects such as myelosuppression and pancreatitis may occur in a subset of patients (22, 23), these side effects appear less severe than those of current chemotherapeutic agents, including gemcitabine. In addition, the well-defined side effects of azathioprine will serve as a valuable tool in monitoring pancreatic cancer patient response and in selecting which patient populations would best respond to azathioprine as an anti-metastatic agent. Further investigation into the properties of Vav1 that are specific to pancreatic cancer cells may guide the development of more selective agents related to azathioprine to minimize off-target effects.

Azathioprine is used clinically as an anti-inflammatory agent. Inflammation is a major factor contributing to the progression and metastasis of most cancers, including pancreatic cancer (24-26). Therefore, it is possible that the anti-metastatic effects of azathioprine could be attributed, in part, to its inhibition of the inflammatory response, in addition to inhibition of Vav1’s pro-invasive function in the isolated tumor cells. Azathioprine likely also has Vav1-independent mechanisms of action that regulate invasion (27). It will be important to define the impact of azathioprine on inflammation associated with pancreatic cancers, as this could be used to guide its therapeutic applications, particularly for patients with early-stage or pre-cancerous inflammatory lesions of the pancreas.
Despite the fact that the metastatic invasion of tumors is the central cause of cancer death, anti-metastatic drugs that specifically target mechanisms of invasion are not utilized as part of a therapeutic program for pancreatic cancer, or any cancer, and are grossly understudied in preclinical models. Critically, specific pharmacologic inhibition of metastasis may not result in a reduction of primary tumor size, and therefore may not significantly alter survival in mice, which become moribund due to the primary tumor burden (28). This has likely masked the beneficial effects of potential anti-metastatic agents in pre-clinical models, where tumor growth is often the primary readout. As such, in human patients, anti-metastatic therapies would need to be used in combination with a means of targeting the primary tumor, such as resection or cytotoxic chemotherapy and/or radiation, and ideally would be coupled with earlier tumor detection. Particularly in PDAC, an additional obstacle to cancer treatment is the access of chemotherapies into a dense, desmoplastic tumor. This obstacle could be bypassed by a specific targeting of metastatic cells, which generally invade as single cells or clusters. Finally, some current methods of cancer treatment are associated with an increase in metastatic dissemination, such as the inflammation following surgical resection or specific chemotherapeutic agents (29-31). These findings further underscore the critical strategy of blocking metastatic spread as a part of cancer therapy.

As anti-metastatic strategies move into clinical trials, it will be critical to designate which patients would benefit most from this intervention. Anti-metastatics, defined here as agents that inhibit mechanisms of invasion but that might not affect primary tumor growth, should be strongly considered for individuals at early stages of disease, even prior to detectable metastasis, and for patients following resection to minimize further spread or recurrence. For azathioprine specifically, our findings suggest that patients with Vav1-positive pancreatic tumors would have
greatest benefit. However, even patients with seemingly Vav1-negative primary tumors may develop metastases from a subset of Vav1-positive cells. Clearly, a more complete understanding of the mechanisms underlying pancreatic cancer metastasis will be required to better define approaches to inhibit tumor dissemination.

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References:


Figure Legends

Figure 1. Azathioprine inhibits transwell invasion by Vav1-expressing pancreatic tumor cells. (A) Vav1 is expressed in metastatic tumors from human PDAC patients. Q-PCR was used to analyze Vav1 transcripts (normalized to 18S) in liver metastases from 35 patients. Vav1 is expressed in nearly half of metastatic tumors, and is highly expressed in a subset of patients. (B) Depletion of Vav1 inhibits invasive transwell migration. DanG cells were depleted of Vav1 by siRNA, then transwell migration was assessed using a blind-well chamber. A representative immunoblot showing Vav1 knockdown is shown. (C-D) Vav1-positive (Panc04.03, DanG, or CFPAC, C), or Vav1-negative (BxPC3, L3.6, PANC1, D) cells were pre-treated with vehicle (black bars) or azathioprine (5 μM, white bars) for 2 days, then seeded in a transwell invasion assay for 20 hours. Azathioprine reduced invasive migration by the Vav1-positive cells, but not the Vav1-negative cells. (E) PANC1 cells were transfected with empty mCherry vector or mCherry Vav1, treated with vehicle or azathioprine for 2 days, then seeded in a transwell migration assay. Vav1 expression increased invasive migration of PANC1 cells, and this was completely inhibited by azathioprine treatment. For (B-E), the percent of cells invaded across the filter was scored and normalized to vehicle-treated cells. Graphed data represent the mean +/- SEM of 3 independent experiments. ** p<0.01, ns: no statistically significant difference (p>0.05).

Figure 2. Azathioprine inhibits Vav1-dependent matrix degradation by tumor cells. (A) Vav1-positive (DanG, CFPAC) or Vav1-negative (BxPC3, CA1D) tumor cells were pre-treated with azathioprine for 2 days, then plated on fluorescently-tagged gelatin in the presence of
azathioprine for 7 (DanG) or 24 (CFPAC, BxPC3, CA1D) hours. Blackened regions indicate areas of matrix degradation. (B) The percent of cells capable of degrading the matrix was scored for each cell type. (C) For the DanG cells, the area of matrix degradation was quantified and normalized to the total cell area. (D) Vav1 was depleted in DanG cells by siRNA, and the cells were treated with azathioprine as described above. The percent of cells degrading the matrix was scored. Vav1 depletion reduced matrix degradation, and there was no further inhibition by azathioprine treatment. (E) Vav1 knockdown in the DanG cells was confirmed by immunoblotting. Graphed data indicate the mean +/- SEM of at least three independent experiments. * p<0.05, ** p<0.01, ns: no statistically significant difference (p>0.05).

Figure 3. Azathioprine reduces activation of Rac and Cdc42 in Vav1-expressing tumor cells. (A-B) DanG pancreatic tumor cells, which express Vav1, or PANC1 cells, which do not express Vav1, were treated with azathioprine (Aza) for 2 days, then Rac activity was assessed by GST-PBD pulldown and immunoblotting for Rac. Levels of active Rac were quantified and normalized to total Rac for each sample, and then normalized to the vehicle-treated control cells for each experiment. (C-D) DanG or PANC1 cells were treated as described in (A) but immunoblotted for Cdc42 to detect Cdc42 activity. (E) DanG cells were transfected with a nontargeting control siRNA or siRNAs targeting Cdc42 or Rac1, then treated with azathioprine for 2 days prior to assessing matrix degradation by plating on fluorescent gelatin for 7 hours. The percent of cells degrading the matrix was quantified (n>100 cells per condition). Knockdowns were verified by immunoblotting. (F) DanG cells were transfected with empty vector or myc-tagged active Cdc42 Q61L or active Rac Q61L, treated with azathioprine for 2 days, and plated on a fluorescent gelatin substrate for 7 hours. Transfected cells were visualized by
immunofluorescence for the myc epitope tag. The percent of cells degrading the matrix was quantified (n>100 cells per condition). Graphed data represent the mean +/- SEM of at least three independent experiments.

Figure 4. Azathioprine inhibits metastasis in mouse models of pancreatic cancer. (A-B) Orthotopic xenograft tumor model. Vav1-positive (DanG, A) or Vav1-negative (L3.6, B) pancreatic tumor cells were implanted in the head of the pancreas of nude mice, and then the mice were treated with azathioprine or vehicle three times per week via IP injection for 3 weeks (DanG) or 4 weeks (L3.6). Upon necropsy, the number of macroscopic metastatic tumors was scored and graphed as the mean +/- SEM. (C-H) Genetic mouse model of pancreatic cancer. Mice with the genotype (p48<sup>Cre</sup>+, LSL-KRas<sup>G12D/+</sup>, p53<sup>FloX/+</sup>) were treated with azathioprine three times per week beginning at 5 weeks of age until sacrifice (mean survival 22 weeks). (C) Control mice formed multiple metastatic lesions, particularly to the intestinal mesentery (arrows), whereas metastasis was reduced in the azathioprine-treated mice. (D-E) Upon necropsy, the number of macroscopic metastatic tumors was scored for all mice (D), or mice with Vav1-positive primary tumors (E). Very few of the drug-treated mice with Vav1 expressing tumors exhibited metastatic dissemination. Red points: primary tumors with high Vav1, blue points: primary tumors with low/no detectable Vav1, black points: Vav1 status unknown. Bars indicate the mean number of metastases. (F) Azathioprine had no effect on the primary tumor weight. (G-H) Kaplan Meier survival plot demonstrating improved survival with azathioprine treatment for all mice (G) or mice with Vav1-positive primary tumors (H). Red lines: control 0 mg/kg, black lines: 5 mg/kg, blue lines: 10 mg/kg. Graphed data in A, B, and F represent mean +/- standard error. * p<0.05.
Figure 2

A. Vav1-positive
DanG  CFPAC

Azathioprine:
0 μM
5 μM

Vav1-negative
BxPC3  CA1D

B. Bar graphs showing the percentage of cells degrading matrix for different cell lines and treatments:

- **DanG**
  - 0 μM: 80%
  - 5 μM: 60%
- **CFPAC**
  - 0 μM: 35%
  - 5 μM: 20%
- **BxPC3**
  - 0 μM: 70%
  - 5 μM: 70%
- **CA1D**
  - 0 μM: 70%
  - 5 μM: 70%

C. Bar graph showing the area of degradation (% of cell) for DanG:

- 0 μM: 30%
- 5 μM: 10%

D. Bar graph showing the percentage of cells degrading matrix for DanG under different siRNA conditions:

- Control: 80%
- Vav1: 30%

E. Western blot showing protein expression levels for Vav1 and Actin:

- Control: Vav1 and Actin levels
- Vav1: Increased Vav1 levels but no change in Actin levels
Figure 3

A B C D
DanG PANC1 DanG PANC1
Aza: 0 μM 5 μM Aza: 0 μM 5 μM
Active Rac Active Rac Active Cdc42 Active Cdc42
Total Rac Total Rac Total Cdc42 Total Cdc42

E F
siRNA: Control Cdc42 siRNA: Control Rac
siRNA: Control Rac
Actin Actin

% of cells % of cells
degrading matrix degrading matrix
siRNA: Control Cdc42 Rac1
siRNA: Control Cdc42 Q61L Rac1 Q61L

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Targeting pancreatic cancer metastasis by inhibition of Vav1, a driver of tumor cell invasion


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