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EFFECT OF DIALLYL TRISULFIDE ON HEDGEHOG SIGNALING IN PANCREATIC CANCER CELLS

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EFFECT OF DIALLYL TRISULFIDE ON HEDGEHOG SIGNALING IN PANCREATIC CANCER CELLS

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
ADAM	a disentigrin and metalloproteinase
APC	anaphase promoting complex
ATR	ataxia telangiectasia and Rad3 related
CI	confidence interval
DATS	diallyl trisulfide
Disp	Dispatched
GRX	Glutaredoxin
Hh	Hedgehog
Hhat	Hedgehog acyltransferase
kDa	kilodaltons
MMP	matrix metalloproteinase
OR	odds ratio
OSC	organosulfur compound
PDAC	pancreatic ductal adenocarcinoma
p-H3	Serine 10 phosphorylated Histone H3
Ptch	Patched

PC	pancreatic cancer
ROS	reactive oxygen species
Smo	Smoothened
Shh	Sonic Hedgehog
ShhN	Sonic Hedgehog N-terminal peptide
SuFu	Suppressor of Fused Homolog
TRX	Thioredoxin
VEGF	vascular endothelial growth factor

ABSTRACT

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With an increasing global cancer burden, new methods of cancer prevention are desired to improve overall survival. In particular, pancreatic cancer (PC) is in desperate need of new strategies to improve the dismal 8% overall 5-year survival rate. Studies have shown aberrant activation of the Hedgehog pathway to be associated with very poor prognosis in multiple cancer types, including pancreatic. Prevention strategies utilizing dietary agents have been shown to regulate multiple cancer hallmark pathways, but little is known regarding their effect on Hedgehog. Here we observed diallyl trisulfide (DATS), an anti-cancer agent found in garlic, reduced colony formation and inhibited Hedgehog signaling in PC cells. Treatment of AsPC-1 PC cells with DATS resulted in inhibition of mRNA and protein expression of the transcription factor Gli1. Protein levels of the Sonic Hedgehog pathway initiating ligand as well as the Patched receptor were increased following DATS treatment. Additionally, DATS reduced ectopic expression of Gli1. These results give further support for the anti-cancer role of DATS and provide rationale for future investigation into its use as a chemopreventive agent in PC.

CHAPTER 1. INTRODUCTION

In the United States, an estimated 1.6 million people will be diagnosed with cancer leading to 595,000 deaths in 2016 [1]. The American Cancer Society has estimated that the global cancer burden will grow from 12.3 million cases and 7.6 million deaths in 2007 [2] to nearly 22 million new cases and 13 million deaths by the year 2030 [3]. A 2007 report by the World Health Organization also estimated that by the year 2010, cancer would overtake ischemic heart disease as the leading cause of death globally [4]. In 2016, cancer has become the leading cause of death in 21 states in the United States [1], but remains the second leading cause of death in high income countries, and the third leading cause of death in developing countries [3]. So while over 1.7 million cancer-related deaths have been averted since 1991 in the United States alone [1], cancer remains a serious present and future public health concern.

Pancreatic cancer (PC), with just an 8% overall 5-year survival rate, is a large source of cancer related mortality in the United States and around the world [1]. While PC is estimated to represent just over 3% of new cancer cases, the 41,780 deaths estimated to be attributed to PC in 2016 ranks third behind lung cancer and colorectal cancer [1]. Of the various types of PC, pancreatic ductal adenocarcinoma (PDAC) represents about 90% of cases and is nearly always fatal [5]. In general, PC tumors are classified as resectable, locally advanced (which is further divided into borderline resectable and non-resectable groups), or advanced [6–9]. The median overall survival ranges from 20-24 months for primarily resectable tumors [7] to less than 12 months from the time of diagnosis for patients with advanced PC [8]. According to the 2016 Cancer Statistics Report, over 50% of patients present after distant metastasis has occurred while just 9% of cases are diagnosed early enough that surgery, currently the

most successful curative option, is a viable prospect [1, 9]. In patients who undergo surgery, adjuvant chemoradiation and/or chemotherapy using gemcitabine based drugs or polychemotherapy regimens may be administered in hopes of curing the patient of their disease or improving their survival [6, 8–10]. Many studies have been conducted investigating the outcomes of these approaches yielding mixed results when comparing overall survival of surgery plus adjuvant therapy with surgery alone [6, 10]. Another approach, neoadjuvant therapy, is an option for patients with resectable or locally advanced cancer. Neoadjuvant therapy is given before surgery in hopes of downstaging the tumor to a point that resection and subsequent adjuvant therapy may be more successful [7]. This approach has been shown to improve overall survival between 1.8 and 10 months depending on tumor classification at the outset [6–9]. Some have debated, though, whether the potential benefits of neoadjuvant therapy may be outweighed by the risks regarding uncertain tumor histology at the outset as well as the possibility of delaying surgery to the point it becomes unfeasible [6, 7]. For patients with advanced PC, palliative care aimed at prolonging survival is the main treatment option. In September 2013, the FDA approved the addition of nab-paclitaxel to gemcitabine chemotherapy regimens for treatment of metastatic PC as the combination increased median survival by 1.8 months while having an acceptable toxicity profile [8]. In spite of the advances made in PC treatment, overall 5-year survivorship has changed very little since 1975 [1]. An 80-85% rate of relapse persists and long term effective solutions for advanced disease are largely absent [9].

Because of this, novel chemopreventive strategies aimed at inhibiting the advance of PC have become the subject of significant research. Whereas the goal of primary prevention is to stop cancer from developing, many studies investigate secondary and tertiary prevention techniques focused on being able to “block, retard, or reverse the carcinogenic process” [11]. The discovery and development of such chemopreventive agents may improve overall survival of PC by reducing aspects of an individual’s cancer burden including but not limited to tumor growth and spreading.

For centuries, whole foods including fruits, vegetables, and spices have been used to prevent and treat a variety of maladies such as wounds, inflammation, and infection. More recently, characterization of bioactive agents derived from these whole foods have been shown to display anti-microbial, anti-inflammatory, and antioxidant effects [12]. Consumption of many of these same foods have also been shown to be correlated with reduced cancer risk of various types including those of the colon, breast, liver, lung, and pancreas [13–18]. Investigation of the anti-cancer effects of bioactive compounds derived from fruits, vegetables, and spices is of great interest as their hopeful clinical application may prove a valuable tool in cancer prevention. One bioactive compound, diallyl trisulfide (DATS), is derived from *Allium* vegetables which have specifically been shown to be associated with reduced cancer risk of various types [19–28]. To date, though, few clinical studies have been completed using DATS. Rather, cell based and preclinical studies provide ample evidence that DATS regulates multiple cancer hallmark pathways including cell cycle, apoptosis, angiogenesis, invasion, and metastasis [29]. DATS has been shown to arrest cancer cells at multiple stages of the cell cycle with the G2/M transition arrest being the most widely studied. Induction of G2/M arrest via regulation of proteins favoring reduced G2/M passage and transition exit has been shown following DATS treatment of multiple cancer types [30]. Additionally, increased pro-apoptotic capacity as a result of regulating intrinsic and extrinsic apoptotic pathway components has been observed following treatment with DATS [30]. Angiogenesis, invasion, and metastasis, three of the largest contributors to cancer mortality, represent emerging targets of DATS that support its continued investigation as a cancer preventive compound [30].

Another pathway associated with cancer progression, the Hedgehog (Hh) pathway, lies at the crossroads between aforementioned cancer hallmarks and stem cell characteristics including plueripotency, enhanced tumorigenicity, and drug resistance [31]. Studies have shown that Hh activity is associated with increased expression of anti-apoptotic protein Bcl-2, cell cycle progression protein CyclinD1, and angiogenesis promoting factor VEGF among many other cancer-associated factors [32]. Additionally,

Hh signaling supports stem cell persistence by modulating expression of stemness regulators Oct4 and Sox2 [33] as well as drug resistance proteins ABCB1 and ABCG2 [32]. In one study, it was determined that, of the 24 PC tumors analyzed, each one contained mutations in Hh pathway related genes suggesting the Hh pathway plays an important role in PC etiology [34]. While the Hh pathway has been shown to be regulated by multiple bioactive compounds including sulforaphane from cruciferous vegetables [35, 36], curcumin from turmeric [37–41], and genistein from soy [37, 42], no studies to date have been published examining the effect of DATS on the Hh pathway. Because of this gap in scientific knowledge, the role of Hh as a regulator of important cancer related pathways, and the apparent importance Hh plays in pancreatic tumorigenesis, we investigated the role of DATS in regulating Hh signaling in PC cells.

Characterization of the Hh pathway was first completed in *Drosophila* in 1980 where it was found to regulate polarity and segmentation of the developing embryo [43]. In vertebrates, Hh pathway activity is largely limited to embryonic development, but has been shown to have a role in pancreas tissue repair in mice [44, 45]. During human development, Hh signaling is vital in regulating the formation of limbs, the central nervous system, organs of the gastrointestinal tract, and craniofacial structures [46–51]. Though the precise mechanisms are yet unknown, the Hh pathway has been shown to be aberrantly activated in multiple cancer types, including pancreatic [35, 52–55]. Increased expression of Hh pathway constituents including Shh, Ptch, Smo, and Gli1 have been shown in PC tissues compared with normal pancreas tissues [56–58]. Furthermore, expression of these constituents is associated with adverse cancer outcomes including larger tumor size [58], presence of lymph node metastasis [58], advanced TNM stage (a combined measure of primary tumor growth, lymph node involvement, and presence of metastases) [56, 58], and reduced survival [56, 59].

A schematic diagram of Hh pathway signaling is presented in Figure 1.1. In humans, signal transduction of the Hh pathway begins with synthesis of Hh ligands in ligand secreting cells. Sonic Hedgehog (Shh) is the most widely studied Hh ligand in cancer etiology while Desert and Indian Hedgehog are largely associated with male

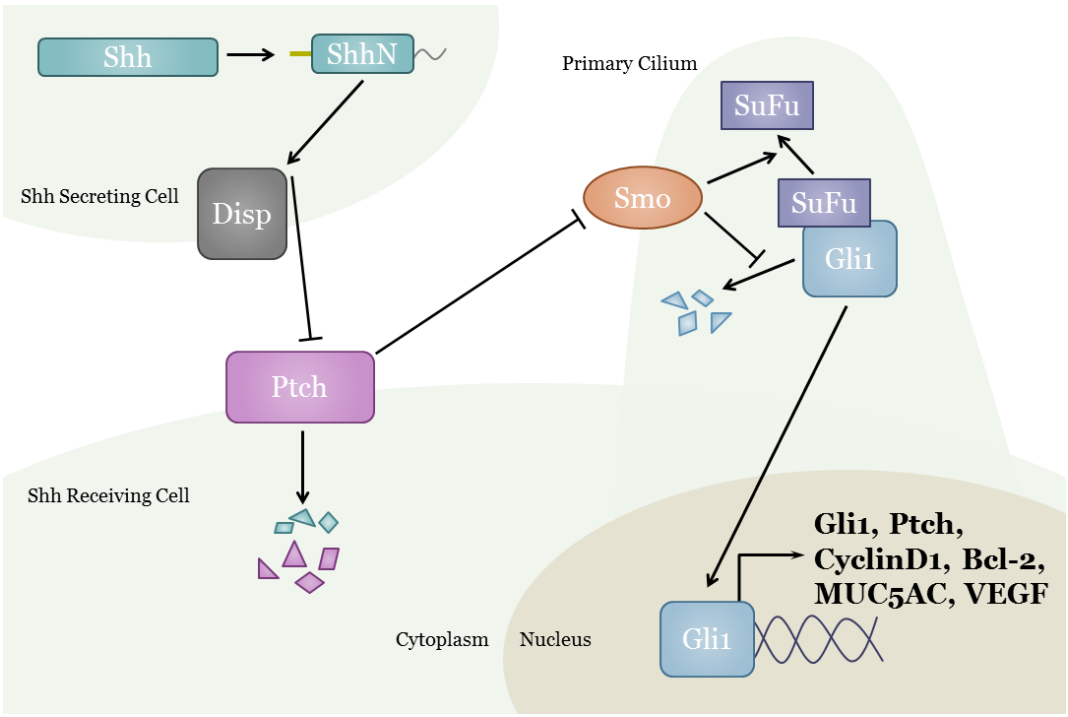

Figure 1.1: Schematic diagram of Hh pathway signaling

germ cells and cartilage development respectively [50]. Shh is translated as a 45 kilodalton (kDa) precursor protein that is auto-proteolitically cleaved into a 26 kDa C-terminal peptide and a 19 kDa N-terminal peptide (ShhN). The C-terminal fragment catalyzes the cleavage and acts in the addition of a cholesterol moiety to the carboxyl terminus of ShhN. Further modification of ShhN by Hedgehog acyltransferase (Hhat) adds a palmitate group to the amino terminal cysteine. The resulting dually lipidated molecule has greatly enhanced signaling activity compared to unlipidated and monolipidated ShhN likely due in part to its ability to multimerize on the cell surface [60]. Shh release is controlled by the protein Dispatched (Disp) as well as multiple members of the a disentigrin and metalloproteinase (ADAM) family including ADAM10 and ADAM17 [61]. Solubilized Shh travels within the tissue microenvironment and interacts with the 12-transmembrane domain receptor protein Patched (Ptch) largely in a paracrine fashion. Following ligand-receptor binding, Ptch is ubiquitinated, the complex is internalized, and subsequently degraded by the lysozyme [62]. Interaction of

Shh with Ptch also releases Ptch-mediated inhibition of the 7-transmembrane domain, G-protein coupled receptor protein Smoothened (Smo). A conformational change in Smo results in its translocation to the primary cilia where it releases the transcription factor Gli1 from suppression by Suppressor of Fused Homolog (SuFu). Gli1 then translocates to the nucleus where it binds promoter sequences and enhances transcription of downstream targets involved in cell cycle progression, evasion of apoptosis, stem cell character, angiogenesis, and epithelial-mesenchymal transition.

We hypothesized that DATS exerts anti-cancer effects on PC cells by down regulating the Hedgehog pathway. We found that treatment of MIAPaCa-2 and BxPC-3 PC cell lines with DATS resulted in reduced colony formation. Additionally, AsCP-1 cells displayed reduced Gli1 mRNA and protein expression following DATS treatment. Protein expression of Ptch and Shh were increased upon incubation with DATS. Overexpression of Gli1 did not show a significant effect on cell cycle and apoptosis pathways although this study require further investigation to better understand how DATS regulates these pathway components. But, the observed reduction in expression of pathway effector Gli1 suggests that DATS may ultimately inhibit the Hh pathway.

CHAPTER 2. THE ANTI-CANCER EFFECT OF DIALLYL TRISULFIDE

2.1 Introduction

Plants of the *Allium* genus have long been known to have medicinal qualities. As far back as the ancient Chinese in 2700 BC, people have used onions and garlic to prevent and treat a variety of ailments [63, 64]. Research has determined that organosulfur compounds (OSCs) are the bioactive agents responsible for the observed beneficial effects. DATS, the most prominent fat soluble OSC found in garlic, is known to regulate disease states including cancer, infection, and metabolic syndrome [65]. Diet derived bioactive compounds represent attractive anti-cancer preventive strategies as they are generally inexpensive, readily available across the world, and considered to have minimal side effects. In recent years, epidemiologic studies have linked consumption of *Allium* vegetables to decreased incidence of cancer. As a result, much research in cell culture and animal models has been directed at investigating the precise molecular mechanism by which these compounds exert their beneficial effects. Results indicate that DATS modulates pathways involved in cancer progression including chemical metabolism, cell cycle, apoptosis, angiogenesis, and migration and invasion. To date, one study investigating DATS as a clinical intervention for cancer prevention has been completed. The following chapter reviews findings of DATS-related cancer research and discusses its use as an anti-cancer agent.

2.2 Epidemiological Studies

In 2007, the World Cancer Research Fund and American Institute for Cancer Research reported that garlic “probably” protects against development of colorectal

cancer [66]. In support of this claim, multiple meta-analyses and epidemiological studies correlate *Allium* vegetable or garlic intake with reduced risk for myeloma [22] as well as gastric [21, 26–28], colorectal [19, 25], endometrial [20], lung [23], and prostate [24] cancer. Conversely, there are instances of null findings in some stand-alone cohort studies [67, 68] and in cohort specific evaluations of meta-analyses [24, 25]. Two cohort studies reporting a null association between garlic intake and colorectal cancer analyzed the Nurse's Health Study, Health Professionals Follow-Up Study, and Cancer Prevention Study II Nutrition Cohort [67, 68]. It is worth noting that both used self-administered food frequency questionnaires and considered a serving of garlic as one clove or 4 shakes of garlic powder or garlic salt [67, 68]. The reported use of garlic powder and garlic salt within these studies is not disclosed, but it is possible that use of these ingredients accounted for intake without providing the same protective effect as fresh garlic cloves [67, 69]. The use of mailed food frequency questionnaires may also partially explain the null findings observed in the aforementioned cohort studies. Results of one meta-analysis of *Allium* vegetable intake and prostate cancer risk reported more significant risk reductions in studies utilizing face-to-face interviews with nutrition experts (odds ratio (OR)=0.70, 95% confidence interval (CI): 0.59-0.84) compared with those using self-administered food frequency surveys (OR=0.89, 95% CI: 0.78-1.02) [24]. Likewise, a case-control study employing face-to-face interviews indicated risk ratios of 0.92 (95% CI: 0.79-1.08) and 0.56 (95% CI: 0.44-0.72) for lung cancer corresponding to raw garlic consumption <2 times/week and ≥2 times/week respectively compared to individuals who never consumed raw garlic [23].

Consuming 20g of *Allium* vegetables per day is associated with an OR of 0.91 (95% CI: 0.88-0.94) for gastric cancer [21]. Comparing highest and lowest garlic intakes, two recent meta-analyses reported a risk ratio of 0.49 (95% CI: 0.38-0.62) for gastric cancer [27] and 0.85 (95% CI: 0.72-1.00) for colorectal cancer [25]. In one meta-analysis, high and low intake were gauged on such diverse measures as yearly, weekly, and daily consumption as well as subject-assessed ratings of "high" or "low" [27]. Such disparity in

intake quantification may partially explain the mixed results of previously mentioned epidemiological studies. Cultural and ethnic differences must also be considered. Greater risk reduction associated with garlic intake was observed in Asian and South American populations than in European populations in some studies [24, 27] while similar results across continents were observed in another investigation of total *Allium* vegetable intake [21]. The potential protective effect of garlic supplementation has also been studied. Similar to *Allium* vegetable intake, data regarding garlic supplementation use have shown an inverse association with cancer risk (hazard ratio = 0.55, 95% CI: 0.34-0.87) when comparing high use (≥4 times per week) with non-users [70] as well as null results [67, 68, 71]. Little description is given about the type of garlic supplement used (powder, aged garlic extract, etc.) which may explain the mixed findings.

2.3 Synthesis, Metabolism, and Pharmacokinetics

Fresh garlic contains a mixture of water, fiber, carbohydrates, protein, and fat as well as more than 20 vitamins and minerals and at least 33 sulfur containing compounds [72, 73]. One of the primary precursors of OSCs in garlic is γ-glutamyl-S-alk(en)yl-L-cysteine [69] (Figure 2.1). Within the garlic clove, this compound is hydrolyzed and oxidized into S-alk(en)yl-L-cysteine sulfoxide (alliin) which accumulates naturally during storage at cool temperatures [63, 69]. Processing garlic by cutting or chewing ruptures vacuoles containing alliinase [63, 69]. This enzyme converts alliin to allicin and other thiosulfinates responsible for the odiferous nature of garlic [63, 69]. The allicin yield is about 2.5mg/g of garlic which correlates to 5-20mg per clove [72]. Allicin quickly decomposes into products including diallyl sulfide, diallyl disulfide, and DATS with yields of 30-100, 530-610, and 900-1100 µg/g respectively [69, 72].

In pharmacokinetic studies, DATS was shown to be 99% absorbed in rabbits [74] while its precursor, allicin, was 79% absorbed in animals within 30-60 minutes [75]. Following ingestion of 730 µmol of DATS in human subjects, breath acetone increased reaching a maximum at around 20 hours [76]. Allyl methyl sulfide, a known component

of breath and putative breakdown product of OSCs, was also increased reaching maximum concentration around the 5 hour time point [76]. While the precise mechanism of DATS metabolism remain elusive, these data suggest DATS is rapidly metabolized but not before causing acetone production to increase by some unknown mechanism. No pharmacokinetic studies in humans have been able to detect DATS in urine or plasma following garlic ingestion. Following a 10 mg dose of DATS into the jugular vein of rats, though, DATS plasma concentration peaked at 5.5 µg/mL (31 µM) within 1 minute followed by a steady return to baseline levels within 24 hours [77]. Using a microemulsion intravenous injection technique in rats to deliver 30 mg/kg DATS, plasma concentration peaked at 7.06 µg/mL (40 µM) within 3 hours [78]. Compared to the current technique of using Tween 80 to emulsify DATS, microemulsion resulted in a slower clearance rate possibly because of delayed penetration and distribution to

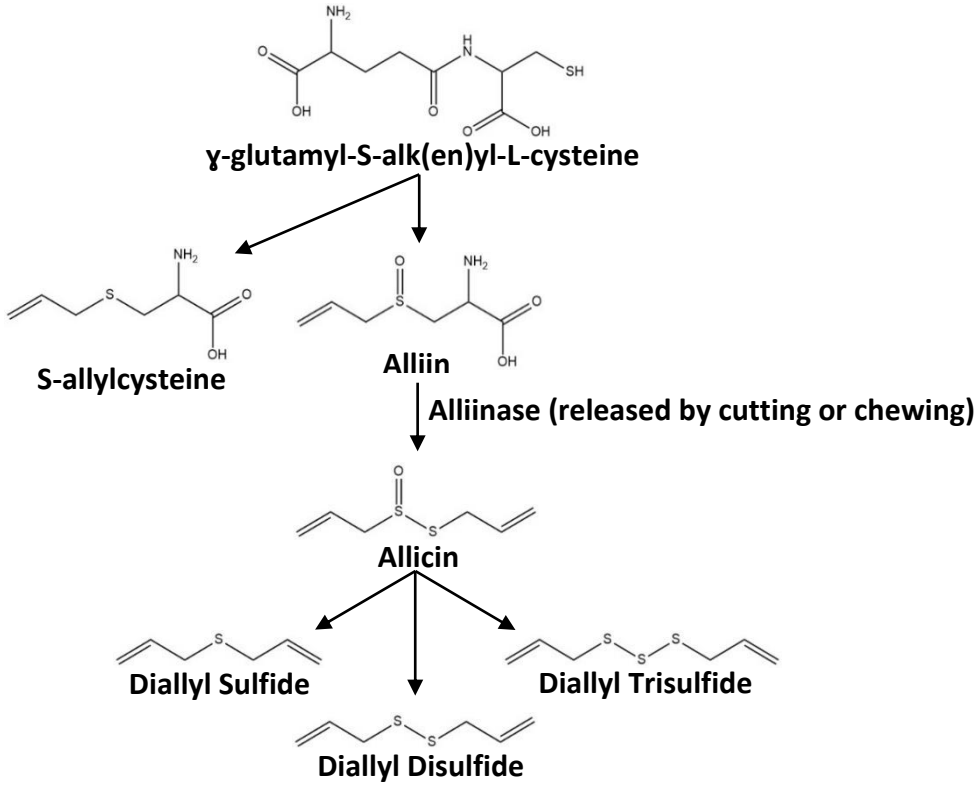

Figure 2.1 Synthesis pathway of OSCs in *Allium* vegetables

tissues [78]. Using intravenous doses of DATS avoids the first pass effect of the liver which may explain why researchers were able to detect plasma DATS in these studies. Future pharmacokinetic studies of DATS are vital for its investigation as a chemopreventive agent. Understanding achievable concentrations following intravenous dose as well as oral ingestion are paramount. In order to achieve accurate results, compound stability must also be considered. Researchers have shown that stability of DATS in rat blood is influenced by purification technique, storage temperature as well as duration, and freeze-thaw cycles [77].

No focused metabolic studies in humans using DATS have been published at this time. Recently, DATS was shown to directly interact with the antioxidant response axis by reacting with the Nrf2 regulator Keap1 [79]. Multiple cysteine residues of β-tubulin are also targeted by DATS with implications in microtubule polymerization [80]. The pharmacokinetics of DATS are poorly understood as multiple metabolic studies have instead focused on SAC, another OSC derived from garlic [81]. As is the case with many bioactive metabolites, it is believed that the kidneys as well as the liver participate in conjugation reactions with DATS resulting in excretion, reabsorption, or further metabolism [82]. In order to effectively study anti-cancer mechanisms in a clinical setting, DATS-specific metabolic and pharmacokinetic studies should be a focus of future research.

2.4 *In vitro* Mechanism of Action

Much of the past and current research into the DATS mechanism of action concerns induction of cell cycle arrest, increased programmed cell death, inhibition of migration and invasion, and reduced angiogenesis. The results of many *in vitro* studies utilizing DATS are summarized in Table 2.1. A schematic of *in vitro* and *in vivo* molecular mechanisms are depicted in Figure 2.2.

2.4.1 *In Vitro* Cell Cycle Arrest

Dysregulation of important cell cycle regulatory mechanisms is an initiating event in cancer development allowing for uncontrolled cell cycle progression and rapid tumor growth. A common theme in re-gaining proper cell cycle regulation is maintaining normal levels of active cyclins, cyclin dependent kinases, and regulatory units that act on these proteins. Many have shown DATS to induce G2/M arrest [80, 83–100] and further elucidation of the precise molecular mechanisms involved in cell cycle blockade provides additional support for the anti-cancer role of DATS.

DATS has been shown to induce cell cycle arrest by enhancing generation of reactive oxygen species (ROS) [87, 88, 97, 99, 101]. One pathway of ROS generation begins with degradation of the iron storage protein ferritin which has been shown following DATS treatment leading to an increase in labile iron pool (LIP) size [97, 101, 102]. Then, through the Fenton/Haber-Weiss reaction, free ferric iron reacts with superoxide and hydrogen peroxide to form hydroxyl radicals and hydroxide ions [88, 103]. These and other ROS are involved in biological processes such as DNA damage and cell death signal transduction [103]. ROS induced DNA damage may explain the rapid phosphorylation and activation of the DNA damage sensing protein Chk1 [93]. In Chk1 expressing prostate cancer cells, DATS induced accumulation of CyclinB1, securin, and serine 10 phosphorylated Histone H3 (p-H3) corresponding to mitotic arrest [93]. Cells expressing wild type ataxia telangiectasia and Rad3 related (ATR), another DNA damage sensing protein, showed similar accumulation [89]. Prostate and colon cancer cells displayed less accumulation of these targets following Chk1 knockdown and expression of inactive ATR respectively [89, 93]. Together, these data suggest accumulation of cells in G2/M may be partially dependent on DNA damage checkpoint proteins Chk1 and ATR [89, 93].

DATS treatment has been shown to regulate downstream targets of another DNA damage sensing protein, p53, including Cdc25C, Cdk1, and Wee1 [85, 87, 88, 92–94, 96]. These proteins are well characterized cell cycle regulators further supporting the

idea that DATS induces cell cycle arrest through inducing DNA damage. Cdc25C, a regulatory phosphatase that promotes passage into mitosis, has been well characterized by multiple investigators [94, 104]. The reduced Cdc25C protein levels triggered by DATS were reversed with antioxidant pre-treatment suggesting oxidative stress may account for decreased expression [88, 94]. This finding is consistent with data indicating that oxidation of cysteine residues at amino acids 330 and 377 negatively impacted Cdc25C stability [104]. In contrast to these findings, overexpression of wild type Cdc25C as well as expression of protein mutated at key cysteine residues failed to rescue prostate cancer cells from DATS-induced G2/M arrest suggesting a dispensable role of Cdc25C in this model [94].

DATS-mediated arrest specifically during passage into mitosis has been examined by several investigators [80, 85, 87, 89, 92–94, 96, 100, 105, 106]. During the G2/M transition, Cdc25C mediated dephosphorylation of Cdk1 and ensuing Cdk1/CyclinB1 complex formation promote entrance into mitosis as a result of enhanced kinase activity. Although DATS prompted an increase in CyclinB1 protein, treatment also reduced Cdc25C expression and increased levels of tyrosine 15 phosphorylated Cdk1 leading to inactivation of the Cdk1/CyclinB1 complex [80, 85, 87, 89, 92, 93, 96, 100, 105, 106]. Without adequate levels of Cdk1 and CyclinB1 co-localized in the nucleus, cells are unable to move past the G2/M checkpoint. Following the start of DATS treatment, CyclinB1 appeared in the nucleus within 1 hour while Cdk1 was not observed in the nucleus until between hours 2 and 4 [94]. These findings may clarify the transient nature of G2/M arrest as cells eventually regained Cdk1/CyclinB1 complex kinase activity after 8 hours of DATS treatment [94]. Interestingly, a small percentage of treated cells have even shown the ability to escape G2/M arrest during 24 hours of continuous DATS treatment [94]. Another group detailed gradual return to normal cell cycling following cessation of a 12 hour DATS treatment [90]. Based on these findings, future research directions in cell cycle regulation need to focus on clarifying specific time points at

which a DATS treatment regimen may achieve maximum arrest specifically in G2/M phase.

Mitotic exit is dependent on an active anaphase promoting complex (APC) made up of Cdc27, Cdc20, and Cdh1 subunits [93]. DATS promoted inactivation of Cdc27 and Cdh1 by maintenance of phosphorylation [89, 93]. Similarly, phosphorylated Cdh1 and Cdc20 were more prevalent in ATR wild type cells than in cells expressing inactive ATR [89]. The APC substrate securin, which must be degraded for sister chromatids to separate, was increased following DATS incubation [89, 93]. This is thought to be a result of decreased APC activity rather than up regulation of securin protein [89, 93]. After DATS treatment, staining of α-tubulin showed fluorescence exclusively surrounding the nucleus rather than throughout the whole cell as observed in control cells [87, 93]. This data agrees with the finding that DATS disrupted the microtubule network in colon cancer cells [80, 90]. Researchers observed depolymerization of the microtubule network as a result of oxidative modification of cysteine residues in β-tubulin [80]. Taken together, these results suggest DATS may also induce G2/M arrest by reducing the ability of chromosomes to separate during the mitotic phase of the cell cycle.

While multiple reports indicate no change in the fraction of S phase arrested cells [105, 106], evidence exists supporting both increased [105, 107, 108] and decreased [87, 92, 93, 106] G0/G1 fractions following DATS treatment. Cell line specific and technique differences may account for these discrepancies as treatment dose as well as duration play important roles in cell cycle regulation. In PC cells, DATS was shown to have an inhibitory effect on cell cycle progression protein CyclinD1 while simultaneously enhancing protein levels of the Cdk inhibitor CDKN1A ($p21^{Cip1/Waf1}$) [106, 109]. These results corroborate results in prostate [87] and breast [105, 110] cancers following DATS treatment.

2.4.2 Induction of Programmed Cell Death

Various mechanisms exist to protect cells from unrestricted growth. In cancer cells, these mechanisms are dysregulated leading to rapid tumor progression. Enhancing programmed cell death is a promising anti-cancer strategy aimed at reducing tumor advance. Apoptotic processes are divided between caspase-dependent and caspase-independent pathways. Caspase dependent mechanisms can be further separated into intrinsic (mitochondria mediated) and extrinsic (death receptor mediated) processes. A growing body of evidence suggests that DATS acts by prompting cancer cells to obey cell death signals including DNA damage, oxidative stress, and cellular damage.

In addition to its role in cell cycle arrest, ROS generation is known to induce cell death [91, 96, 111–115]. As mentioned previously, ferritin degradation and subsequent increased LIP size is one mechanism by which ROS are generated following DATS treatment. Augmented expression of p66Shc and Itch E3 ligase induced by DATS are part of the mechanism by which ferritin is degraded [97, 101, 102, 116]. These proteins are further implicated in cell death signal propagation as their reduced wild type expression resulted in resistance to the effects of DATS [97, 102]. The role of LIP size in DATS-mediated cell death recently came into question by the finding that iron chelation afforded no protection from DATS-induced cytotoxicity [102]. Further research is required to characterize the role of iron in cancer cells as high iron status may have a substantial impact on promoting neoplastic progression [117].

Results across many cancer cell types have observed activation of the intrinsic apoptotic pathway following DATS treatment. Increased activation of the apoptosis regulating protein JNK has been shown across many studies after DATS incubation [91, 111, 114, 118–121]. One cause of JNK activation may be related to generation of another potent ROS, hydrogen peroxide. Under non-stress conditions the JNK activator ASK1 is held inactive by the ubiquitous redox sensing proteins Thioredoxin (TRX) and Glutaredoxin (GRX). Certain stressors promote oxidation of the intermolecular disulfide bond between TRX and GRX causing dissociation and subsequent activation of ASK1.

Enhanced ASK1 dissociation in addition to JNK phosphorylation following DATS treatment were recently observed in breast cancer cells [120]. Furthermore, ASK1 activation was inhibited following catalase transfection implicating DATS-induced production of hydrogen peroxide as the putative source of TRX:GRX bond oxidation [120].

Increased expression of p53 has been shown to have pro-apoptotic action in pancreatic [106], lung [91], breast [105], and skin [96, 99] cancer cells. DATS treatment increased nuclear translocation of p53 in breast and PC cells and decreased expression of MDM2, a negative regulator of p53 [105, 106]. Downstream targets of p53 include members of the Bcl-2 family of proteins involved in regulating intrinsic apoptosis [122]. Thus, DATS treatment is known to increase apoptosis by regulating expression of pro-apoptotic Bax [92, 95, 98–100, 105, 106, 113, 115, 121, 123–125], Bak [92, 112, 115, 123], Bid [92], Bad [114], PUMA [123, 125], and NOXA [123] as well as anti-apoptotic Bcl-2 [91, 92, 95, 98, 99, 105, 106, 112–114, 119, 121, 123–127] and Bcl-XL [92, 99, 112, 121, 123, 127]. Moreover, the pro-apoptotic protein Bim, which is a downstream target of JNK, displayed increased phosphorylation following DATS incubation thus clarifying one connection between DATS treatment, ROS generation, and induction of apoptosis [120, 123]. It is noteworthy that, although Bcl-2 overexpression did not provide protection from DATS-induced apoptosis in one prostate cancer cell line, Bax and Bak knockdown conferred partial resistance [92, 112]. These data suggest DATS-mediated apoptosis may be attributed to enhanced pro-apoptotic action of Bax and Bak rather than to reduced anti-apoptotic activity of Bcl-2 and Bcl-XL. Results show that enhanced apoptotic action may be brought about by translocation of pro-apoptotic proteins to the mitochondrial membrane due to disruption of the interaction with Bcl-2 [99, 119] and decreased interaction with 14-3-3 proteins [128].

Localization of pro-apoptotic proteins to the mitochondrial membrane leads to mitochondrial membrane depolarization [91, 96, 99, 129, 111–114, 121, 123], calcium release from the endoplasmic reticulum [96, 99, 111, 130], and mitochondrial release of

various apoptosis regulating proteins. Results investigating apoptosis inhibitors consistently support the decrease of XIAP expression [100, 121, 123, 131] following DATS treatment, but cell specific discrepancies exist regarding regulation of cIAP [121, 123, 131] and survivin [91, 123, 131]. Release of Smac [111] and expression of HtrA2 [96, 99] have been shown to be up regulated following DATS treatment signifying reduced inhibition of cell death. Following release from the mitochondria, cytochrome C is known to complex with Apaf-1 to initiate cleavage of pro-caspase-9 thus activating caspase-dependent apoptosis through executioner caspase-3. Reports of enhanced cytochrome C release [84, 99, 111–113, 121, 123, 124, 127], increased Apaf-1 protein[99], enhanced caspase-9 expression and activity [96, 99, 121, 123, 124, 127], and greater cleaved PARP [92, 95, 96, 99, 114, 121, 127, 128] further support the extensive pro-apoptotic capabilities of DATS. Many pro-apoptotic effects have been shown to be partially abolished following superoxide dismutase overexpression [115] or pre-treatment with an antioxidant such as N-acetylcysteine [91, 99, 112, 114] suggesting they are ROS dependent.

In addition to the intrinsic apoptotic mechanism, DATS is known to affect the extrinsic apoptosis pathway. Extrinsic apoptosis is initiated through binding of an extracellular death ligand such as TRAIL or Fas to a death receptor (DR) on the cell membrane. The subsequent signaling cascade results in caspase-8 activation which also activates caspase-3. It is well established that LNCaP prostate cancer cells are resistant to TRAIL-induced apoptosis while PC-3 cells are TRAIL sensitive [123]. DATS treatment was able to sensitize LNCaP cells to TRAIL induced apoptosis and synergize with TRAIL to enhance apoptosis in PC-3 [123]. Greater expression of extrinsic pathway constituents such as DRs as well as their ligands has been observed in other cancer types following DATS treatment [91, 105, 106, 121]. Additionally, downstream events including increased expression and activity of caspase-8 [121, 123, 124, 132] and caspase-3 [80, 84, 93, 96, 99, 100, 108, 111–113, 121, 123, 124, 126, 127] have been observed in many studies.

Components of the endoplasmic reticulum stress-mediated apoptosis pathway including BiP, CHOP, and caspase-4 were shown to be increased in one study following DATS treatment [99]. Addition of a caspase inhibitor did not completely reverse the effects of DATS in a basal cell carcinoma model implying apoptotic action also occurs through caspase independent pathways [99]. In support of this hypothesis, researchers observed increased nuclear levels of DNA fragmentation proteins AIF and EndoG [99]. Autophagy, another caspase independent pathway with potential for causing cell death, has been studied following treatment with DATS precursor allicin, but not with DATS itself [129]. Understanding the cross-talk with apoptosis as well as the prospect of characterizing the dual nature of autophagy necessitate further autophagy-focused research in cancer cell lines following treatment with DATS.

Results from *in vitro* studies have shown DATS to be more potent at reducing cell viability and proliferation than other OSCs including diallyl disulfide and diallyl sulfide [92, 96, 114, 130, 133–135]. Investigation of nine trisulfides found those with three carbon chains provided the most inhibition of cell viability compared to compounds with longer chains [136]. Similar experiments ordered the efficacy of trisulfides at inducing apoptosis as follows: bis(2-methylallyl) trisulfide > DATS ≈ dibutenyl trisulfide ≈ dipentenyl trisulfide [98]. It is important to note that DATS is considered minimally toxic to non-transformed cells at concentrations as high as 160 μM [87, 92, 101, 112, 115, 130]. Though some levels of ROS were generated in the normal breast epithelial cell line MCF-10A following DATS incubation, Bax and Bak expression as well as apoptotic figures remained unchanged [115]. While these data provide an explanation for how MCF-10A cells avoid apoptosis, mechanistic studies may help to elucidate how these cells avoid altering apoptosis-related gene expression in spite of ROS production. Such research may provide insight into how transformed cells evade apoptosis during cancer progression and promote understanding of the mechanisms by which normal cells are protected from the effects of DATS.

2.4.3 Inhibition of Migration and Invasion

Cancer spreading is a result of migration events leading to tumor growth in new areas of organs as well as invasion events leading to growth in other tissues. It is well understood that the spreading of cancer throughout the body is a major cause of mortality. Furthermore, a cancer diagnosis in late stages, after spreading has occurred, represents up to a 13.5 fold decrease in survival compared to diagnosis during early, localized stages [1]. These statistics make apparent the need for anti-cancer strategies aimed at reducing migration and invasion.

Recent *in vitro* work has revealed inhibition of migration and invasion proteins following DATS treatment. Expression and activity of MMP -2, -7, and -9 were inhibited indicating decreased ability to degrade basement membranes [107, 133, 134, 137, 138]. Known inhibitors of MMPs, TIMP-1 and -2, were increased upon DATS treatment leading to enhanced tight junction formation between bladder cancer cells [137]. In this report, claudin expression was shown to decrease following DATS treatment [137]. Expression of different claudins and their relation to an invasive phenotype are highly contextual as lines of evidence exist claiming claudins both promote [139] and inhibit [140, 141] migration, invasion, and metastasis. These results necessitate further research to elucidate the cell-specific relationship between claudin expression and overall invasiveness characterization. Specific upstream metastasis-related proteins were reduced following DATS treatment including iNOS, Grb2, Ras, COX-2, PI3-K, JNK1/2, and p38 [133, 138]. While enhanced JNK1/2 expression was shown to be responsible for apoptosis induction [91, 114, 119, 120], others have shown JNK2 to enhance cell migration [142]. Further research is required to better characterize this apparent dichotomy. Cell surface proteins vimentin and E-cadherin, which are also known to be involved in the epithelial-mesenchymal transition, were regulated following DATS treatment to favor increased adhesion [115]. Regulation of the JAK/STAT pathway may also contribute to migration and invasion of cancer cells as ectopic expression of STAT3 in LNCaP cells resulted in enhanced migration [143]. DATS treatment reduced migration

by blocking STAT3 phosphorylation events related to dimerization and nuclear localization [143].

2.4.4 Reduced Angiogenesis

Because cell culture models contain no vasculature, *in vitro* investigation of angiogenesis often examines protein levels of angiogenesis proteins as well as the effect of treatment on angiogenesis model systems. VEGF secretion is responsible for increasing size, number, and penetrance of tumor vasculature required for each incremental increase in tumor size [138]. DATS treatment has been shown to reduce expression and secretion of VEGF as well as multiple upstream angiogenesis proteins in different cancer cell types [107, 138]. Human umbilical vein endothelial cells treated with DATS also demonstrated decreased VEGF secretion, decreased capillary-like tube formation, and reduced VEGF receptor expression [138, 144]. Incubating these endothelial cells with conditioned media from DATS treated osteosarcoma cells also resulted in decreased capillary-like tube formation compared to incubation with media from untreated cells [107]. It was shown that these effects were not caused by DATS presence in the media but rather by the presence or absence of secreted factors within the conditioned media [107].

2.4.5 Interaction with Hormone Regulated Cancer Types

Hormone signaling by estrogen and androgens are known to be key factors in progression of breast and prostate cancers respectively [110, 145]. Regulation of hormone related signaling in these cancer types not only displays the diverse effects of DATS but provides further evidence that DATS may be a useful anti-cancer agent. In breast cancer, estrogen sensitivity and HER-2 expression are important factors in patient prognosis. Recent research involving breast cancer cell lines differing in estrogen sensitivity and HER-2 status showed diminished cell viability upon DATS treatment [110, 115]. The observed reduction in cell viability was unaltered following ER-α

overexpression in the triple negative breast cancer cell line MDA-MB-231 [110]. These data implicate DATS as an attractive anti-cancer agent because of its broad ability to decrease cell viability in breast cancer cells regardless of ER-α and HER-2 status. DATS treatment also reduced estrogen receptor mRNA, protein levels, and reporter activity in ER-α positive breast cancer cell lines MCF-7 and T47D [110].

In prostate cancer, the effect of androgen dependence has been investigated by comparing the effect of DATS in androgen independent PC-3 and androgen dependent LNCaP cell lines. Both underwent significant apoptosis following DATS treatment [112, 119]. Upon Bcl-2 overexpression, PC-3 cells were protected from apoptosis [119] while apoptosis was unchanged in androgen sensitive LNCaP cells [112]. These data suggest DATS may be an effective anti-cancer agent in prostate cancer but implies a differential role of the androgen receptor in regulating apoptosis. DATS also reduced androgen receptor mRNA level, protein level, transcriptional activity, and PSA secretion in prostate cancer cells [145]. Furthermore, DATS reversed androgen receptor nuclear translocation and cell proliferation induced by the androgen analog R1881 [145].

2.4.6 Other Mechanisms

Regulation of cellular responses to stressors has long been considered an important anti-cancer mechanism. Enhanced mRNA levels of phase II detoxifying enzymes NADPH:quinone oxireductase and heme oxygenase were observed following DATS treatment of various cancer cells [79, 118, 146]. Induction of phase II enzymes is a desirable goal especially in preventing cancer initiating events as cells are able to better inactivate and remove mutation causing carcinogens [147]. The stress-sensing protein Nrf2 and the antioxidant response axis may be partially responsible for this observed effect. In one study, Nrf2 expression was positively correlated with expression of multiple phase II genes [118] while another study observed reversal of phase II gene induction in DATS treated cells transfected with Nrf2 siRNA [79]. Under non-stress conditions, Nrf2 is degraded in the cytoplasm through interaction with Keap1.

Introduction of stressors modifies cysteine residues on Keap1 resulting in Nrf2 accumulation, translocation to the nucleus, and induction of downstream targets. Increased Nrf2 protein levels and augmented antioxidant response element activity have been observed in liver cancer cells following DATS treatment [118]. In fact, DATS may directly be involved in Nrf2 signaling as the mass of a mono-allyl sulfide moiety was recently observed bound to the protein fragment containing cysteine 288 of Keap1 following DATS treatment of gastric cancer cells [79]. This amino acid was shown to be necessary for regulating Nrf2 activity as a mutation resulted in failed induction of target genes [79]. Determining the interaction of DATS with specific transcription factors and their regulators by mass spectrometry represents an exciting area of research as it may provide a direct link between DATS treatment and its downstream effects. The anti-cancer role of DATS may also extend into sensitizing cancer cells to chemotherapy. Differential effects of DATS on drug resistance proteins observed in multiple reports indicate a need for continued research to define these molecular mechanisms [73, 148].

Altered cellular signaling of key pathways involved in growth, differentiation, and development exemplify the breadth of mechanisms affected by DATS. Inhibition of the mTOR, NF-κB, and MAPK cascades provide further evidence that DATS limits the survival capacity of cancer cells [79, 91, 95, 97, 100, 101, 105, 106, 114, 116, 119, 121, 128, 133, 134]. HDAC inhibition as well as increased acetylation of the promoter for IκB-α regulator MT2A in gastric cancer cells provides one mechanism by which DATS may regulate NF-κB [100]. The Notch developmental and stem cell related pathway has also been shown to be affected by DATS in osteosarcoma cells [107]. Decreased levels of transcriptional activator proteins such as Notch-1 intracellular domain, Hes, and Hey were observed upon DATS incubation along with reduced downstream expression of CyclinD1 [107]. In the same study, researchers observed increased expression of miR-143 and miR-145 along with decreased expression of miR-21 [107]. In another stem cell related study, DATS reduced the $CD44^{high}/CD24^{low}/ESA^{+}$ population as well as ALDH activity in breast cancer cells [135]. Researchers proposed that reduced FoxQ1 protein

expression as a result of DATS treatment caused this effect as well as the observed reduction in mammosphere growth, induction of apoptosis, and modulation of cell cycle regulatory proteins [135]. Further investigation is required in these emerging areas of cancer research to characterize the roles of epigenetics, developmental and stem cell pathways, and miRNAs as well as their response to anti-cancer agents.

2.5 *In Vivo* Mechanism of Action

Because of the ability to more adequately re-create a tumor specific microenvironment, examine tumors as they relate to whole organisms, and relate results to the endpoint of survival, *in vivo* models are a desirable next step in cancer research following *in vitro* studies. Common pathways affected are often similar to *in vitro* experiments and results act as rationale for potential future human studies. A summary of *in vivo* results is shown in Table 2.2.

2.5.1 Prevention of Chemical Carcinogenesis

Reduction of chemical related carcinogenesis was among the first anti-cancer strategies investigated using DATS and this has been reviewed by others [30, 149]. Briefly, experiments investigating the activity of chemical metabolizing enzymes in mice following DATS incubation display suppression of phase I enzymes responsible for activation of chemical carcinogens and induction of phase II enzymes responsible for detoxification [150]. Recently, DATS was shown to induce phase II enzymes NADPH:quinone oxireductase and heme oxygenase in the stomach of mice [79]. Additionally, Nrf2 was shown to be indispensable for DATS-mediated phase II enzyme induction as Nrf-/- and Nrf2 siRNA transfected cells from mouse embryos showed abrogated expression of NADPH:quinone oxireductase and heme oxygenase [79].

2.5.2 Reduced Tumor Growth and Increased Apoptosis

Many results from *in vivo* studies of various cancer models detail decreased tumor volumes as well as lower tumor incidence and multiplicity following DATS treatment [100, 114, 123, 124, 131, 135, 138, 148, 151–153]. The DNA binding ability of mitogenic transcription factor AP-1 and activity of cell survival pathway constituent Akt were shown to be reduced following DATS treatment [151]. Additional regulation of c-Myc, phosphorylated mTOR, TNF-α, IL-6, IKK, IκB-α, and NFκB expression observed in multiple models correspond to additional inhibition of growth signaling and survival pathways as a result of DATS treatment [100, 123, 151, 154]. Topical application of 25 μM DATS prior to application of a carcinogen was shown to attenuate expression of cancer progression marker COX-2 [151]. These results support the idea that DATS may serve as useful anti-cancer agents *in vivo* by reducing cancer progression.

Subcutaneous glioblastoma tumors displayed enhanced apoptotic activity through reduced Bcl-2 expression, increased Bax protein levels, and greater caspase-3 activation [153]. DATS treatment of orthotopically implanted prostate cancer cells in nude mice revealed an increased pro-apoptotic:anti-apoptotic signal ratio by modulation of Bax, Bak, Bcl-2, and Bcl-XL along with greater DR protein levels [123]. Further regulation was observed with TRAIL co-treatment demonstrating the interplay between caspase-dependent mechanisms [123] In a TRAMP model of prostate cancer, decreased XIAP protein along with increased survivin have been documented following DATS administration [131].

While hematopoietic cancers do not display solid tumors, reduced cancer progression was evident following DATS treatment of nude mice intraperitoneally injected with murine leukemia cells [108]. Enhanced phagocytic activity and natural killer cell cytotoxicity were observed in DATS treated mice following 10 mg/kg treatment for 14 days [108]. In cancer bearing mice, B-cell proliferation was reduced with DATS treatment [108]. Non-leukemic mice showed no change in phagocytosis or B-cell

proliferation representing another instance of the relative innocuous nature of DATS toward un-transformed cell types [108].

2.5.3 *In Vivo* Cell Cycle Arrest

Increased cell cycle arrest following DATS treatment was recently connected to reduced histone deacetylase activity and enhanced histone H3 and H4 acetylation [153]. Persistent acetylation surrounding negative regulators of the cell cycle may diminish checkpoint passage thereby inhibiting cell cycle progression [153]. Enhanced p53 expression, MDM-2 degradation, reduced Cdc25C, increased CDKN1A ($p21^{Cip1/Waf1}$), and greater phosphorylated Cdk1 induced G2/M arrest following 1 week of DATS treatment of glioblastoma tumors [153]. Increased CyclinB1 and securin protein are viable downstream endpoints that further support a large body of evidence suggesting DATS promotes cell cycle arrest *in vivo* [100, 154].

2.5.4 Inhibition of Migration, Invasion, and Angiogenesis

In vivo models allow investigators to study migration, invasion, and angiogenesis more completely. A recent publication detailed reduced angiogenesis in chick embryos following DATS treatment supplemented with VEGF [138]. This supports prior findings that 4 weeks of daily 40 mg/kg DATS decreased microvessel density along with VEGF and IL-6 expression [123]. In a glioblastoma xenograft model, VEGF expression was reduced following 7 days of DATS treatment [153]. Decreased hemoglobin concentration in tumor sections was observed in colon cancer xenograft and allograft models treated with 50 mg/kg DATS suggesting anti-angiogenic action [138, 152]. However, a 2 mg/kg DATS treatment of TRAMP mice did not decrease number of vessels, diameter of vessels, or expression of angiogenesis marker CD31 [154]. These results indicate that, while DATS treatment may directly influence angiogenesis in some studies, dosage must be considered.

Decreased expression of MMP-2, -7, and -9 as well as p-STAT3 were observed in a mouse models supporting the role of DATS as an inhibitor of migration and invasion [123, 143]. Enhanced differentiation represents an exciting anti-cancer prospect of DATS treatment as cells with an undifferentiated phenotype are often responsible for invasion, migration, and metastasis. Investigators observed a decreased incidence of poorly differentiated prostate carcinoma in dorsolateral prostates of 1 mg and 2 mg DATS treated mice [154]. Increased area of well differentiated carcinoma and prostatic intraepithelial neoplasm were also observed [154]. These data suggest DATS may be able to inhibit the transformation from a well differentiated to poorly differentiated phenotype and reduce lung and lymph node metastases [154]. In a zebrafish model, DATS treatment decreased the number of metastatic foci as well as maximal metastatic distance of triple negative breast cancer cells [134].

2.6 Clinical Studies

Multiple human trials have been undertaken investigating the anti-cancer capacities of garlic-derived compounds [155–160], but only one clinical study has specifically examined the anti-cancer role of DATS [161]. Researchers administered 200 mg synthetic DATS daily along with 100μg selenium every other day for 1 month per year for 3 years in a Chinese population at moderate to high risk for developing gastric cancer based on presence of stomach disorders, family history of cancer, or lifestyle factors such as smoking or alcohol consumption [161]. In the first 5 years of follow-up, the relative risk in the intervention group was 0.67 (95% CI: 0.43 – 1.03) for malignant tumors and 0.48 (95% CI: 0.21 – 1.06) for gastric cancer [161]. The intervention reduced morbidity of all cancers by 22% and reduced gastric cancer morbidity by 47% [161]. Following adjustment for factors including family history of cancer, alcohol consumption, and age, the relative risk associated with the DATS intervention reached significance for malignant tumors (relative risk = 0.51 (95% CI: 0.30 – 0.85)) and gastric cancer (relative risk = 0.36 (95% CI: 0.14 – 0.92)) in men only. Researchers also report this dose of

synthetic DATS was well tolerated. The results of this trial indicate a need for future clinical trials investigating the effects of garlic-derived bioactive compounds, specifically DATS. Dose, duration, cancer type, and cancer stage must also be selectively studied to determine the anti-cancer efficacy of DATS.

2.7 Conclusions

Accumulating evidence has shown DATS to regulate many cancer-related pathways including but not limited to chemical detoxification, cell cycle arrest, cell death, inhibition of invasion and migration, and reduced angiogenesis. Epidemiological studies provide background support for the beneficial health properties of garlic that humans have known for centuries by correlating *Allium* vegetable intake with reduced cancer risk. Many similar outcomes from cell based and preclinical models detail the molecular mechanisms involved in the anti-cancer effects of DATS as well as provide rationale for further investigation. Additionally, the limited use of garlic compounds in clinical cancer research has yielded enough positive data to warrant continued exploration. Similar anti-cancer properties observed in experiments utilizing other diet-derived compounds such as curcumin, sulforaphane, and resveratrol emphasize the potential usefulness of diet in reducing cancer progression [162]. As such, continued elucidation of established and novel molecular mechanisms regulated by DATS must be completed. While all levels of research must be sustained, emphasis of future studies should focus on DATS-related pharmacokinetics and metabolism to better support the design and execution of clinically impactful research.

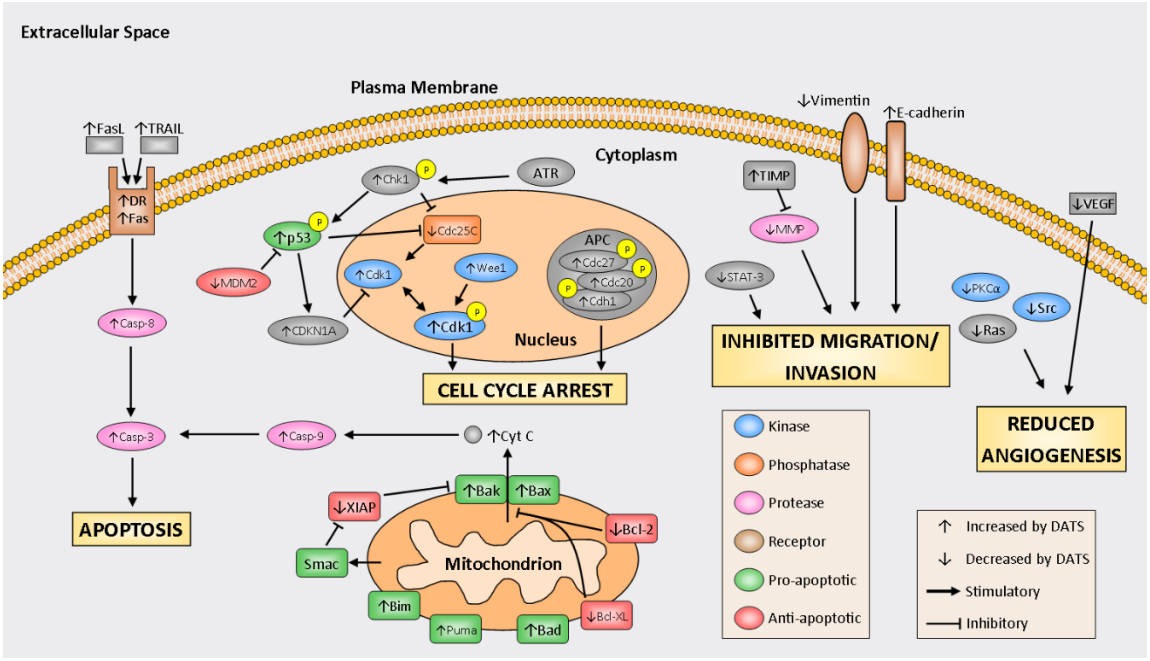

Figure 2.2: Summary of molecular mechanisms regulated by DATS

Table 2.1: Summary of *In vitro* studies utilizing DATS

Cancer Type	Molecular Targets	Mechanism	Model	Reference
Prostate	↑ CyclinB1; ↓ Cdk1; ↑ P-Cdk1; ↓ Cdk1/CyclinB1 kinase activity; ↑ CDKN1A ($p21^{Cip1/Wap1}$); α-tubulin disruption; ↓ Cdc25C, ↑ P-Cdc25C; ↑ Cdc25C:14-3-3β; ↑ ROS	G2/M arrest	PC-3, DU145	[87]
	↓ ferritin L/H (by proteasomal degradation); ↑ LIP; ↑ ROS; ↓ P-Cdc25C	G2/M arrest	PC-3, DU145	[88]
	↓ APC activity (maintain P-Cdc27/ P-Cdc20); ↓ Chk1; ↑ P-Histone H3; ↑ P-Securin; ↑ CyclinB1; ↑ H_2O_2	G2/M arrest	PC-3, DU145	[89]
	α-tubulin disruption; ↑ P-Histone H3; ↑ CyclinB1; ↓ Cdk1; ↑ P-Cdk1; ↓ Cdc25B/C; ↓ APC activity (↑ Cdc27; ↑ P-Cdc27; ↑ P-Cdh1); ↑ securin; ↑ P-Chk1; ↑ Caspase-3 activity; ↑ p53; ↑ P-p53	G2/M arrest, apoptosis	LNCaP	[93]
	↓ P-Akt; ↑ P-ERK1/2; ↑ P-p66Shc; ↓ Ferritin H; ↑ LIP; ↑ ROS	G2/M arrest, apoptosis	PC-3	[97, 101]
	↓ Bcl-2; ↓ Bcl-XL;↑ Bak; ↓ ΔΨm; ↑ cytosolic Cytochrome C; ↑ Caspase-3; ↑ ROS	Apoptosis	LNCaP	[112]
	↓ Ferritin; ↑ LIP; ↑ ROS; ↑ P-Itch	Apoptosis	PC-3	[102]
	↓ Bcl-2; ↑ P-Bcl-2; ↓ Bax:Bcl-2 heterodimer; ↑ Caspase-3/9; ↑ P-JNK; ↑ JNK activity; ↑ P-ERK1/2; ↑ ERK1/2 activity	Apoptosis	PC-3, DU145	[119]
	↑ DR4/5; ↓ Bcl-2; ↓ Bcl-XL; ↓ Mcl-1; ↑ Bax; ↑ Bak; ↑ Bim; ↑ NOXA; ↑ PUMA; ↓ XIAP; ↓ survivin; ↓ cIAP1/2; ↑ Caspase-3/9 activity; ↓ ΔΨm	Apoptosis	PC-3, LNCaP	[123]
	↓ P-Akt; ↓ Akt activity;↓ P-Bad; ↓ IGF-1R; ↓ P-IGF-1R; ↓ PI3-K; ↑ BAD mitochondrial translocation; ↑ Caspase-3; ↑ cleaved PARP; ↓ Bad:14-3-3β	Apoptosis	PC-3, DU145	[128]
	↓ XIAP (by proteasomal degradation); ↑ cIAP1; ↑ survivin; ↑ survivin mRNA	Apoptosis	PC-3, LNCaP	[131]

Table 2.1 (Continued)

	↓ P-JAK2; ↓ P-STAT3; ↓ STAT3 nuclear localization	Apoptosis, inhibited migration and invasion	LNCaP, DU145	[143]
	↓ Androgen receptor mRNA; ↓ AR (not by proteasomal degradation); ↓ P-AR; ↓ AR nuclear translocation; ↓ AR promoter activity; ↓ secreted PSA	Altered hormone signaling	LNCaP, LNCaP-C4-2, TRAMP-C1	[145]
Colon	↑ CyclinB1; ↑ Caspase-3 activity; inhibit spindle formation (β-tubulin oxidation); ↑ P-Histone H3	G2/M arrest	HCT-15, DLD-1, HT-29	[80, 90]
	↑ P-Histone H3; ↑ CyclinB1; ↓ Cdk1; ↑ P-Cdk1; ↓ ACP activity (↑ P-Cdh1); ↑ securin; ↑ P-Chk1; ↑ Caspase-3 activity	G2/M arrest, apoptosis	HCT-116	[93]
	↑ ROS; ↓ ΔΨm; ↑ Caspase-3/9 ; ↑ Caspase-3/9 activity; ↑ Cytochrome C; ↑ Bax; ↓ Bcl-2	Apoptosis	Primary colorectal cancer cells	[113]
	↓ MMP-2/7/9; ↓ PKC; ↓ ERK1/2; ↓ COX-2; ↓ Ras	Inhibited migration and invasion	colo205	[133]
	↓ MMP-2/7/9 mRNA; ↓ MMP-2/7/9; ↓ VEGF mRNA; ↓ VEGF secretion; ↓ iNOS; ↓ COX II; ↓ uPA; ↓ H/K/N-Ras; ↓ RhoA; ↓ FAK; ↓ SoS, ↓ PI3-K; ↓ MKK7; ↓ MEKK3; ↓ ERK1/2; ↓ P-ERK; ↓ JNK1/2; ↓ p38; ↓ ROCK-1; ↓ Src; ↓ PKCα	Reduced angiogenesis, inhibited migration and invasion	HT-29, HUVEC	[138]
Breast	↑ CyclinB1; ↑ CDKN1A ($p21^{Cip1/Waf1}$); ↑ Bax mRNA; ↑ Bax; ↑ p53 mRNA; ↑ p53; ↑ p53 nuclear translocation; ↑ Fas mRNA; ↑ Fas; ↓ Bcl-2 mRNA; ↓ Bcl-2; ↓ MDM2; ↓ Akt mRNA; ↓ Akt; ↑ CyclinD1 mRNA; ↑ CyclinD1	Apoptosis	MCF-7	[106]

Table 2.1 (Continued)

	↓ ER-α mRNA; ↓ ER-α; ↓ nuclear ER-α; ↓ ER-α promoter activity; ↓ pS2; ↓ CyclinD1; ↓ Pin-1	Apoptosis, altered hormone signaling	MCF-7, MDA-MB-231, T47D	[110]
	↓ ΔΨm; ↑ Caspase-7; ↑ cleaved PARP; ↓ Bcl-2; ↑ P-Bcl-2; ↓ P-Bad; ↓ ERK1/2 activity; ↓ P-Akt; ↑ P- JNK; ↑ JNK activity; ↑ ROS; ↑ AP-1 DNA binding activity (↑ c-Jun; ↑ P-c-Jun; ↑ c-Jun nuclear translocation)	Apoptosis	MCF-7	[114]
	↑ ROS;↑ Bax; ↑ Bak; ↑ E-cadherin; ↓ vimentin; ↑ HO-1	Apoptosis, inhibited migration and invasion, chemical metabolism	MCF-7, MDA-MB-231	[115]
	↑ ROS; ↑ GRX:TRX oxidation;↑ ASK1 dissociation; ↑ P-JNK; ↑ P-Bim	Apoptosis	MDA-MB-231	[120]
	↓ MMP-2/9 mRNA; ↓ MMP-2/9; ↓ MMP-2/9 activity; ↓ P-IκBα; ↓ p65 nuclear localization; ↓ NF-κB promoter activity; ↓ P-ERK	Inhibited migration and invasion	MDA-MB-231, HS 578T	[134]
	↓ mammosphere formation; ↓ ALDH activity; ↓ $CD44^{high}/CD24^{low}$/ESA+; ↓ Bmi-1 mRNA; ↓ Bmi-1; ↓ uPAR; ↓ FoxQ1 mRNA; ↓ FoxQ1; ↓ nuclear FoxQ1; ↑ DACH1 mRNA; ↓ Myc mRNA	Reduced stemness	MCF-7, SUM159	[135]
Lung	↑ ROS; ↓ ΔΨm; ↑ P-ERK1/2; ↑ P-JNK1/2; ↑ p53; ↓ Bcl-2; ↑ survivin	G2/M arrest, apoptosis	A549	[91]
	↓ Bcl-2; ↓ Bcl-XL; ↑ Bax; ↑ Bak; ↑ Bid; ↑ cleaved PARP; ↑ CyclinB1; ↓ Cdk1; ↓ P-Cdk1; ↓ Cdc25C	G2/M arrest, apoptosis	H358, H460	[92]

Table 2.1 (Continued)

	↓ Bcl-2; ↑ Caspase-3/8/9 mRNA; ↑ Caspase-3/8/9 activity; ↑ Bax mRNA; ↑ Bax	Apoptosis	A549	[124]
	↑ Intracellular Ca^{2+}	Apoptosis	A549	[130]
Skin	↓ Cdc25C; ↓ Cdk1; ↑ Caspase-3/9; ↑ Caspase-3/9 activity ↑ ROS; ↑ intracellular Ca^{2+}; ↓ ΔΨm; ↑ P-H^2AX; ↑ P-p53; ↑ CDKN1A ($p21^{Cip1/Waf1}$); ↑ Wee1; ↑ CyclinB1; ↑ cleaved PARP	G2/M arrest, apoptosis	A375, BCC	[96]
	↑ ROS; ↑ ɣ-H^2AX; ↑ P-p53; ↑ CDKN1A ($p21^{Cip1/Waf1}$); ↓ ΔΨm; ↑ intracellular Ca^{2+}; ↑ GRP78/BiP; ↑ CHOP/GADD153; ↑ Caspase-3/4/9; ↓ Bcl-2; ↑ P-Bcl-2; ↓ Bcl-XL; ↑ Bax; ↑ Cytochrome C release; ↑ Apaf-1; ↑ cleaved PARP; ↑ cytosolic $HtrA^2$/OMI; ↑ cytosolic AIF; ↑ nuclear AIF; ↑ nuclear Endo G	G2/M arrest, apoptosis	BCC	[99]
	↓ Bcl-2; ↓ Bcl-XL; ↑ Cytochrome C release; ↑ Caspase-3 activity; ↑ cleaved PARP	Apoptosis	A375, M14	[127]
Bladder	↑ cleaved PARP; ↓ P-Akt; ↓ P-PDK1; ↓ Bcl-2; ↑ Bax	G2/M arrest, apoptosis	T24	[95]
	↑ Caspase-3/8/9; ↑ Caspase-3/8/9 activity; ↑ cleaved PARP; ↑ DR4/5; ↑ Fas; ↑ FasL; ↑ TRAIL; ↑ Bax; ↓ Bcl-2; ↓ Bcl-XL; ↓ ΔΨm; ↓ XIAP; ↓ cIAP1/2; ↓ P-Akt; ↑ P-ERK; ↑ P-JNK	Apoptosis	T24	[121]
	↓ MMP-2/9 mRNA; ↓ MMP-2/9; ↓ MMP-2/9 activity; ↑ TIMP-1/2 mRNA; ↑ TIMP-1/2; ↑ tight junctions; ↓ claudins	Inhibited migration and invasion	5637	[137]
Osteosarcoma	↓ Mdr1 mRNA; ↓ P-gp	Altered drug resistance	Saos-2	[73]
	↑ Chk1 activity; ↑ CyclinB1; ↑ P-securin; ↑ P-histone H3; ↓ APC activity (↑ P-Cdc20, ↑ P-Cdh1); ↑ ATR activity	G2/M arrest	U2OS	[89]

Table 2.1 (Continued)

	↓ VEGF mRNA; ↓ VEGF secretion; ↓ Notch-1 mRNA; ↓ Notch-1; ↓ Hes-1 mRNA; ↓ Hes-1; ↓ Hey-1/2 mRNA; ↓ CyclinD1,; ↓ MMP-2/9 mRNA; ↓ MMP-2/9; ↓ MMP-2/9 activity; ↑ miR-143; ↑ miR-145; ↓ miR-21; ↑ miR-34a	Inhibited migration and invasion, decreased angiogenesis	U2OS, SaOS-2, MG-63	[107]
Glioblastoma	↑ ROS; ↓ ΔΨm; ↑ Caspase-9 activity	Apoptosis	T98G, U87MG	[111]
Liver	↑ CyclinB1; ↓ Cdk1	G2/M arrest	J5	[85]
	↑ NQO1 mRNA; ↑ HO-1 mRNA;↑ ARE promoter activity; ↑ Nrf2; ↑ P-JNK; ↑ P-ERK	Chemical metabolism	HepG2	[118]
	↑ QR mRNA; ↑ GSTA5 mRNA; ↑ EH-1	Chemical metabolism	H4IIE	[146]
Pancreas	↑ Bax mRNA; ↑ Bax; ↓ Bcl-2 mRNA; ↓ Bcl-2; ↑ p53 mRNA; ↑ p53; ↑ p53 nuclear translocation; ↓ MDM2; ↓ Akt; ↑ Fas; ↓ CyclinD1 mRNA; ↓ CyclinD1; ↑ CyclinB1; ↑ CDKN1A ($p21^{Cip1/Waf1}$)	Apoptosis	Capan-2, H6C7	[106]
Gastric	↓ Bcl-2; ↓ Caspase-3; ↓ Caspase-3 activity	Apoptosis	BGC823	[126]
	↑ PDCD5; ↑ PDCD5 nuclear translocation	Apoptosis	BGC823, MGC803, SGC7901, PAMC82, N87	[163]
	↑ CyclinB1; ↑ caspase-3; ↑ IκB-α; ↓ p- IκB-α; ↓ CyclinD1 mRNA; ↓ CyclinD1; ↑ Bax; ↓ XIAP; ↓ p-p65; ↑ cytoplasmic p65; ↓ nuclear p65; ↑ MT2A mRNA; ↑ MT2A; ↑ H3K9ac; ↑ H4K5ac; ↓ HDAC1/2; ↑ IκB-α promoter activity	G2/M arrest, apoptosis, epigenetic regulation	BGC823, SGC7901, AGS	[100]
Leukemia	↑ Caspase-3 activity	G0/G1 arrest, apoptosis	WEHI-3	[108]
Ovarian	↑ PUMA mRNA; ↑ PUMA; ↑ Bax mRNA; ↑ Bax; ↓ Bcl-2 mRNA; ↓ Bcl-2	Apoptosis	SKOV-3/DDP	[125]

Table 2.1 (Continued)

Nasopharyngeal	↑ Caspase-8 activity; ↑ P-p38	Apoptosis	CNE2	[132]

Table 2.2: Summary of *In vivo* studies utilizing DATS

Cancer Type	Effects	Mechanism	Dose	Model	Reference
Prostate	↓ tumor growth; ↑ DR4/5; ↓ Bcl-2; ↓ Bcl-2; ↓ Bcl-XL; ↑ Bax; ↑ Bak; ↓ microvessel density; ↓ VEGF; ↓ IL-6; ↓ IKK activity; ↓ P-Akt; ↓ MMP-2/7/9; ↓ MT-1 MMP	Inhibited cancer progression, apoptosis, inhibited migration and invasion, reduced angiogenesis	40mg/kg; 5x per week; 4 weeks	BALB/c orthotopic (PC-3)	[123]
	↓ XIAP; ↑ survivin	Inhibited cancer progression, apoptosis	2mg; 3x per week; 13 weeks	TRAMP	[131]
	↓ STAT-3; ↓ P-STAT3	Inhibited migration and invasion	2mg; 3x per week; 13 weeks	TRAMP	[143]
	↓ androgen receptor	Altered hormone signaling	2mg; 3x per week; 13 weeks	TRAMP	[145]
	↓ poorly differentiated carcinoma; ↑ well differentiated carcinoma and prostatic intraepithelial neoplasm; ↓ pulmonary and pelvic lymph node metastases; ↓ proliferation; ↓ neuroendocrine differentiation; ↑ cyclinB1; ↑ securin	Inhibited cancer progression, reduced metastasis	2mg; 3x per week; 13 weeks	TRAMP	[154]
Colon	↓ tumor growth; ↓ hemoglobin	Inhibited cancer progression, reduced angiogenesis	10 and 50mg/kg; daily; 4 weeks	BALB/c xenograft (HT-29)	[138]
	↓ tumor growth; ↓ hemoglobin	Inhibited cancer progression, reduced angiogenesis	10 and 50mg/kg; every 4 days; 32 days	BALB/c allograft (CT26)	[152]

Table 2.2 (Continued)

Breast	↓ tumor volume; ↑ survival (after 12 weeks)	Inhibited cancer progression	5µmol/kg; 2x per week; 1 month	BALB/c xenograft (MCF-7)	[114]
	↓ metastatic foci; ↓ maximal metastatic distance	Inhibited migration and invasion	0-20µM for 24h	zebrafish	[134]
	↓ tumor incidence; ↓ ALDH activity	Inhibited cancer progression, reduced stemness	2mg; 3x per week; 55 days	SCID xenograft (SUM159)	[135]
Lung	↓ tumor incidence; ↓ tumor volume	Inhibited cancer progression	6µM; every other day; 30 days	BALB/c xenograft (A549)	[124]
Skin	↓ COX-2; ↓ AP-1 DNA binding (↓ c-Jun; ↓ c-Fos);↓ P-JNK; ↓ JNK activity; ↓ P-Akt; ↓ Akt activity; ↓ tumor incidence and multiplicity (DMBA initiated papillomas)	Inhibited cancer progression	5 and 25µM topically prior to TPA application	ICR	[151]
Glioblastoma	↓ tumor growth; ↓ HDAC activity; ↑ histone H3/4 acetylation; ↓ Cdk1; ↓ Cdc25C; ↑ CDKN1A ($p21^{Cip1/Wap1}$); ↑ p53; ↓ MDM-2; ↓ survivin; ↑ mcalpin; ↓ P-Akt; ↓ c-Myc; ↓ mTOR; ↓ VEGF; ↑ calpain; ↑ Bax; ↓ Bcl-2; ↑ caspase-3; ↑ ICAD degradation	Inhibited cancer progression, G2/M arrest, apoptosis	10ug/kg – 10mg/kg; daily; 1 week	NOD/Prkdc scid/J xenograft (U87MG)	[153]
Gastric	↑ Nrf2; ↑ nuclear Nrf2; ↑ NQO-1; ↑ HO-1	Chemical metabolism	0.5 and 2mg/kg; every other day; 2 weeks	C57BL/6	[79]

Table 2.2 (Continued)

	↓ tumor size; ↓ tumor weight; ↑ MT2A; ↑ IκB-α; ↑ CyclinB1; ↓ CyclinD1; ↓ p-↑ IκB-α	Inhibited cancer progression, G2/M arrest, apoptosis	20mg/kg; every 4 days; 20 or 24 days	Balb/c xenograft (BGC823)	[100]
Leukemia	↑ cell surface markers (CD11b, Mac-3); ↑ macrophage activity; ↑ NK cell activity; ↓ B-cell proliferation	Inhibited cancer progression	1 and 10mg/kg; daily; 2 weeks	BALB/c xenograft (WEHI-3)	[108]

CHAPTER 3. EFFECT OF DIALLYL TRISULFIDE ON HEDGEHOG SIGNALING IN PANCREATIC CANCER CELLS

3.1 Introduction

Pancreatic Cancer (PC) is among the deadliest cancer types with an overall 5-year survival rate of only 8% [1]. A large contributor to PC mortality stems from the fact that over half of cases are diagnosed after distant metastasis has occurred [1]. In the case of late diagnoses, adjuvant treatment strategies of chemo- and/or radiation therapy have not proven successful over the past 40 years. Even in the case of curative resection, 80-85% of patients experience metastasis and local relapse [9]. Current research in the area of chemoprevention aims to increase survival at all stages of PC by reducing disease progression.

Bioactive compounds found in fruits and vegetables are an attractive source of putative chemopreventive agents due to their wide applicability and association with low toxicity. Evidence showing an inverse correlation between cancer risk and fruit and vegetable intake provided the first evidence that consumption of these foods may have chemoprotective effects. Further characterization of specific components from fruits and vegetables and their application in cell based and preclinical cancer models suggests certain bioactive compounds in whole foods have anti-cancer capabilities. Diallyl trisulfide (DATS), a compound derived from *Allium* vegetables such as garlic and onions, has been shown to regulate multiple cancer hallmark pathways including apoptosis, evasion of cell cycle arrest, angiogenesis, and invasion and metastasis [29, 30]. Previous work has shown accumulation of PC cells in G2/M phase, increased expression of phosphorylated Histone H3 (p-H3), enhanced expression of p21, and phosphorylated CyclinB1 [109]. These results indicate maximum cell cycle arrest following 16 hours of

DATS treatment which has been shown to cause apoptosis [109, 164]. Previous studies have also shown reduced survival as well as increased cytoplasmic histone associated DNA fragmentation in PC cells following DATS treatment [165]. Induction of the apoptosis inducing proteins Bim and cleaved caspase-3, enhanced protein expression of inactive apoptosis inhibitor Bcl-2, as well as increased levels of apoptosis marker cleaved PARP have also been observed [109, 165].

Another pathway, the Hedgehog (Hh) pathway, is of great interest for PC prevention in light of its role in pancreatic carcinogenesis [32]. Hh signaling is activated when a Hh ligand such as Sonic Hedgheog (Shh) binds to the Patched (Ptch) receptor. This relieves inhibition of Smoothened (Smo) leading to its shuttling to the primary cilia. Smo present in cilia induces release of the Gli1 transcription factor from its repressor Suppressor of Fused Homolog (SuFu) leading to Gli1 nuclear translocation. There, it enhances transcription of target genes with known roles in enhancing cancer hallmark pathways. Hh signaling is tremendously important during normal embryonic development but has been shown to be aberrantly activated in many cancer types. Furthermore, aberrant activation of this pathway is associated with reduced survival of PC patients. If a compound is able to inhibit the Hh pathway, it may prove invaluable for reducing PC progression and improving survival.

Multiple PC cell lines representing a spectrum of mutations and characteristics were used in this study. All cell lines are poorly differentiated but were derived from different locations [166]. AsPC-1 cells were derived from the ascites while BxPC-3, MIA PaCa-2, and PANC-1 cells lines were derived from the primary pancreatic tumor [166]. These cell lines differ in the status of Kras as well as p16, two genes estimated to be mutated in 95% of PDAC tumors [5, 167]. BxPC-3 possesses wild type Kras while the other cell lines are mutated at amino acid 12 [166]. MIAPaCa-2 and PANC-1 PC cells possess a homozygous deletion in p16 while conflicting evidence exists regarding the status of p16 in AsPC-1 and BxPC-3 [166]. These cell lines also exhibit varying degrees of Hh pathway component expression. AsPC-1 and PANC-1 PC cells display the highest

levels of Gli1 mRNA expression so they were chosen for investigation of the Hh pathway [168].

The Hh pathway is a desirable target in PC prevention because of its regulatory overlap with multiple cancer hallmark pathways, high degree of mutation in PC, and additional involvement with cancer stem cell characteristics. This study was undertaken to investigate Hh regulation by DATS as well as to elucidate the role of Hh pathway signaling in PC cells. The first goal of this work was to characterize the effect of DATS on Hh pathway components. We hypothesized that DATS treatment of PC cells would result in downregulation of the Hh pathway. Another goal of this study was to elucidate the mechanistic significance of Gli1 as it relates to cancer hallmark pathways. To this end, we chose to overexpress Gli1 and observe the downstream consequences in the cell cycle and apoptosis. Specifically, protein expression of mitotic cell marker p-H3 and apoptosis indicator cleaved PARP were examined with and without DATS treatment.

In the present study, we observed reduced colony formation of two PC cell lines following DATS treatment. We provide the first evidence that DATS regulates the Hh pathway constituent Gli1 by inhibiting mRNA and protein expression in AsPC-1 cells. DATS is also shown to enhance protein expression of Hh pathway components Shh and Ptch.

3.2 Materials and Methods

Chemicals And Reagents

Diallyl trisulfide was purchased from LKT Laboratories (St. Paul, MN). DMSO was obtained from Fisher Scientific (Pittsburg, PA). Gli1 (cs2534s), p-H3 (cs3377s), and PARP (9548s) antibodies were purchased from Cell Signaling (Danvers, MA). Mouse β-actin (A5441) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Shh (sc1194), Patched (sc9016) and anti-goat (sc2020) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse (95017-336) and anti-rabbit (95017-556) secondary antibodies were purchased from GE Healthcare (Radnor, PA). Goat anti-rabbit AlexaFluor 568 antibody (A-1101) was purchased from Invitrogen (Carlsbad, CA).

Cell Culture

MIAPaCa-2, BxPC-3, AsPC-1, and PANC-1 cell lines were purchased from American Type Culture Collection (Manassas, VA). MiaPaCa-2 were cells were grown in DMEM (Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS) and 2.5% donor horse serum. BxPC-3 cells were grown in RPMI-1640 (Corning) with final concentration of 4.5 g/L glucose (Corning), 10mM HEPES (Corning), and 1mM sodium pyruvate (Corning) in addition to 10% FBS. AsPC-1 cells were grown in RPMI-1640 supplemented with 10% FBS. PANC-1 cells were grown in DMEM supplemented with 10% FBS. All media were additionally supplemented with 1% penicillin/streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin). Cells were maintained at 37°C with 95% air and 5% CO_2.

Clonogenic Survival Assay

Clonogenic assay was completed as described previously [169]. To summarize, cells were plated in 100-mm plates and allowed to attach overnight. Cells were treated with either DMSO (vehicle) or DATS (20, 40, 60 μmol/L) for 24 hours. Cells were trypsinized and re-seeded in 6-well plates at 300 cells/well in normal growth media lacking DATS. Medium was replaced every 3 days for 10 (MIAPaCa-2) or 14 (BxPC-3) days. Colonies were fixed with methanol and stained with 0.5% (w/v) crystal violet (Amresco, Bridgeport, NJ) in 20% methanol. Only colonies larger than 50 densely populated cells were counted.

Western Blot Analysis

Western blotting procedure was completed as described elsewhere [169]. In short, cells were plated in 100-mm plates and allowed to attach overnight. Cells were treated with either DMSO (vehicle) or DATS (20, 40, 60 μmol/L) for 16 or 24 hours. Cells were collected by cell scraping and pellets were frozen at -80°C. Cell pellets were then lysed with buffer containing final concentrations of 1% Triton X-100, 0.1% SDS, 50mM Tris, and 150mM NaCl with added 1x protease and phosphatase inhibitor cocktails

(Sigma-Aldrich, St. Louis, MO). Protein concentration was determined by Bradford assay (Bio-Rad, Philadelphia, PA). Protein samples were loaded and separated by SDS-PAGE according to expected protein weight. Proteins were transferred to PVDF membranes (Perkin Elmer, Waltham, WA) by wet electrotransfer. Following blocking for 2 hours at room temperature, membranes were incubated in primary antibody overnight at 4°C. Membranes were washed 3 times in Tris-buffered saline with Tween-20 and incubated in secondary antibody at room temperature for 1 hour. The membranes were then washed 3 times for 15 minutes each. Proteins were visualized on X-ray film using the enhanced chemiluminescence detection system (GE Healthcare, Pittsburgh, PA).

Real Time Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated and purified from frozen cell pellets following manufacturer's protocol using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA) and PureLink DNase Kit (Invitrogen, Carlsbad, CA). cDNA was synthesized using the BioRad iScript cDNA Synthesis Kit (Hercules, CA). Primers for Gli1 (Forward: 5'-CAT CAG GGA GGA AAG CAG AC-3', Reverse: 5'-CAT TGC CAG TCA TTT CCA CAC-3'), Shh (Forward: 5'- CTC GTA GTG CAG AGA CTC CT-3', Reverse: 5'- GAG CGG ACA GGC TGA TG-3'), Smo (Forward: 5'-GAA GAT CAA CCT GTT TGC CAT G-3', Reverse: 5'-TTT GGC TCA TCG TCA CTC TG-3') and GAPDH (Forward: 5'-GGA GCG AGA TCC CTC CAA AAT-3', Reverse: 5'-GGC TGT TGT CAT ACT TCT CAT GG-3') were purchased from Integrated DNA Technologies (Coralville, IA). Quantitative real-time PCR was performed using a LightCycler 96 system (Roche, Basel, Switzerland). Target sequences were amplified at 95°C for 10 minutes, followed by 45 cycles of a 3-step amplification consisting of 95°C incubation for 15 seconds, 60°C for 10 seconds, and 72°C for 30 seconds. Fold change was calculated relative to GAPDH control using the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence

Immunofluorescence was carried out as described previously [170]. Briefly, cells were plated on coverslips and allowed to attach overnight at 37°C (5% CO_2). Cells were

then incubated with DMSO (vehicle) or DATS (40 and 60 µmol/L) for 16 hours. Following media removal, cells were washed with PBS and fixed with 2% paraformaldehyde overnight at 4°C. Cell membranes were permeabilized with 0.5% Triton X-100 in PBS for 15 minutes at room temperature and blocked for 1 hour with buffer containing final concentrations of 0.5% BSA and 0.15% glycine. Coverslips were incubated in primary antibody diluted in blocking buffer for 2 hours at room temperature followed by 3 washes with blocking buffer. Anti-rabbit AlexaFluor 568 secondary antibody was diluted in blocking buffer and incubated on coverslips for 1 hour. Cells were counterstained with 500 ng/mL DAPI (Invitrogen, Carlsbad, CA) and visualized on a Nikon Eclipse TE300 microscope using SPOT Advanced software.

Transient Transfection and Gli1 Overexpression

AsPC-1 cells were plated in 6 well plates at 100,000 cells per well 24 hours prior to transfection with 1 µg plasmid DNA. A 1:3 plasmid:FuGene6 transfection reagent ratio was used (Qiagen, Valencia, CA). pCMV6-Gli1 (RC201110) and pCMV6-Entry (PS100001) plasmids were purchased from Origene (Rockville, MD). Following 24 hours of transfection, cells were treated with DMSO (vehicle) or DATS (60 µmol/L) for 16 hours. Cells were collected, lysed, and analyzed by Western Blot as described.

Statistical Analysis

Protein band quantification utilized UN-SCAN-IT software (Silk Scientific, Orem, UT). Statistical analysis was done using Graph Pad Prism 9.1 (La Jolla, CA). One-way ANOVA followed by Dunnett's multiple comparisons test was used to calculate significant differences for all experiments except transfections. For transfection experiments, unpaired Student's t-test was performed to determine significant differences between groups. Values are presented as mean ± SEM. A *P*-value of less than 0.05 was considered statistically significant.

3.3 Results

DATS reduced colony formation of pancreatic cancer cells

To determine the effect of DATS on PC cell colony formation, we performed a clonogenic assay. Treatment of MIAPaCa-2 and BxPC-3 cells with DATS for 24 hours caused a dose dependent decrease in clonogenic survival after 10-14 days of colony growth (Figure 3.1). Treatment with 20, 40, and 60 µmol/L DATS reduced colony formation of MIAPaCa-2 cells by 15, 27, and 42% respectively (Figure 3.1 A). BxPC-3 colony formation decreased by 19, 39, and 50% with similar DATS treatments (Figure 3.1 B). Additionally, smaller average colony were observed size with increasing DATS concentration in both cell lines (Figure 3.1 A and B).

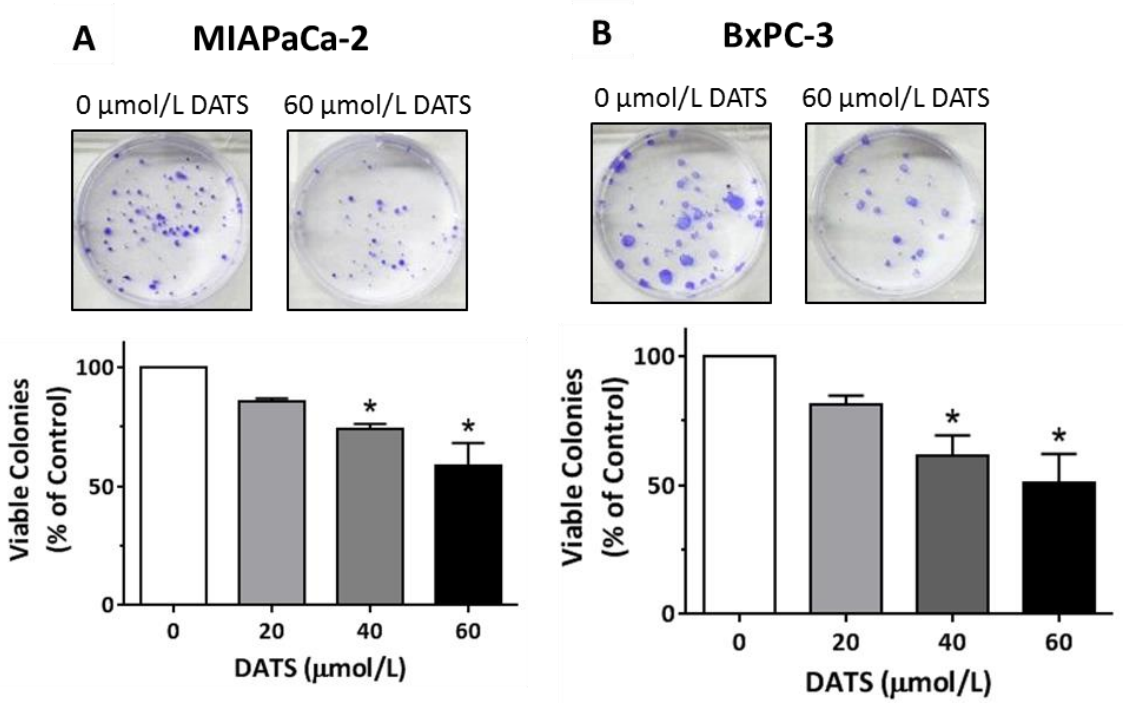

Figure 3.1 DATS inhibits colony formation of pancreatic cancer cells

Clonogenic survival of (A) MIAPaCa-2 and (B) BxPC-3 pancreatic cancer cells. Column represents mean (n=2); bar represents SEM. *, significantly different from vehicle treated, $P<0.05$, by one way ANOVA followed by Dunnett's multiple comparisons test.

DATS decreased the protein and mRNA expression of Gli1 in AsPC-1 pancreatic cancer cells

We analyzed the effect of DATS on Gli1 expression in AsPC-1 and PANC-1 PC cells lines. In AsPC-1 cells, we observed reduced Gli1 protein expression by Western blot following DATS treatment for 16 and 24 hours (Figure 3.2 A). All protein expression

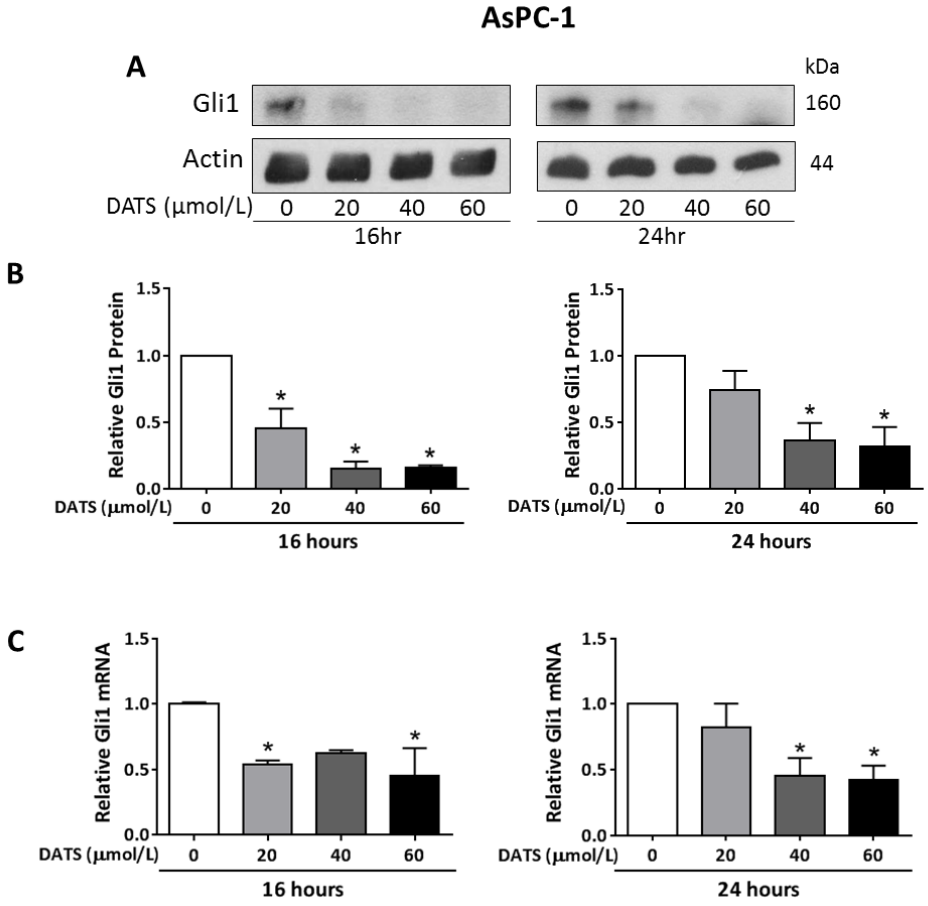

Figure 3.2 DATS treatment reduces levels of Gli1 protein and mRNA in AsPC-1 pancreatic cancer cells

(A) Western blot of Gli1 in AsPC-1 cells following 16 and 24 hours of DATS treatment. Data of representative experiment are shown. (B) Densitometry analysis of Western blots. Column represents mean (n=2); bar represents SEM. *, significantly different from vehicle treated of specified time point, $P<0.05$, by one way ANOVA followed by Dunnett's multiple comparisons test. (C) qPCR analysis of Gli1 in AsPC-1 cells following 16 and 24 hours of DATS treatment. Column represents mean (n=2); bar represents SEM. *, significantly different from vehicle treated of specified time point, $P<0.05$, by one way ANOVA followed by Dunnett's multiple comparisons test.

levels in 16 hour treated AsPC-1 cells were significantly different compared to vehicle treatment following densitometry analysis. Gli1 protein expression was reduced up to 85 and 69% compared to control after 16 and 24 hours of 60 µmol/L DATS treatment respectively in AsPC-1 cells (Figure 3.2 B). These results were supported by a reduction in Gli1 protein fluorescence observed comparing vehicle and 40 µmol/L DATS treated AsPC-1 cells (Figure 3.3). Gli1 mRNA displayed a 55 and 58% reduction in Gli1 mRNA expression following 60 µmol/L DATS treatment for 16 and 24 hours respectively (Figure 3.2 B). Treatment of PANC-1 cells with DATS for 16 and 24 hours caused no significant change in Gli1 protein or mRNA expression level compared to vehicle treated cells. (Figure 3.4 A – C).

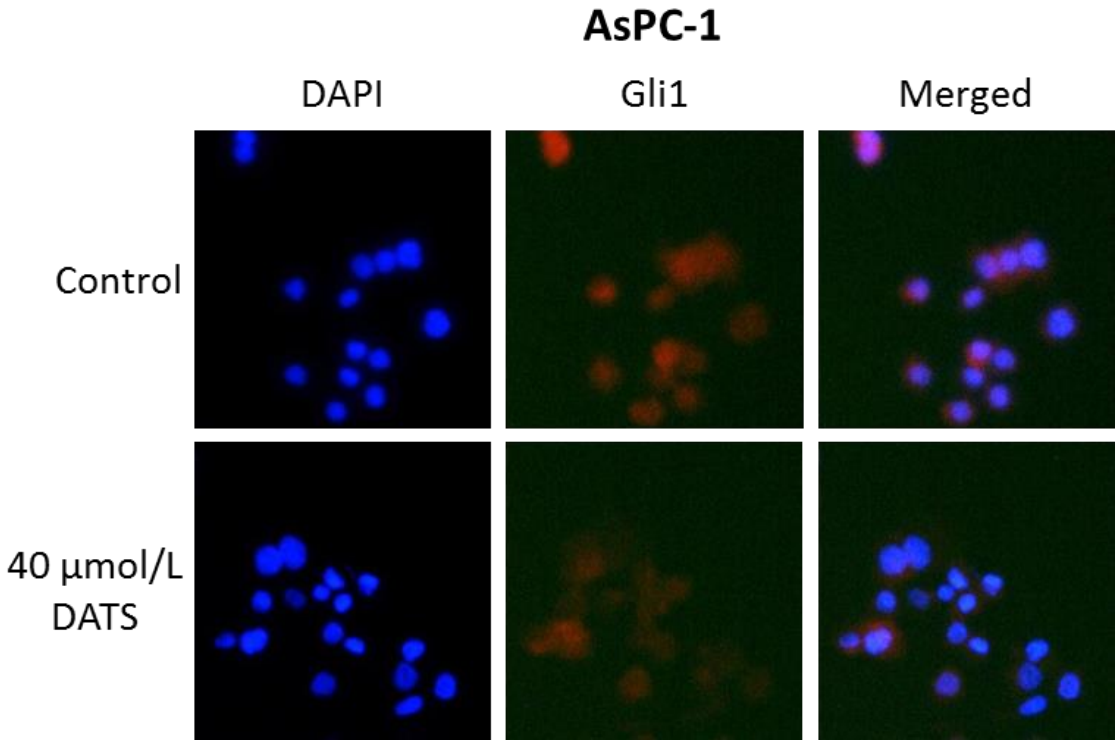

Figure 3.3 DATS treatment reduces Gli1 immunofluorescence in AsPC-1 pancreatic cancer cells

Images showing fluorescence of nuclear DNA (blue) and Gli1 (red) in AsPC-1 cells following treatment with vehicle and 40 µmol/L DATS for 16 hours. Representative images are shown.

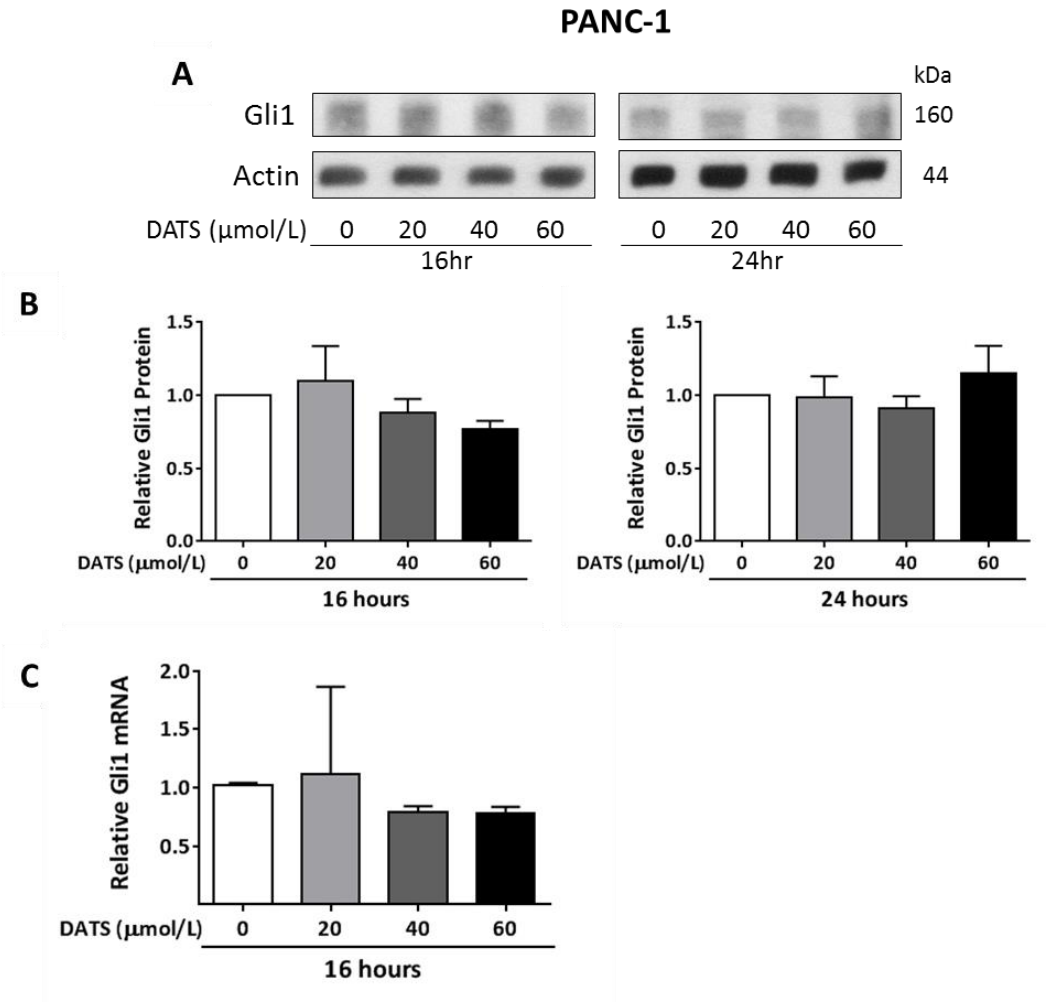

Figure 3.4 Gli1 protein expression is minimally affected in PANC-1 cells

(A) Western blot of Gli1 in PANC-1 cells following 16 and 24 hours of DATS treatment. Data of representative experiment are shown. (B) Densitometry analysis of Western blots. Column represents mean (n=2); bar represents SEM. Statistics analyzed by one way ANOVA followed by Dunnett's multiple comparisons test. (C) qPCR analysis of Gli1 in PANC-1 cells following 16 hours of DATS treatment. Column represents mean (n=2); bar represents SEM. Statistics analyzed by one way ANOVA followed by Dunnett's multiple comparisons test.

DATS modulated protein expression of other Hh pathway constituents

Analysis of Shh, Ptch, and Smo protein level in response to DATS treatment was done by Western blot. In AsPC-1 cells, full length Shh protein increased greater than 3-fold with 60 µmol/L DATS treatment at both 16 and 24 hour time points compared with

untreated cells (Figure 3.5 A). Levels of the N-terminal, physiologically active peptide were also increased following DATS treatment by 2.8 (±1.4) and 1.9-fold (±0.45) following 60 µmol/L treatment for 16 and 24 hours respectively when compared with vehicle treated cells (Figure 3.5 A). DATS treatment increased Ptch protein expression in AsPC-1 cells by up to 2.1-fold (±0.66) following 16 hour treatment and 3.5-fold (±1.9) following 24 hour treatment relative to control (Figure 3.5 B). Expression of Smo was largely unaffected in all treatments at both 16 and 24 hour time points (Figure 3.5 C)

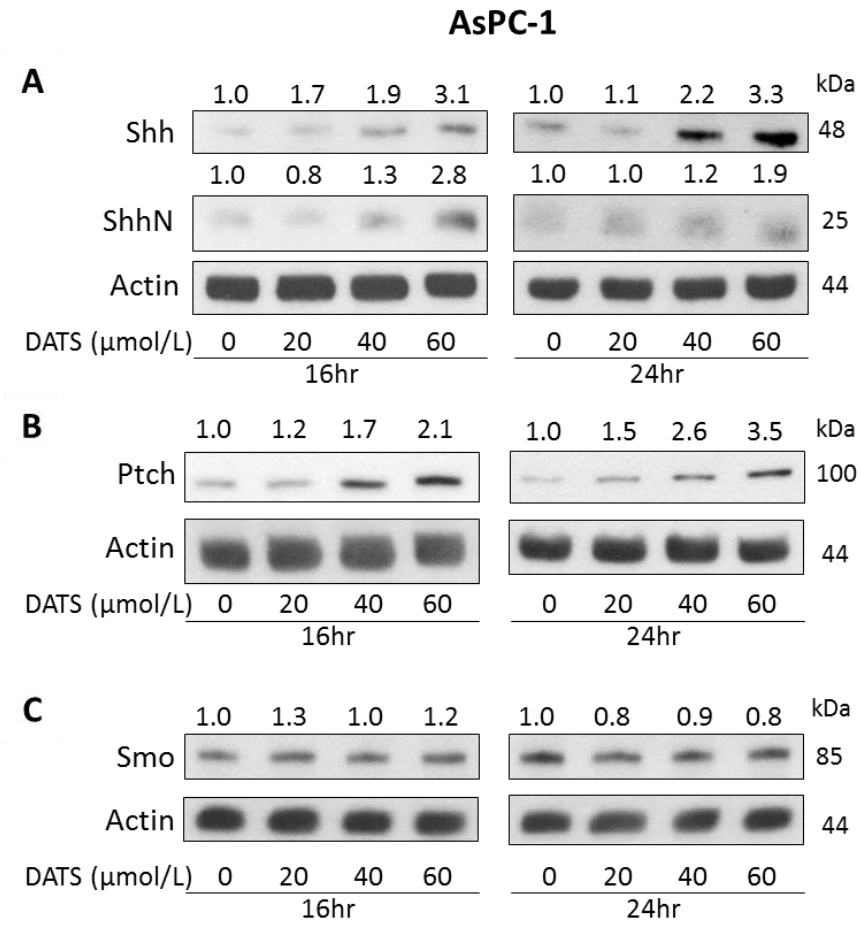

Figure 3.5 Treatment with DATS modulates expression of other Hedgehog pathway components in AsPC-1 cells

Western blot s of (A) Shh, (B) Ptch, and (C) Smo in AsPC-1 cells following 16 and 24 hours of DATS treatment. Data of representative experiment are shown. Relative expression values above blot images represent mean expression of 2 independent experiments.

Effect of Gli1 overexpression on mitotic cell marker p-H3 and apoptosis marker cleaved PARP

To investigate the mechanistic role of Gli1 in PC cells, AsPC-1 cells were transiently transfected with Gli1 using the pCMV6-Gli1 plasmid. An average 4.4-fold (±0.50) increase in the 160kD isoform of Gli1 was achieved. Following 60 µmol/L DATS treatment for 16 hours, Gli1 protein expression was reduced to 27% of untreated levels in empty vector transfected cells (P = 0.007). In Gli1 transfected cells, DATS treatment

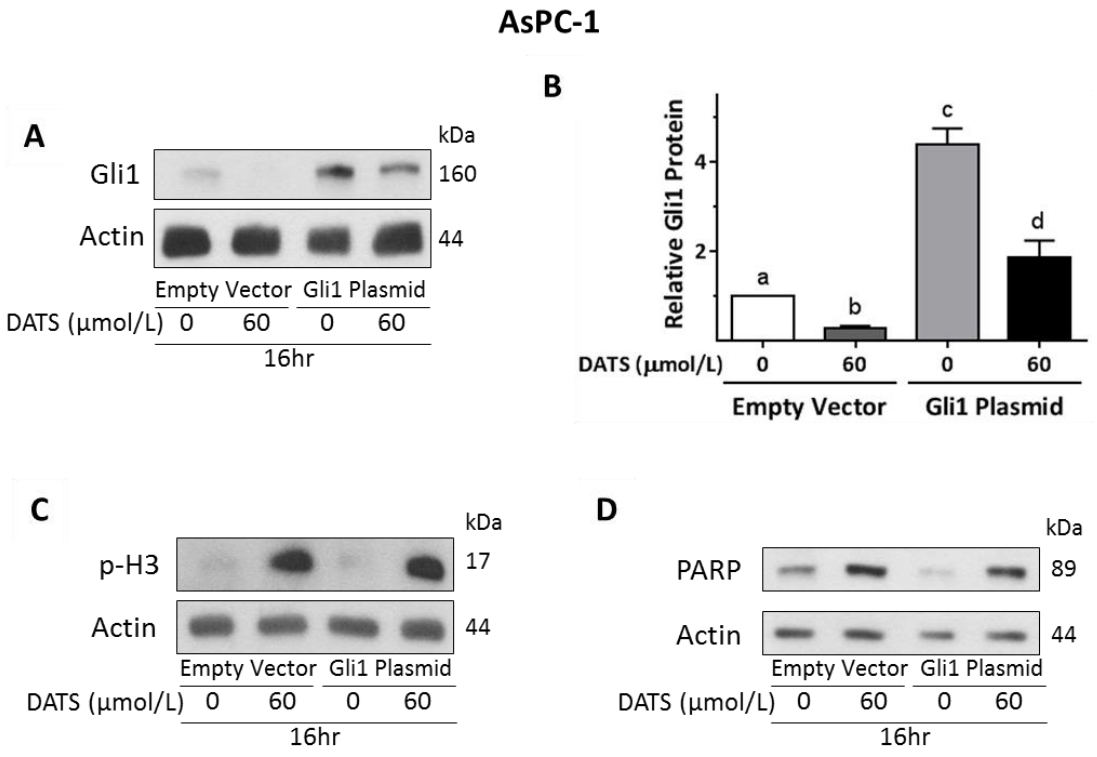

Figure 3.6 Gli1 overexpression shows a trend toward decreased p-H3 and cleaved PARP expression following DATS treatment

(A) Western blot of Gli1 in AsPC-1 cells following transfection with pCMV6-Entry (Empty Vector) or pCMV6-Gli1 (Gli1 Plasmid) and treatment with 60 µmol/L DATS for 16 hours. (B) Densitometry analysis of Western blots. Column represents mean (n=2); bar represents SEM. Different letters signify significant differences from Empty Vector, vehicle treatment, P<0.05, by unpaired, two-tailed Student's t-test. Western blots of (C) p-H3 and (D) PARP from Gli1 overexpression experiments. Data of representative experiment are shown.

resulted in a 58% reduction of Gli1 protein (P = 0.039) (Figure 3.6 A and B). Protein levels of mitotic cell marker p-H3 were significantly increased in empty vector and Gli1 transfected cells after 16 hours of 60 µmol/L DATS treatment (Figure 3.6 C). Protein expression of apoptosis marker cleaved PARP was also determined by Western blot. DATS treatment increased cleaved PARP expression in empty vector and Gli1 plasmid transfected cells (Figure 3.6 D).

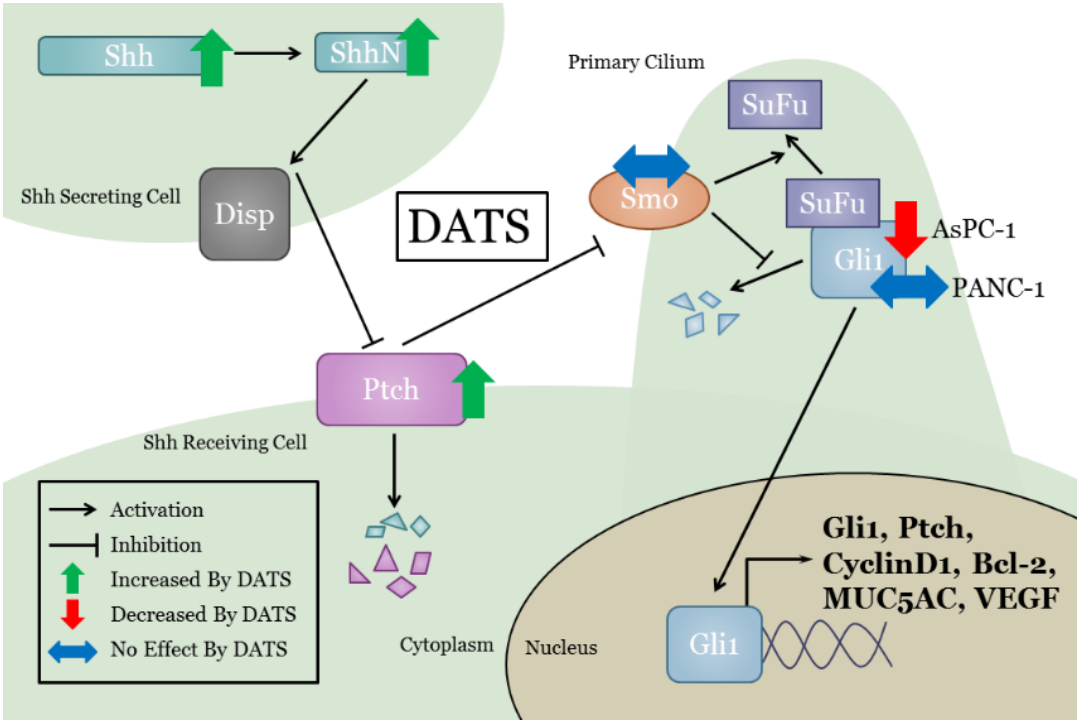

Figure 3.7 Summary of the effects of DATS on Hh signaling in pancreatic cancer cells

3.4 Discussion

In this study, PC cells displayed reduced clonogenic survival following DATS treatment. Here we also provide the first evidence of regulation of the Hh pathway in PC cells following DATS treatment. Expression of the transcription factor Gli1 was inhibited following DATS incubation of AsPC-1 cells although PANC-1 cells were less effected. Shh and Ptch protein expression were increased upon DATS treatment of AsPC-1 cells while protein levels of pathway mediator Smo were largely unaffected. A summary of these

effects is shown in Figure 3.7. Following Gli1 overexpression, DATS was shown to target exogenous Gli1 protein. Additionally, G2/M marker p-H3 was as well as apoptosis marker cleaved PARP were induced upon DATS treatment. Gli1 overexpressing cells exhibited a trend toward reduced p-H3 as well as cleaved PARP expression suggesting Gli1 may have mechanistic involvement with cell cycle and apoptosis mechanisms though these results are overall inconclusive.

The PC cell lines used in these experiments represent a variety of key mutations, invasive capabilities, migration capacities, and tumorigenic potential. Many sources of cancer tumor heterogeneity exist including between patients, between metastases, and within primary as well as metastatic tumors [171]. Multiple PC cell lines were used in this study to better understand how DATS affects cells representing a spectrum of origins, mutations, and characteristics – a vital aspect of determining the potential of DATS as a chemopreventive agent.

MIAPaCa-2 cells have been characterized as having high colony formation ability, which is consistent with our findings in the clonogenic survival assay [172]. BxPC-3 has been characterized as having no colony formation ability [173] which is in opposition to our observations. Differences in clonogenic survival assay procedure including growing conditions and allotted growth time may account for this discrepancy. Reductions in both colony size and number with increasing DATS treatment suggest DATS inhibits growth and survival of MIAPaCa-2 and BxPC-3 PC cells. Because cells were grown in media lacking DATS for 10-14 days following a 24 hour DATS incubation, the fact that colonies from treated cells were smaller and less numerous suggests DATS permanently or semi-permanently alters programs in PC cells to favor reduced clonogenic survival. Inhibition of cell cycle progression as well as induction of apoptosis by DATS are possible explanations for these results. Previous data from our lab have shown DATS-induced G2/M arrest as well as increased p-H3 expression in MIAPaCa-2 and PL45 PC cells [109]. Additionally, it has been shown that DATS reduced viability and induced apoptosis in PC cell lines Capan-2 and H6C7 [106]. Previous data indicated MIAPaCa-2 cells exhibit

reduced survival following DATS treatment as well as modulation of Bim and phosphorylated Bcl-2 protein expression [109, 165].

Inhibition of the Hh pathway is a desirable goal because of its involvement in carcinogenesis including propagation of cancer hallmark pathways as well as cancer stem cell characteristics [32]. Others have shown bioactive agents including sulforaphane, curcumin, and genistein to regulate the Hh pathway in various cancer cell types [37], but the effect of DATS on Hh signaling in PC cells has never been investigated. In general, results regarding Hh pathway modulation by dietary agents in cancer cells have shown reduced mRNA and protein expression of Hh pathway constituents Shh, Ptch, Smo, and Gli1 [37]. Observed reductions in Gli1 nuclear translocation, reporter activity, and downstream target expression including CyclinD1 and Bcl-2 show that these agents not only inhibit Hh pathway constituent expression, but overall pathway activity [37]. Additionally, induction of downstream effects such as increased cell cycle inhibition and apoptosis markers indicate that dietary agents such as curcumin, resveratrol, and epigallocatechin 3-gallate have potential as chemopreventive Hh inhibitors [37]. Mechanistic understanding of how bioactive compounds specifically inhibit the Hh pathway is largely lacking. In this study, we aimed to understand how DATS regulates multiple Hh pathway constituents on the mRNA and/or protein level as well as elucidate the mechanistic significance of Gli1 in PC cells.

In the present study, DATS was shown to inhibit mRNA and protein expression of the Hh pathway transcription factor Gli1 at 16 and 24 hour time points in AsPC-1 PC cells. Because Gli1 is the downstream effector of the pathway, these findings suggest DATS may ultimately cause Hh pathway inhibition. These results are noteworthy when considering the chemopreventive applicability of DATS as recent clinical trials of Smo inhibitor IPI-926 plus gemcitabine significantly reduced median survival in metastatic PC patients when compared with gemcitabine treatment alone [174]. It is not mentioned in the study whether tumors were able to evade Smo inhibition or if the drug combination induced disease progression. Because DATS targets the downstream effector Gli1, the likelihood that a tumor may circumvent its effect or that DATS would contribute to

disease advancement is low. PANC-1 PC cells exhibited more resistance to DATS treatment supporting the need for continued research regarding a variety of cell types. It is possible, though, that enhanced drug efflux capacity is responsible for this effect. While the DATS concentrations used in this study ranged from 20 – 60 μmol/L, other studies utilizing upwards of 100 μmol/L DATS have been carried out [106, 134]. The applicability of such a concentration is yet to be tested *in vivo*, but it is possible that PANC-1 cells would exhibit a response on the Gli1 mRNA and protein level under such conditions.

The presented data are particularly striking in light of the observed increases in Shh and Ptch protein expression as well as relatively unchanged Smo expression. To date, very few studies have reported increased or unchanged Hh pathway constituent expression following treatment with a chemopreventive dietary agent. One study suggested that Med283 medulloblastoma cells and HSR-GBM1 neurosphere cells were largely resistant to curcumin treatment as an explanation for the minimal change in Ptch and Gli1 mRNA expression that showed a mild tendency towards increased expression in some treatments [175]. Because a reduction in Gli1 mRNA and protein were observed in AsPC-1 cells, overall cell line resistance is unlikely. Another study of medulloblastoma cells reported reduced Shh protein expression following 8 hours of curcumin treatment that increased greater than 5-fold by the 24 hour time point [38]. This could presumably be a result of stem cell enrichment as observed in another study of PC cells treated with gemcitabine [172]. Again, because DATS targets the eventual effector of a stem cell related pathway, this likely does not explain the present results. Expression of Shh is driven by multiple transcription factors including p63, NF-κB, TAp73β, and ERα [32]. Because little is known about regulation of p53 family members TAp73β and p63 in PC cells, induction of Shh via these pathways following DATS treatment cannot be ruled out. In one study, treatment of ovarian cancer cells with the chemotherapeutic drug paclitaxel induced expression of TAp73β [176]. If this effect was observed in PC cells, it is plausible that Shh expression would be increased as observed in this study. DATS has been shown to reduce expression of ERα and NF-κB in multiple cancer cell types making

the action of these pathways an unlikely explanation for the observed increased Shh protein expression [100, 110]. Outside of being a product of Gli1 activation, few studies have examined regulation of Ptch expression. Some have declared Ptch to be a tumor suppressor because of its constitutive inhibition of Smo. Interestingly, though, reduced Gli1 expression did not lead to reduced Ptch presence in this study. Elucidation of the mechanism by which Ptch protein expression increases is required to more fully understand Hh pathway regulation.

Another goal of this study was to characterize the mechanistic significance of Gli1 downregulation in PC cells. To this end, AsPC-1 PC cells were transiently transfected with a pCMV6-Gli1 plasmid. Our data show that DATS targets exogenous Gli1 further supporting its role as a Gli1 inhibitor. Because Hh regulates multiple cancer hallmark pathways, we sought to gain insight into its role in cell cycle progression. During mitosis, DNA is condensed by wrapping around histone proteins. One protein, Histone H3, exists in the phosphorylated form beginning during chromosome condensation through metaphase and is rapidly dephosphorylated beginning in anaphase. Therefore, increased p-H3 is a quality marker for cells in G2/M transition. Following DATS treatment of vector and Gli1 transfected AsPC-1 cells, we observed significant increases in p-H3 protein suggesting cells were arrested in G2/M phase. These results support previous findings from our lab that DATS significantly induced G2/M arrest and induced p-H3 expression in MIAPaCa-2 cells following 8 and 16 hours of DATS treatment [109]. We observed that Gli1 overexpressing cells treated with DATS exhibited reduced p-H3 expression compared to vehicle treated, mock transfected cells although this difference did not reach statistical significance. Examining protein expression of cleaved PARP is a quality marker for apoptosis because PARP is a substrate for executioner caspases thus indicating the degree of apoptosis pathway induction. In this study, we observed an increase in PARP expression following DATS treatment in both empty vector and Gli1 plasmid transfected cells. As others have reported, this indicates DATS is a potent inductor of apoptosis [99, 114, 121]. Investigation of the mechanistic significance of Gli1 downregulation on PARP expression yielded no significant effect and requires further

investigation to more precisely elucidate the mechanism by which Gli1 interacts with these and other cancer hallmark pathways.

The hypothesis that DATS down regulates the Hh pathway is supported by the finding that Gli1 expression is inhibited in AsPC-1 cells. Observed increased expression of Shh and Ptch protein levels necessitate further research in this area to determine the mechanism by which DATS exerts its effects in PC cells. Further investigation into the mechanistic significance of Gli1 down regulation in PC cells is also required.

CHAPTER 4. CONCLUSION

In this study, we tested the hypothesis that DATS down regulates the Hh pathway in PC cells. This would provide the first evidence as to the modulation of the Hh pathway in cancer cells using DATS, a bioactive compound derived from *Allium* vegetables with known anti-cancer capabilities. We chose to target the Hh pathway in PC cells because of its significance in PC etiology as a known regulator of important pathway in both transit amplifying cells as well as cancer stem cells. The results of a clonogenic survival assay suggest DATS has a lasting effect on cell growth in MIAPaCa-2 and BxPC-3 PC cells. While growth and survival are endpoints for many upstream signals, it is possible that Hh inhibition by DATS is partially responsible for these effects. We chose to focus on AsPC-1 and PANC-1 PC cells for determination of the effect of DATS on Hh pathway constituents because they exhibit high expression of various pathway components [168]. In this study, we observed markedly reduced mRNA and protein expression of the Hh pathway component Gli1 in AsPC-1 cells while the effect of DATS on overall Hh pathway activity requires further investigation. This effect was not observed in PANC-1 cells suggesting they may be more resistant to DATS. Interestingly, we observed increased protein expression of two proteins upstream in the Hh pathway, Shh and Ptch along with very little change in Smo expression. These findings necessitate more research as they may display a novel mechanism by which DATS may prevent the advance of cancer.

Because little is known regarding the mechanism of Hh regulation in PC cells, Gli1 was overexpressed in AsPC-1 cells and its effect on p-H3 and cleaved PARP protein expression was examined. No significant effect of Gli1 overexpression on p-H3 or PARP

protein levels were observed although this study requires further investigation. This study supports future investigation into the mechanism of chemoprevention by DATS in PC cells.

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