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Nutraceutical Inhibition of Hedgehog Signaling in Pancreatic Cancer

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Abstract

Pancreatic cancer (PC)is the fourth leading cause of cancer-related deaths in the United States. PC is predominantly diagnosed at distant stage and is characterized as being highly chemoresistant and metastatic. Aberrant activation of the Hedgehog (Hh) signaling pathway is implicated with cancer cell proliferation, chemoresistance, and metastasis. Dietary nutraceuticals have been identified which show inhibition of the Hh signaling pathway in PC cells, an increase in apoptosis, and a decrease in cell growth and cancer stem cell characteristics.

Diallyl trisulfide (DATS) is a bioactive compound present in *Allium* vegetables such as garlic and onion. Our lab has previously shown that DATS inhibits cell growth and colony formation of PC cells and modulates the Hh signaling pathway including downregulation of the transcription factor Gli-1 in AsPC-1 cells. The present study expanded upon this work, further implicating DATS as a potential chemopreventive dietary agent capable of inhibiting the Hh signaling pathway in PC cells.

Core components of the Hh signaling pathway (Gli-1, Gli-3, SMO, and Shh) were assessed by Western blotting, following a 24-hour treatment with 0 – 100μmol/L DATS in PC cell lines AsPC-1, BxPC-3, and MIA PaCa-2. SMO and Gli-3 protein expression in BxPC-3 cells were further assessed via immunocytochemistry. Gli-3 mRNA expression was evaluated by qRT-PCR analysis. DATS was shown to differentially modulate Hh signaling in PC cell lines. A significant reduction in the levels of Hh signaling proteins was detected in AsPC-1 (Gli-1) and BxPC-3 (Gli-1, and Gli-3) cells suggesting inhibition of the pathway. This study supports further investigation of DATS as a potentially chemopreventive dietary agent for PC.

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Abbreviations

BCC – Basal cell carcinoma

BMJ – Bitter melon juice

DAPI - 4’,6-diamidino-2-phenylindole

DATS – Diallyl trisulfide

EGCG - (-)-epigallocatechin-3-gallate

EMT – Epithelial to mesenchymal transition

Gli – Glioma-associated oncogene

HDAC – Histone deacetylase

Hh – Hedgehog

Kif7 – Kinesin family member 7

KRAS – Kristen rat sarcoma viral oncogene homolog

OSC – Organosulfur compound

PanIN – Pancreatic intraepithelial neoplasia

PC – Pancreatic cancer

PDAC – Pancreatic ductal adenocarcinoma

PDX-1 - Pancreatic and duodenal homeobox-1

PKA – Protein kinase A

PMJ – Pomegranate juice extract

PTCH – Patched homolog

qRT-PCR – Quantitative real time polymerase chain reaction

ROS – Reactive oxygen species

SMO – Smoothened

SuFu – Suppressor of fused homolog

TRPV1 – Transient receptor potential vanilloid 1

Chapter 1. Introduction

Cancer is the second leading cause of death after heart disease in the United States. It was estimated to be the cause of death of 608,570 Americans in 2021 equating to more than 1,600 deaths per day [1]. The World Health Organization has estimated the global economic cost of cancer to be approximately $1.16 trillion per year [2]. While many cancers have had a steady increase in treatment options and survival rates, advances in the treatment of PC have remained slow and the mortality rate high. PC ranks 10th in U.S. cancer incidence but is the 4th leading cause of cancer-related death with an estimated 60,430 new cases and an estimated 48,220 related deaths in 2021 [1]. This makes PC one of the deadliest forms of cancer. Cancer of the pancreas has the lowest 5-year relative cancer survival rate for all cancers and stages combined at ~10% [1].

While there has been a steady increase in the development of treatments and survival rates for most cancer types, advancements have been slow for PC. This is due in part to the distant stage at diagnosis which inhibits significant clinical research and intervention [3]. Symptoms of PC most commonly present as loss of appetite, indigestion, weight loss, abdominal pain, nausea, jaundice, steatorrhea, and back or belly pain [3]. These generally less intrusive symptoms often result in a distant stage diagnosis. There is no cure for PC outside of surgical resection. While the perioperative mortality rate for surgical resection of PC is low, less than 15% of patients have cancers which are resectable at the time of diagnosis [3]. The only currently available treatments

for PC are neoadjuvant treatments to diminish the size of primary tumors, with adjuvant chemoradiation and/or chemotherapy [3].

The deadliest form of PC, pancreatic ductal adenocarcinoma (PDAC) is considered one of the most aggressive and lethal of all solid tumors [4]. These tumors are characterized as being hypovascular with significantly reduced tumor perfusion which leads to the development of a thick stromal wall which results in poor drug delivery and increased chemoresistance [4]. Within this stroma is a small sub-population of cancer stem cells (CSCs) with an unlimited potential for cell division which have been implicated in the onset of metastasis and post-surgical relapse [4, 5, 6]. One characteristic of CSCs is the aberrant regulation of embryonic development-related signaling pathways, such as Hh signaling [5, 7, 8]. The aberrant re-activation of Hh signaling in mature PC stem cells has been implicated in facilitating the creation of a tumor microenvironment which promotes the continued proliferation and increases epithelial to mesenchymal transition (EMT) of cancer cells [4, 5, 7, 9]. This implies that PC cure is reliant on the complete inactivation or removal of all CSCs.

Initially, aberrantly reactivated Hh signaling was only implicated in cancers of the skin, skeletal, muscle, and brain [10, 11]. Emerging studies have implicated the Hh signaling pathway to be aberrantly re-activated in many other solid tumors including breast [9, 12], prostate [13], digestive tract [14], liver [15], lung [16], colorectal [17], and pancreas [18, 19, 20-23] and CSCs of multiple myeloma [24], osteosarcoma [25] and leukemia [26]. This has resulted in prolific research into Hh signaling inhibitors as

chemotherapeutic agents with the potential to treat or prevent the growth and proliferation of numerous types of cancer cells.

Inhibition of the Hh pathway has been shown to decrease proliferation, increase apoptosis, and decrease stem cell characteristics of PC cells *in vitro* [27-46]. Numerous compounds of natural, but non-dietary origin have also been identified which reduce the proliferation or metastasis of PC cells through the inhibition of the Hh signaling pathway [47-54]. These compounds are, however, limited in their applications as they can only be prescribed as adjuvant chemotherapy. An alternative approach to slow or potentially arrest the development, progression, and or metastasis of PC is through the inhibition of aberrant Hh signaling pre-diagnosis. One avenue to this goal is through the ingestion of dietary bioactive compounds capable of Hh signaling inhibition.

The ingestion of foods containing bioactive dietary compounds as a treatment for illness has been in use throughout human history. Records exist of the use of garlic (*Allium sativum*) for medicinal purposes in ancient Egypt, Rome, India, China, and Japan as well as throughout the European Middle Ages, Renaissance, and early American colonialism [55]. Modern mechanistic studies have validated some of these medicinal uses including antibacterial, antifungal, and antiatherosclerotic properties but have also indicated antitumorigenic potential [56-58]. Epidemiological evidence demonstrates a close association between *Allium* vegetable consumption and decreased PC risk [59-61].

Increased *Allium* vegetable consumption is strongly correlated with a decrease in cancer incidence and progression [62-67]. The complex pathogenesis and long latent

period of PC progression, however, presents considerable limitations to nutritional epidemiology [60]. The observed effects may be due to phytochemical ingestion, or the displacement of foods positively correlated with disease pathogenesis such as a high fat diet [68, 69]. Cancer development is also influenced by a host of factors related to genetic predispositions, activity level, environmental exposures, and dietary intake spanning a lifetime [60, 62, 70, 71]. Self-reported intake is often unreliable and inconsistently designed between studies [56, 62]. This is further complicated by food frequency questionnaires frequently grouping all *Allium* vegetable consumption together (garlic, onion, garlic powder, leek, etc.) despite significant biochemical differences [56, 61, 62, 71]. While epidemiological evidence is mounting implicating a protective role of *Allium* vegetables in mitigating cancer risk, the precise bioactive compounds and mechanisms of action are poorly characterized [67]. In order to validate the observations of epidemiological studies, mechanistic studies investigating the association between organosulfur compound (OSC) consumption and PC proliferation and metastasis are required.

The most biologically active OSCs identified in *Allium* vegetables are allyl derivatives [63]. The term “Allyl” (derived from *Allium*) was proposed in 1844 by the German chemist Theodor Wertheim to refer to the chemical structure CH2=CHCH2 [57]. Many compounds which include an allyl group have a pungent odor [57]. One of the most odoriferous of the culinary *Allium* vegetables, garlic (*Allium sativum*) also contains the highest concentration of volatile OSCs [72]. One such OSC is the fat-soluble

compound diallyl trisulfide (DATS) (CH2=CH-CH2-S-S-S-CH2-CH=CH2) present in numerous plants of the *Allium* genus [73].

DATS has been shown to increase apoptosis and induce G2/M cell cycle arrest in a dose dependent manner in PC cells [44]. DATS induced G2/M cell cycle arrest has also been reported in several other types of cancers including but not limited to gastric, liver, colon, prostate, lung, bladder, and skin [59, 73]. Other OSCs containing allyl groups are also present in significant concentrations in garlic oil with diallyl sulfide (1.9–9.5%), diallyl disulfide (20.8–27.9%), and diallyl trisulfide (16.8–33.4%) respectively [74]. Although garlic and onion (*Allium cepa*) represent the primary dietary sources of DATS, many culinary species of plant of the *Allium* genus contain DATS such as leek (*Allium porrum*), scallion (*Allium fistulosum*), shallot *(Allium ascalonicum*), and chive (*Allium schoenoprasum*) [58].

Biochemical synthesis of DATS in garlic begins with glutathione in the undisrupted vacuole of the garlic clove. Glutathione is S-alk(en)ylated before a glycyl group is removed to form γ-glutamyl-S-alk(en)yl-L-cysteine [75]. γ-glutamyl-S-alk(en)yl-L-cysteine is then further deglutamylated and oxygenated to yield S-alk(en)yl-L-cysteine sulfoxide (alliin) [75]. Alliin is the odorless precursor of DATS. Upon tissue disruption (such as cutting or chewing) of garlic, the vacuole is broken and the endogenous vacuolar enzyme cysteine sulfoxide lyase (alliinase) comes in contact with alliin to yield di(prop-2-enyl)thiosulfinate (allicin) [57, 73, 75-77]. Allicin then rapidly degrades into

the final allyl derivatives, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) [73, 75, 76].

Thiosulfinate formation is largely dependent upon the activity of alliinase. If freshly cut garlic or onion is allowed to stand for ~10 minutes alliinase can yield allyl sulfur compounds such as DATS. If freshly cut garlic or onion is not allowed to stand and is instead heated there is a denaturation of alliinase and inhibition of the formation of allyl sulfur compounds [70]. Although some DATS may still be formed in the body despite rapid denaturation of allinase, the overall synthesis of DATS is greatly reduced [77]. This suggests that handling and processing of *Allium* vegetables can influence DATS concentration in foods, which in turn may influence epidemiological studies and *in vivo* research.

Despite the growing epidemiological evidence and previously established pharmacological applications of *Allium* derived bioactive compounds, the relationship between such compounds and PC has yet to be fully elucidated. Further mechanistic studies investigating the impact of DATS on PC are required. The present study evaluated the impact of DATS upon the Hh signaling pathway in human PC cell lines AsPC-1, BxPC-3, and MIA PaCa-2. This study supports DATS as a potential chemopreventive nutraceutical capable of inhibiting the Hh signaling pathway in PC cancer cells.

Chapter 2. Dietary Agents Impact on Hedgehog Signaling in Pancreatic Cancer

2.0 Abstract

Pancreatic cancer (PC) has a poor prognosis and a high mortality rate related to distant stage diagnosis. Aberrant activation of the Hedgehog (Hh) signaling pathway has been implicated in the maintenance, growth, and stem cell characteristics such as epithelial to mesenchymal transition (EMT) of PC cells. Dietary bioactive compounds capable of inhibiting Hh signaling in PC cells offer a unique treatment approach as these compounds may inhibit PC proliferation pre-diagnosis and may have pharmacological application in the treatment of PC post-diagnosis. Several such compounds have been identified which inhibit Hh signaling in PC cells *in vitro* (bitter melon, crocetinic acid, capsaicin, curcumin, resveratrol, sulforaphane, vitamin D3, (-)-epigallocatechin-3-gallate, baicalein, quercetin, and pomegranate juice) as well as *in vivo* (crocetinic acid, capsaicin, sulforaphane, baicalein, quercetin, ellagic acid). This suggests that consumption of nutraceuticals may inhibit the growth of PC cells though the inhibition of the aberrantly activated Hh signaling pathway.

2.1 Introduction

PC develops when cells in healthy mucosa accumulate mutations in key regulatory genes which leads to unregulated growth, altered intercellular signaling, and altered phenotype [56, 78]. Clones of these cells can form non-invasive pre-cancerous lesions which possess the ability to become cancerous [56]. As a result of the

accumulation of mutations, PC and pre-cancer cells are morphologically and antigenically distinct from other cells in the human body [56]. In time, these cells can form precursor lesions called pancreatic intraepithelial neoplasia (PanIN) and are characterized by specific histologic changes to the duct epithelium that correlate with the accumulation of genetic mutations in the Kristen rat sarcoma viral oncogene homolog (KRAS) oncogene and inactivation of tumor suppressor genes p16, p53, and CDKN2A [4, 78-86]. It is suspected that mutations in the pancreatic and duodenal homeobox-1 (PDX-1) promoter region in cooperation with oncogenic KRAS lead to the aberrant reactivation of the Hh signaling pathway in PC [18, 81, 82, 86-88]. The aberrant re-activation of Hh signaling in pancreatic progenitor cells is implicated in their continued stem cell characteristics such as self-renewal, differentiation, and tumorigenicity [89, 90].

The Hh signaling pathway is active during pancreatic organogenesis, but is largely suppressed in adult tissues [82, 91]. Hh signaling is limited to beta cells of the pancreas and functions to regulate insulin secretion and regeneration of pancreatic tissue after injury [10, 23]. In the healthy adult human pancreas, there is no detectable levels of Hh signaling in the islets, acini or ductal epithelium [18, 92]. There is an abundant expression of Hh signaling in ~70% of all human PC cells sampled and is detectable as early as PanIN-1 [18]. As PanIN progresses in degrees of atypia (1 to 3) there is a higher expression of Hh signaling with continued expression in every stage of disease progression [18, 91]. This suggests that the re-activation of the Hh signaling pathway in

PanIN lesions plays a key role in the pathogenicity of PC. This highlights the potential therapeutic value of Hh signaling inhibitors in the prevention and treatment of both invasive and pre-invasive pancreatic epithelium.

Cyclopamine, extracted from the California false hellebore lily (*Veratrum Californicum*) was the first identified Hh signaling inhibitor [93]. Cyclopamine has been investigated as a treatment for PC but has since been largely abandoned due to poor oral bioavailability, high teratogenicity, and development of resistance mechanisms in PC cells [93]. The first FDA approved Hh inhibitor for cancer treatment was vismodegib. Approved by the FDA in 2012, vismodegib is a smoothened (SMO) antagonist currently prescribed for the treatment of non-resectable basal cell carcinoma (BCC) [94]. Vismodegib is indicated to decrease BCC tumor size prior to surgery or to treat symptoms of patients unsuitable for surgery or radiation [95]. Vismodegib has shown inhibition of the Hh pathway in PC cell lines: AsPC-1, PANC-1, and Mia PaCa-2, as evidenced by a reduction in glioma-associated oncogenes (Gli) Gli-1 and Gli-2 (the final effectors of the Hh signaling pathway) resulting in a decrease in proliferation and an increase in apoptosis [96, 97]. Despite these initially promising results, SMO antagonists like vismodegib are rather disappointing *in vivo*. After initial response to treatment, patients become insensitive to the drug due to emergent mutations within the CSC populations [90]. Mutations which are downstream of SMO, such as Gli-1 or Gli-2 amplification or a loss of SuFu proteins, render SMO antagonists ineffective. This then leads to tumor regrowth within a few months [90, 98].

Vismodegib has been utilized in combinatorial treatment with the standard chemotherapeutic drug prescribed for PC, gemcitabine [99, 100]. In both studies vismodegib and gemcitabine treatment yielded no benefit relative to gemcitabine treatment alone. In one study, Hh signaling was found to be elevated in the biopsies of patients with metastatic PC. Following 3 weeks of gemcitabine and vismodegib treatment there was a decrease in Gli-1 and PTCH1 mRNA levels. Despite evidence of Hh signaling inhibition, there was no detectable increase in overall survival or a decrease in the CSC population [99]. Another study measured Hh signaling and progression-free survival in patients with PDAC. After initial treatment with gemcitabine and nab-paclitaxel (a common PC chemotherapy drug) to reduce primary tumor size, patients received an additional treatment with the Hh inhibitor vismodegib [100]. There was no significant increase in progression-free survival with vismodegib treatment relative to prior studies evaluating gemcitabine and nab-paclitaxel in PDAC progression. The study also reported an increase in Gli-1 and PTCH1 despite the presence of the SMO antagonist [100]. Neither study supports the use of vismodegib in the treatment of metastatic PC. On average SMO antagonist treatments provide only a few more weeks of life to patients with PC. This coupled with severe side effects such as: dysgeusia, alopecia, fatigue, nausea, diarrhea, weight loss, and the high risk of teratogenicity makes these drugs far from ideal [90].

Gli transcription factors are the final effectors of the Hh signaling pathway and are therefore less susceptible to the same forms of resistances which diminish the

viability of SMO antagonists [90]. Inhibition of Hh signaling through Gli antagonists has the additional benefit of inhibiting the activation of the pathway through both canonical and non-canonical regulation [101]. For this reason, most research is currently being directed at inhibiting the action of or diminishing the concentration of the final pathway activators Gli-1 or Gli-2. The compounds under investigation can be categorized depending upon their mechanism of action. Compounds can either inhibit Gli proteins directly through direct binding to Gli proteins which block their transcriptional ability to bind with DNA, or Gli antagonists inhibit Gli proteins indirectly through proteolytic degradation or post translational modification [90, 102].

Dietary phytochemicals, also known as nutraceuticals, have been shown to inhibit growth and proliferation of PC cells through inhibition of Hh signaling without developing significant resistance mechanisms with prolonged treatment [103]. In one study, BxPC-3 cells were treated with frequent and repeated cycles with the dietary phytochemicals sulforaphane, quercetin, or the chemotherapy drug gemcitabine for >1 year. BxPC-3 cells developed resistance to gemcitabine but did not develop apparent resistance to treatment with quercetin or sulforaphane and still demonstrated significantly decreased proliferation *in vitro* and decreased tumorgenicity *in vivo* [103]. This suggests that the mechanisms by which bioactive dietary agents diminish cancer growth are not only unique but may be superior long-term treatments than their more cytotoxic counterparts (such as gemcitabine) which predominate current PC treatments.

Natural compounds have been long standing sources for drug discovery. A range of compounds of both dietary and non-dietary origins have been identified which modulate or inhibit the Hh signaling pathway at multiple levels including Gli inhibition [89, 104, 105]. While both dietary and non-dietary compounds have potential pharmacological applications as treatments post-diagnosis, dietary agents can be consumed prophylactically, whereby they can modulate progression and incidence of disease pre-diagnosis.

2.2 Hedgehog Signaling Pathway

The Hh signaling pathway is a key regulator of tissue patterning during embryogenesis and homeostatic mechanisms such as tissue repair in some adult epithelium [106, 107]. Slight differences exist between vertebrate and invertebrate Hh signaling, but the pathway is well conserved is all metazoan species [106]. Dysregulation or aberrant activation is associated with the development of disease from congenital malformations to carcinogenesis [108]. Aberrant activation of this pathway in PC facilitates the creation of the tumor microenvironment which influences the creation of a favorable environment for PC development and chemoresistance among cancer and pre-cancer cells [4, 109]. Activation of the Hh signaling pathway occurs through the binding of Hh signaling ligands and is regarded as the canonical Hh signaling pathway. Figure 2.1 provides an overview of the canonical Hh signaling pathway in vertebrates as it is currently understood.

Hh signaling occurs in the primary cilium of the cell, a microtubule-based extension of the cellular membrane [23, 106, 109]. The Hh signaling pathway is initiated when a neighboring cell secretes one of the Hh signaling ligands. Mammals and birds have three separate Hh ligands: Sonic Hedgehog (Shh) Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). The most extensively studied ligand and the ligand most associated with tumorgenicity is Shh [106]. These ligands are secreted by the action of the transmembrane protein Dispatched. These ligands are trafficked over adjacent cells through interactions with cell surface proteins until they bind to the transmembrane proteins patched homolog 1 (PTCH1) and or patched homolog 2 (PTCH2) along with the co-receptors Cdon, Boc, and Gas1 [110]. PTCH1 and PTCH2 share structural and functional similarities but form separate hetero-complexes with the co-receptors (PTCH2/Gas1 and PTCH1/Boc/Cdon) both of which can mediate Hh ligand response [106]. While PTCH1 and PTCH2 serve overlapping roles in Hh signaling, PTCH1 has been shown to be the primary Hh ligand receptor with PTCH2 serving a redundant and potentially muted role [110]. When Hh ligands are not bound to PTCH1 the action of SMO is suppressed. When PTCH1 is bound to Hh ligands, the action of PTCH1 is suppressed. SMO then moves to the primary cilium, while PTCH1 moves to the cytoplasm and undergoes proteolytic degradation. Once SMO has moved to the plasma membrane it prevents Protein Kinase A (PKA), a known inhibitor of the Hh signaling pathway, from phosphorylating Gli proteins [106, 108, 111]. There are 3 Gli proteins, all of which bind to DNA through five zinc-finger domains that recognize the consensus sequence 5’-TGGGTGGTC-3’ [71]. Gli-1 is a constitutive activator of the pathway leading

to Hh signaling amplification [106, 108]. Gli-2 has a repressor domain at the N-terminal and an activator domain at the C-terminal. When the Hh pathway is inactive, the C-terminal of Gli-2 is removed and Gli-2 becomes the pathway repressor Gli-2R [112]. Gli-3 has predominantly been considered to be a constitutive repressor of the Hh signaling pathway [106, 108]. Growing evidence has demonstrated the existence of two isoforms of Gli-3, an activator (Gli-3A) and a repressor (Gli-3R) [113]. When the Hh pathway is inactive, Gli-1, Gli-2, and Gli-3 are bound to suppressor of fused homolog (SuFu) proteins and kinesin family member 7 (Kif7) to restrain their effects in the absence of SMO. SuFu is one of the main negative regulators of the Hh signaling pathway [101, 106]. SuFu interacts with Gli-1 at both the C-terminal and N-terminal and inhibits pathway activation by retaining Gli-1 in the cytoplasm and repressing Gli-1 transcriptional activity in the nucleus [101, 107]. Upon pathway activation SMO interacts with Kif7, and Kif7 dissociates from the SuFu-Gli protein complex and moves into the cytoplasm. Kif7 also functions in the formation and maintenance of the primary cilium [114]. Gli-1 and Gli-2 then undergo post-translational modification and become Gli-1 Active (Gli-1A) and Gli-2 Active (Gli-2A). Gli-1A and Gli-2A then move to the nucleus where they bind to DNA and promote the transcription of Hh genes including Gli-1, Gli-2, Gli-3, and PTCH1 [66, 106]. PTCH1 and Gli-3 inhibit the Hh pathway and serve as negative feedback inhibition [106, 108]. Gli-3 behaves in a similar fashion to Gli-1 and Gli-2 with the Gli-3 full length protein (Gli-3FL) initially bound to SuFu. When Hh signaling is active Gli-3FL dissociates from SuFu into the cytoplasm and is

phosphorylated and truncated into the transcriptional activator Gli-3A. Gli-3A then translocates to the nucleus and promotes the transcription of Hh signaling genes [113].

In the absence of Hh ligand secretion and binding to PTCH1 and co-receptors the Hh signaling pathway remains inactivated. PTCH1 suppresses the action of SMO by preventing entry into the cilium and SMO remains in the cytoplasm bound to an endosome [106, 108, 115]. In the absence of SMO in the primary cilium, Kif7 and SuFu remain bound to the Gli-1, Gli-2, and Gli-3 proteins. The Gli proteins bound to SuFu and Kif7 are then phosphorylated by PKA and undergo proteasomal modification. Gli-2 and Gli-3 become pathway repressors Gli-2R and Gli-3R through proteasome-mediated carboxyl cleavage and move to the nucleus where they inhibit the transcription of Hh genes. Gli-1 and the remaining segment of Gli-2 which was not modified into Gli-2R are then degraded by a proteasome in the cytoplasm [106, 108]. With Gli-1 transcriptionally repressed there is a subsequent inactivation of the pathway [108, 115].

In short, several components of this pathway function either as a tumor suppressor (PTCH1, SuFu) or proto-oncogenes (Shh, SMO, Gli-1, Gli-2) [10, 116]. Signs of canonical Hh signaling pathway inhibition are a decrease in: Gli-1, Gli-2, SMO and a decrease in Hh ligands Shh, Ihh, and Dhh [101, 106, 108, 109, 115]. SMO and Gli-1 protein concentrations function as a significant prognostic tool for overall survival and disease-free survival for patients with post-surgical resection of PC [117]. Lower concentrations of Shh and Gli-1 were associated with a higher overall survival and disease-free survival [117].

Although a decrease in the level of Gli-1 has been a primary biomarker for treatment response and Hh signaling inhibition [27-42, 47-53], what constitutes inhibition of this pathway with regard to other core Hh proteins lacks a coherent consensus. This gap in knowledge is most significant for the proteins Gli-3 and PTCH1 which function in feedback inhibition of the Hh signaling pathway. While both Gli-3 and PTCH1 function to inhibit the pathway, there is growing evidence that these proteins play a wider role in Hh signaling and crosstalk with other pathways. PTCH1 has been implicated in exerting influence upon other oncogenic pathways such as Bcl-2, AKT, and CCND1 [118]. The two forms of Gli-3 (Gli-3A and Gli-3R) both share the same DNA binding motifs and a result the two forms of Gli-3 compete for Gli binding sites which can influence the transcriptional activity of Gli proteins. Thus, the ratio of Gli-3A to Gli-3R plays a critical role in Hh signaling activation or inhibition [118]. In one study loss of Gli-3R was enough to activate the Hh signaling pathway independently of Hh ligands or SMO [119]. Additionally, a reduction in Gli-3R protein has been associated with an increase in Hh signaling [113]. Due to the complex and poorly characterized nature of PTCH1 and Gli-3, an increase or decrease in the levels of these proteins in response to treatment can be interpreted in several ways. While a decrease in PTCH1 or Gli-3 indicates a decrease in Hh gene transcription and inhibition of the Hh signaling pathway, the absence of PTCH1 and or Gli-3 can potentially influence other oncogenic pathways or induce aberrant Hh signaling reactivation.

Figure 2.1 *Active and Inactive States of the Hedgehog Signaling Pathway*

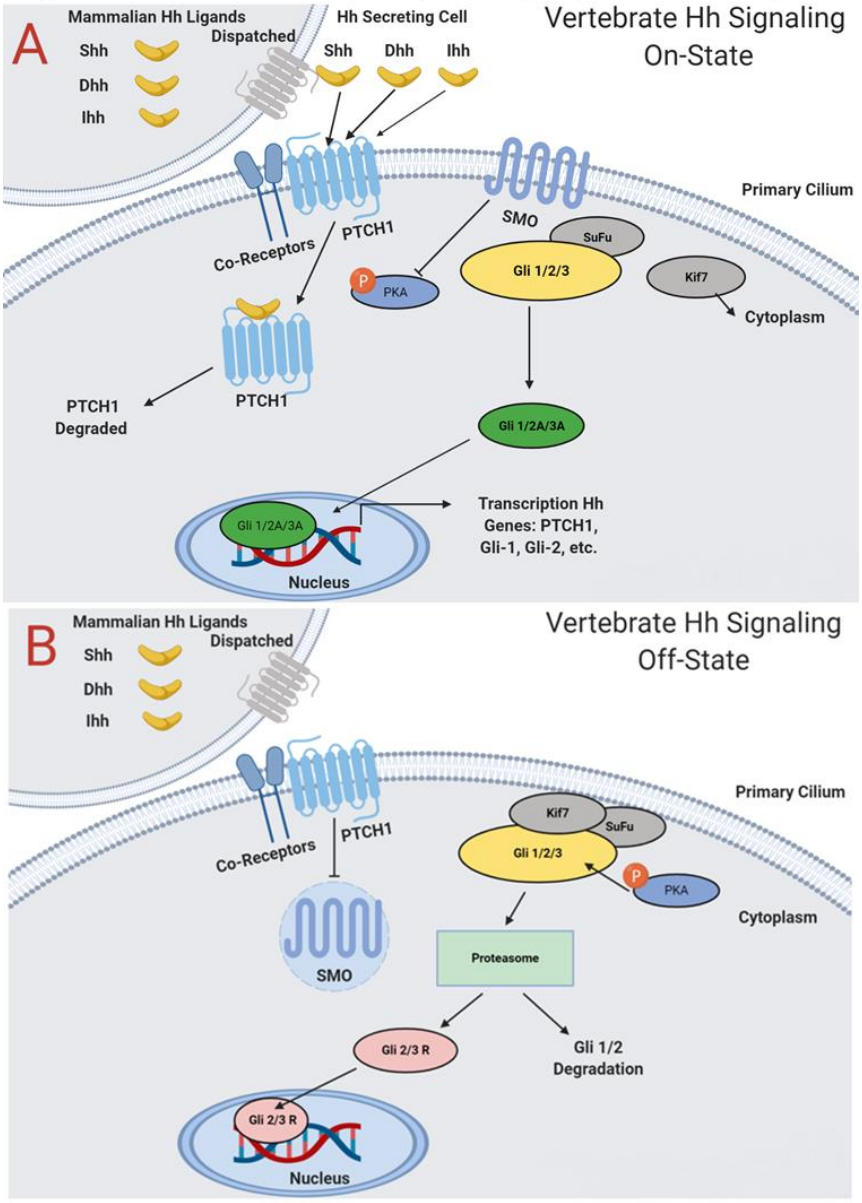

Figure 2.1 *Active and Inactive States of the Hedgehog Signaling Pathway.* (A) Active (B) inactive canonical Hh signaling pathway in vertebrates, respectively. (Image created in BioRender.com)

2.3 Dietary Agent Inhibitors of Hedgehog Signaling in Pancreatic Cancer

The consumption of foods, herbs, and spices as an approach to treat disease has been in use for centuries [55, 105]. Modern medicine has confirmed the efficacy of some of these treatments through the identification of bioactive compounds. Numerous dietary agents have shown inhibition of the Hh signaling pathway both *in vitro* and *in vivo* [27-42]. These findings indicate potential dietary chemopreventive and chemotherapeutic applications. The following summarizes the findings of fourteen such nutraceuticals and their inhibitory potential upon the Hh signaling pathway in PC (Table 2.1).

Table 2.1 *Dietary Agent Inhibitors of Hedgehog Signaling in Pancreatic Cancer*. This table summarizes the findings of all currently identified dietary compounds which have shown inhibition of Hh signaling in PC cells *in vitro* and in *vivo.*

Compound	Dietary Source	Treatment	Experimental Model	Mechanism of Action	Effect	References
Bitter Melon Juice (BMJ)	Bitter Melon *(Momordica charantia)*	2% and 4% v/v BMJ *In Vitro*	MIA PaCa-2	↓Gli-2	↓ Proliferation ↓ Stemness ↑ Apoptosis	[27]
Crocetinic Acid	Saffron *(Crocus sativus)*	10μM *In Vitro* 0.5mg/kg *In Vivo*	MIA PaCa-2, PANC-1, MIA PaCa-2 Tumor Xenograft	↓Gli-1↓SMO ↓SuFu ↑PTCH1	↓ Proliferation ↑ Apoptosis	[28]
Capsaicin	Chili Peppers	20 p.p.m *In Vivo*	Caerulein-Induced Pancreatic Intraepithelial Neoplasia	↓Shh ↓Gli-1	↓Inflammation ↓ Proliferation	[29]
Curcumin	Turmeric *(Curcuma longa)*	20μM *In Vitro*	PANC-1	↓Shh ↓Gli-1 ↓SMO	↓ Proliferation ↓ EMT	[30]

Resveratrol	Skin of grapes, Blueberries, Raspberries	25μM *In Vitro* 50μM *In Vitro*	PANC-1, BxPC-3, AsPC-1 PANC-1, BxPC-3	↓SMO ↓Gli-1 ↓PTCH1 ↓Shh ↓Gli-1 ↓SMO	↓ Proliferation ↑ Apoptosis ↓ EMT ↓ Stemness ↓ Proliferation	[31] [32]
Sulforaphane	Cruciferous Vegetables	20μM *In Vitro* 20mg/kg *In Vivo*	AsPC-1, PANC-1 Human Pancreatic CSCs (CD133+/CD44+/CD24+/ESA+)	↓SMO ↓Gli-1 ↓Gli-2 ↓SMO ↓Gli-1 ↓Gli-2	↓ Proliferation ↑ Apoptosis ↓ EMT ↓ Proliferation	[33] [34]
Vitamin D3	Fish, Fortified Products	10μM *In Vitro* 5μg *In Vivo*	MIA PaCa-2, BxPC-3, PANC-1, HS766-T 10.7	↓SMO	↓ Proliferation *In Vitro* No Effect *In Vivo*	[35]
Quercetin	Numerous Fruits, Vegetables, and Nuts	10μM - 100μM *In Vitro* 75mg/kg *In Vivo*	PANC-1, Patu 8988 PANC-1 Tumor Xenograft	↓ Shh ↓ Gli-2 ↓ SMO ↑ PTCH1 No Impact Gli-1 or Ihh	↓ Proliferation ↑ Apoptosis ↓ EMT ↓ Tumor Growth	[36]
(-)-epigallocatechin-3-gallate (EGCG)	Green Tea *(Camellia sinensis)*	20μM - 60μM *In Vitro*	Human Pancreatic CSCs ($CD133^+/CD44^+/CD24^+/ESA^+$)	↓SMO ↓Gli-1 ↓Gli-2 ↓PTCH1 ↓PTCH2	↓ Proliferation ↑ Apoptosis ↓ EMT ↓ Stemness	[37]
Baicalein	Chinese Skullcap Thyme, Onions	32-256μM *In Vitro* 20 or 60 mg/kg *In Vivo*	PANC-1 PANC-1 Tumor Xenograft	↓Gli-1 ↓Gli-2 ↓Gli-3 ↓PTCH1 ↓Shh ↓SMO ↓Gli-2 ↓PTCH1 ↓Shh ↓SMO	↓ Proliferation ↑ Apoptosis ↓ Tumor Growth	[38]
Pomegranate Juice Extract (PMJ)	Pomegranate *(Punica granatum)*	25 - 50μg/ml *In Vitro*	COLO 357	↓Gli-1	↓Proliferation	[39]
α-Mangostin	Mangosteen (*Garcinia mangostana*)	2.5 - 10μM *In Vitro*	Human Pancreatic CSCs (CD133+/CD44+/CD24+/ESA+)	↓Gli-1 ↓Gli-2 ↓SMO ↓PTCH1 ↓PTCH2	↓ Proliferation ↑ Apoptosis ↓ EMT ↓ Stemness	[40]
Z-ajoene	Garlic *(Allium sativum)*	5μM - 20μM *In Vitro*	PANC-1	↓Gli-1 ↓Gli-2 ↓SMO	↓ Proliferation	[41]
Ellagic Acid	Berries and Nuts	40 mg/kg *In Vivo*	PANC-1 Tumor Xenograft	↓Gli-1 ↓Gli-2	↓ Proliferation ↓ EMT	[42]

2.3.1 Bitter Melon

Bitter melon juice (BMJ) is isolated from bitter melons (*Momordica charantia*) and is a vegetable commonly consumed throughout Asia, Africa, and some parts of South America. In recent studies bitter melon has shown potential therapeutic applications towards a range of conditions including inflammation, hypoglycemia, and various cancers [27]. In one study, bitter melons of the Chinese variety were deseeded, juiced, centrifuged at 3000g and supernatant collected to generate a purified extract of BMJ. PC cell lines MIA PaCa-2, PANC-1 and AsPC-1 were then treated with (2-4%, v/v) BMJ for 72 hours. Following treatment of BMJ cell proliferation decreased by 69 – 97% relative to controls. There was also a statistically significant reduction in Gli-2 mRNA concentration after 72-hour treatment with BMJ 2% v/v [27].

2.3.2 Crocitinic Acid

Crocitinic acid is a carotenoid present in the spice saffron from the plant *Crocus sativus*. Crocitinic acid has been shown to inhibit nucleic acid and protein synthesis as well as PC cell lines *in vitro* [28]. In one study, freshly extracted crocitinic acid demonstrated inhibition of cell proliferation and enhanced apoptosis is PC cell lines MIA PaCa-2 and PANC-1 at an IC_{50} of 10µM [28].

There was a statistically significant increase in apoptosis in PANC-1 cells with 1µM crocitinic acid measured by flow cytometry. Western blot also showed that 10µM crocitinic acid led to a reduction in the concentration of Hh signaling proteins Shh, Gli-1,

SuFu, SMO, PTCH1, and a product of active Hh gene transcription Cyclin D1 in MIA Pa-Ca-2 cells. Crocitinic acid also suppressed growth of MIA PaCa-2 tumor xenograft growth at 0.5mg/kg/day in athymic female mice [28]. While freshly extracted crocitinic acid inhibited PC cell growth at doses as low as 1μM, commercially purchased crocetin had no effect on cell proliferation or apoptosis [28]. This suggest that supplementation with commercially produced crocitinic acid is ineffective and only crocitinic acid extracted from saffron exerts inhibitory effects on PC cell growth.

2.3.3 Capsaicin

Capsaicin is a transient receptor potential vanilloid 1 (TRPV1) antagonist responsible for the sensation of heat and pain when consuming chili peppers. Extensive studies have shown capsaicin to inhibit cell proliferation and induce apoptosis in several cancer cell lines *in vitro* with only a few studies investigating the chemopreventive effect of capsaicin *in vivo* [29].

One study induced PanIN in LSL $Kras^{G12D}$/Pdx1-Cre mice and wild-type mice by injecting one dose of caerulein (250μg/kg) intraperitoneally at age 4 weeks. One-week post injection, animals were randomly assigned into two treatment groups which consumed a standard diet containing either 10 ppm or 20 ppm capsaicin. Following treatment, a significant and dose-dependent reduction in the progression of PanIN and inflammation was detected [29]. A 50% decrease in mRNA expression of Shh and Gli-1 was then measured *via* quantitative real-time polymerase chain reaction (q-RT-PCR) from freshly collected pancreatic tissues. These results suggest inhibition of the Hh

signaling pathway and a potential application of capsaicin in chemoprevention of PC [29].

2.3.4 Curcumin

Curcumin, a polyphenol present in the spice turmeric, has been shown to inhibit hypoxia-induced epithelial to mesenchymal transition (EMT) by suppressing the Hh signaling pathway [30]. In one study with PANC-1 cells, the Hh proteins Shh, SMO, and Gli-1 were shown to increase in hypoxic culturing conditions leading to an increase in cell proliferation and EMT characteristics as measured by Western blotting, wound healing assay, and transwell matrigel invasion assay. After 1-hour treatment with 20μM curcumin, there was a significant decrease in proliferation of PANC-1 cells and hypoxia-induced EMT characteristics [30]. This suggests curcumin can inhibit the Hh signaling pathway in PC in a dose and time-dependent manner.

2.3.5 Resveratrol

Resveratrol is a polyphenol present in large quantities in the skin of grapes and to a lesser extent in some berries and nuts. Resveratrol has been shown to be a potent antioxidant conferring cardioprotective and neuroprotective effects. In two separate studies resveratrol demonstrated inhibition of the Hh signaling pathway in PC cell lines *in vitro* [31, 32]. At a dose of 25μM for 24 hours, resveratrol showed Hh signaling inhibition through a decreased level of Hh proteins PTCH1, Gli-1, and SMO in AsPC-1, PANC-1 and BxPC-3 cell lines via Western blotting. This resulted in a decrease in PC cell

proliferation and an increase in apoptosis [31]. In a separate study, resveratrol demonstrated similar results in PANC-1 and BxPC-3 cell lines at an IC_{50} of 50µM. At this dose there was a significant reduction in PC cell proliferation, decrease concentration of Shh, Gli-1, and SMO protein level measured by Western blotting. There was also a significant reduction in EMT and stem cell characteristics [32].

2.3.6 Sulforaphane

Sulforaphane is an OSC present in cruciferous vegetables such as broccoli, cauliflower, cabbage, and kale. Epidemiological studies have demonstrated an inverse relationship with the consumption of cruciferous vegetables and the incidence of PC [33]. This inverse relationship has been validated by two separate studies demonstrating sulforaphane's ability to inhibit the Hh signaling pathway in PC cell lines *in vitro* and *in vivo* [33, 34]. In one study, PANC-1 and AsPC-1 cells at a dose of 20µM sulforaphane showed a statistically significant reduction in SMO, Gli-1, and Gli-2 mRNA and protein levels as measured by q-RT-PCR and Western blotting, respectively. This was associated with a decrease in cell proliferation and increase in apoptosis as measured by flow cytometry [33]. Sulforaphane further showed its potential applications as a potential chemopreventive dietary agent by inhibiting cell proliferation of CSCs *in vivo*. When human pancreatic CSCs ($CD133^+/CD44^+/CD24^+/ESA^+$) were implanted into NOD/SCID/IL2Rgamma mice and administered by oral gavage (20 mg/kg sulforaphane 5 days a week for 6 weeks) there was a 45% reduction in tumor growth and EMT. There was also a significant reduction in Gli-1, Gli-2, and SMO [34]. This suggests the dietary

agent sulforaphane is a potential inhibitor of the Hh signaling pathway *in vitro* and *in vivo*.

2.3.7 Vitamin D3

Vitamin D3 (Vit D3), also known as cholecalciferol, has been identified as a SMO antagonist capable of similar levels of efficacy as cyclopamine in PC cell lines: BxPC-3, MIA PaCa-2, PANC-1, and Hs766T *in vitro* [35]. A dose of 10μM Vit D3 significantly reduced cell proliferation and led to a decrease in the concentration of SMO protein level as measured by Western blotting. Despite promising results *in vitro*, Vit D3 demonstrated no effect on the proliferation of PC cells *in vivo*. The PDAC cell line 10.7 was injected into immunocompromised mice and allowed to form tumors. Mice in the treatment groups were then injected with 5μg Vit D3 twice weekly for 34 days. Following treatment there was no significant effect upon tumor growth [35]. The proposed mechanism responsible for the differential effects observed between *in vitro* and *in vivo* treatments is a short half-life and subsequently low serum concentrations *in vivo*. This suggests that while Vit D3 is a potent SMO antagonist *in vitro*, it is not indicated as a potential treatment or chemopreventive agent to PC *in vivo.*

2.3.8 Quercetin

Quercetin is a bioflavonoid present in numerous fruits and vegetables including apples, cherries, *Brassica* and *Allium* vegetables [120]. Quercetin possesses antioxidant and anti-inflammatory activities and has been implicated as a neuroprotective,

antiarthritic and antidiabetic agent [120]. One study showed decreased cell proliferation (measured by real-time cellular analysis) and increased apoptosis (measured by flow cytometry) in PC cell lines PANC-1 and Patu 8988 *in vitro* and decreased tumor growth of PANC-1 in nude mice *in vivo* [36].

Male BALB-c nude mice were injected with PANC-1 cells subcutaneously then received daily intragastric injections of quercetin (75mg/kg a day) for 30 days. Following treatment there was a significant reduction in tumor growth [36]. To further characterize the mechanism of quercetin induced PC apoptosis, the researchers investigated the Hh signaling pathway in PANC-1 and Patu 8988 cells following quercetin treatment at a dose of 10μM or 100μM *in vitro*. There was a dose dependent decrease in Shh, Gli-2, SMO and a dose dependent increase in PTCH1 protein and mRNA levels, however, there was no impact on the level of Ihh or Gli-1 protein or mRNA in both cell lines (measured by Western blot analysis and qRT-PCR respectively) [36]. This suggests that quercetin can inhibit PC proliferation *in vitro* and *in vivo* in part through the inhibition of Hh signaling. In addition, this suggests that quercetin exerts these effects by decreasing Shh and is dependent upon Gli-2, not Gli-1 as has been seminally reported.

2.3.9 (-)-epigallocatechin-3-gallate (EGCG)

The polyphenol (-)-epigallocatechin-3-gallate (EGCG) is present in large quantities in green tea. The flavonoid quercetin is present in a wide range of fruits and vegetables such as onion, apples, kale, berries, and some teas. Both EGCG and quercetin

have demonstrated anti-inflammatory, antioxidant, and anti-tumor properties in numerous studies [37]. One study evaluated the synergistic effect of EGCG and quercetin on the inhibition of Hh signaling on human pancreatic CSCs ($CD133^+$ /$CD44^+$ /$CD24^+$ /ESA^+). EGCG alone (20µM - 60µM for 36 hours) decreased cell proliferation, and decreased mRNA expression of SMO, PTCH1, Gli-1, and Gli-2. EGCG also diminished EMT and stem cell characteristics such as survival, and differentiation. These effects were increased two-fold following concomitant treatment with 20µM quercetin relative to EGCG treatment alone [37]. This suggests EGCG could be a potent inhibitor of the Hh signaling pathway as well as suggesting a statistically significant synergistic effect between EGCG and quercetin.

2.3.10 Baicalein

Baicalein is a flavonoid present in some traditional Chinese medicines and is highly concentrated in the roots of Chinese Skullcap (*Scutellaria baicalensis*) and to a lesser extent in thyme and Welsh onions [38, 121]. Baicalein has been reported to exert a host of pharmacological applications including use as an antibacterial, anti-inflammatory, and antioxidant [38]. One study showed inhibition of PANC-1 cells via inhibition of the Hh signaling pathway. There was a dose dependent decrease in cell proliferation and increase in apoptosis following 48 to 72 hours of exposure to 32-256µM baicalein [38]. Western blotting indicated a decrease in protein levels of Gli-1, Gli-2, Gli-3, SMO, PTCH1, and Shh following baicalein exposure. Baicalein also showed reduced tumor growth *in vivo*. PANC-1 cells were implanted in BALB/c nude mice and

allowed to grow for 7 days. Mice then received treatment of 20 or 60 mg/kg baicalein by oral gavage daily for 14 days [38]. A significant and dose dependent decrease in the size of tumors was recorded. This suggests that baicalein can inhibit PC cell proliferation through the inhibition of the Hh signaling pathway *in vitro* and *in vivo*, in addition to significant oral bioavailability in mice.

2.3.11 Pomegranate Juice Extract (PMJ)

PMJ is isolated from pomegranate fruit (*Punica granatum*). PMJ contains several polyphenolic compounds including but not limited to Quinnic acid, Ellagic acid, Punicalin, and Punicalagin [39]. In one study, PMJ from the commercially available product "POM Wonderful" was shown to inhibit PC cell proliferation. Western blotting revealed a dose-dependent decrease in the expression of Gli-1 protein in COLO 357 cells following 48-hour treatment with PMJ at a dose of 10 - 50µg/ml *in vitro* [39]. Gli-1 protein expression diminished by ~40% following 10µg/ml treatment of PMJ relative to control, with ~95% reduced expression of Gli-1 and no band detected following 25µg/ml and 50µg/ml treatments respectively [39]. This study suggests that a dietary agent/agents present in PMJ may possess potential chemopreventive and chemotherapeutic applications to PC treatment through the modulation of Hh signaling.

2.3.12 α-Mangostin

The phytochemical α-mangostin is a xanthonoid derived from mangosteen fruit (G*arcinia mangostana*) [40]. Several studies have demonstrated α-mangostin as an

antioxidant, anti-inflammatory agent, as well as inhibiting PC growth *in vitro* and *in vivo* [40, 122, 123]. In one study, α-mangostin was shown to inhibit PC proliferation, increase apoptosis, diminish EMT, and reduce CSC characteristics such as colony formation and pluripotency in AsPC-1, PANC-1, and human pancreatic CSCs (CD133+/CD44+/CD24+/ESA+) [40]. Western blotting revealed α-mangostin to inhibit Hh signaling in a dose dependent manner (2.5 – 10μM for 48 hours) via a reduction in protein expression of core Hh signaling proteins Gli-1, Gli-2, SMO, PTCH1, and PTCH2 in human CSCs *in vitro*. Inhibition of Hh signaling was further supported by luciferase assay which demonstrated a similar dose dependent reduction in Gli reporter activity [40]. This suggests that α-mangostin is a potent inhibitor of Hh signaling with potential therapeutic applications for PC.

2.3.13 Z-ajoene

Z-ajoene is an OSC derived from garlic (*Allium Sativum*) [41]. Z-ajoene decreased PC proliferation and demonstrated inhibition of the Hh signaling pathway by down regulating the protein expression of Gli-1, Gli-2, and PTCH1 in a dose dependent manner (5 - 20μM for 20 hours) in PANC-1 cells *via* western blot analysis [41]. Inhibition of Hh signaling was further supported by luciferase assay and qRT-PCR which demonstrated a similar dose dependent reduction in Gli reporter activity and level of Gli-1 mRNA respectively. Z-ajoene was also shown to induce G2/M cell cycle arrest in PANC-1 cells measured by flow cytometry [41].

2.3.14 Ellagic Acid

Ellagic acid is a phenolic lactone phytochemical present in various berries (strawberries, cranberries, and raspberries) and nuts (walnuts and pecans). Ellagic acid has been implicated in having a range of health benefits to include antimutagenic and hepatoprotective activity [124]. In one study, BALB-c nude mice were injected with PANC-1 cells subcutaneously then received an oral gavage of ellagic acid (40mg/kg a day) 5 days a week for 6 weeks. Following treatment there was a significant reduction in PC cell proliferation and EMT in PANC-1 tumor xenografts [42]. Western blotting and immunohistochemistry also revealed a decrease in both Gli-1 and Gli-2 following treatment with ellagic acid [42]. This suggests the decrease in PC cell proliferation and EMT is due, in part, to inhibition of aberrantly activated Hh signaling.

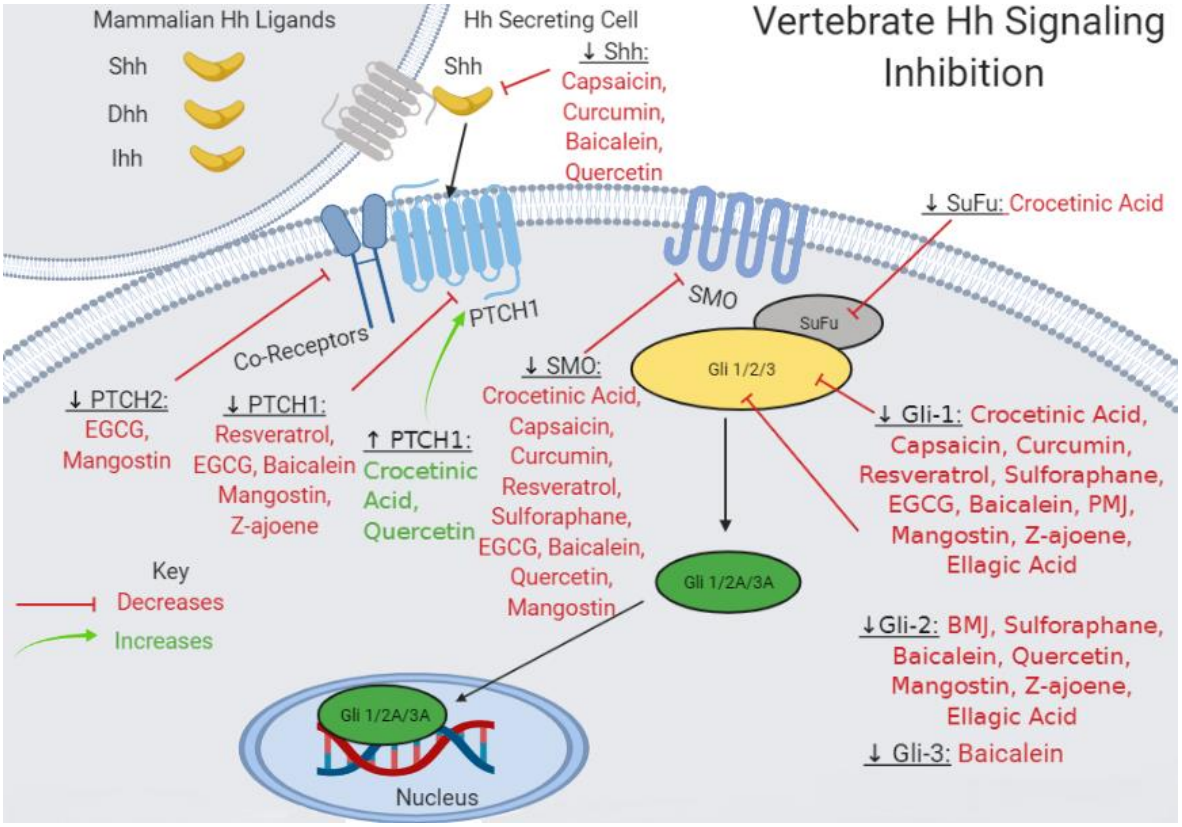

Figure 2.2 *Effects of Dietary Agent Inhibition of Hedgehog Signaling in PC.* This figure illustrates the summary findings of bioactive dietary agents which inhibit the Hh signaling pathway in PC cells. Effects on Hh signaling molecular targets protein and/or mRNA levels were measured via Western blot analysis, immunohistochemistry, and qRT-PCR in human pancreatic CSCs (CD133+/CD44+/CS24+/ESA+) PANC-1, AsPC-1, BxPC-3, MIA PaCa-2, HS766-T, 10.7, Patu 8988, COLO 357, and caerulein-induced pancreatic intraepithelial neoplasia. (Image created in BioRender.com)

2.4 Conclusion

PC is one of the most devastating of all human malignancies with a 5-year survival rate of ~10% [1]. Due to the general lack of early symptoms, PC is frequently diagnosed late stage and metastatic rendering many PC tumors inoperable. Surgical resection and current therapeutic options are insufficient to address the increasing prevalence of PC in the United States [1]. Due to the difficulties associated with treatment of PC post-diagnosis, chemopreventive therapies are currently being investigated. Bioactive dietary agents which inhibit the Hh signaling pathway present a unique approach to PC chemoprevention. When taken prophylactically and consumed in sufficient quantities, these dietary agents could potentially diminish PC proliferation and metastatic spread pre-diagnosis. This, in turn, could provide additional time for early detection of PC and increase likelihood of surgical resection success.

This chapter summarized the findings of bioactive dietary agents which have been shown to decrease cell proliferation of PC through the inhibition of the Hh signaling pathway (Figure 2.2) [27-42]. All compounds investigated *in vitro* showed a reduction in the expression of Hh signaling proteins and a decrease proliferation of PC cells [27-42]. Vitamin D3, capsaicin, sulforaphane, baicalein, quercetin, and ellagic acid were also investigated *in vivo* and with the exception of vitamin D3, all compounds investigated showed a similar dose-dependent decrease in PC tumor growth [29, 34, 35, 36, 38, 42]. Results following exposure to Hh signaling inhibitors were mostly consistent with a decrease expression of SMO, Shh, Gli-1, and

Gli-2. Results differed with regard to the expression of PTCH1 following treatment with suspected Hh signaling inhibitors. Crocetinic acid and quercetin indicated an increase in PTCH1 while resveratrol, EGCG, and α-mangostin showed a decrease in PTCH1, despite all five compounds showing a dose-dependent decrease in the expression of SMO [28, 34, 36, 37, 40]. Similar results were observed following quercetin treatment, with no change in Gli-1 protein expressions despite a dose-dependent decrease in Shh, SMO, and Gli-2 [36]. The level of core Hh signaling proteins PTCH1 and Gli-3 following pathway inhibition is poorly characterized and inconsistent between studies [28, 31, 36-38, 40]. While dietary agents capable of Hh signaling inhibition have been identified, the mechanism of action and crosstalk with other pathways is currently largely unknown.

Chapter 3. Impact of Diallyl Trisulfide on Hedgehog Signaling in Pancreatic Cancer Cells

3.0 Abstract

Pancreatic cancer (PC) is one of the most malignant and deadliest of cancers. Aberrant activation of the Hedgehog (Hh) signaling pathway is present in numerous cancer cell types including PC, with higher levels of Hh signaling protein expression in PC stem cells. Hh signaling has been implicated in sustaining cancer cell proliferation, metastasis, and maintenance of a chemoresistant tumor microenvironment in PC. Bioactive garlic-derived organosulfur compounds present in *Allium* vegetables have been identified which inhibit the proliferation and induce apoptosis of PC cells. One of the most biologically active of these compounds is diallyl trisulfide (DATS).

Prior research by our lab has shown DATS to inhibit colony formation and Hh signaling in pancreatic cancer, including inhibition of Gli-1 in AsPC-1 cells [125]. This study expanded upon that research and showed that DATS reduced the expression of Gli-1 and Gli-3 in BxPC-3 cells. Components of the Hh signaling pathway (Gli-1, Gli-3, SMO, Shh) were assessed by Western blotting analysis following 24-hour treatment with 0 – 100µmol/L DATS. Gli-3 in BxPC-3 and SMO in AsPC-1, BxPC-3, and MIA PaCa-2 cells were further assessed via immunocytochemistry. Gli-3 mRNA expression was further assessed via quantitative real time polymerase chain reaction (qRT-PCR).

Following *in vitro* treatment with DATS, Western blotting revealed a dose-related change in Hh signaling proteins with a decrease in Gli-1 in AsPC-1 and BxPC-3, and Gli-3

in BxPC-3, while also revealing an increase in Shh in AsPC-1. This suggests that DATS differentially impacts the Hh signaling pathway in PC cell lines. This effect may be due in part to mutational differences between the cell lines tested, or through non-canonical Hh signaling.

3.1 Introduction

PC is one of the leading causes of cancer-related death globally and the 4th highest cause of cancer-related death in the United States [1, 3]. This high mortality rate is related to the late stage of diagnosis and high chemoresistance to current pharmaceutical therapies [3]. The only cure for PC is radical resection of the pancreas [3]. A diet rich in nutraceuticals has been implicated to be a primary method of cancer chemoprevention [62, 73, 126].

Mature PC cells possess mutations in core signal transduction pathways which enable their continued proliferation and metastatic spread [7]. Neo-adjuvant chemotherapies (gemcitabine, 5-FU, mitomycin C, and platinum compounds) have been shown to reduce tumor size, enhance resectability, and reduce metastasis in PC [1, 8, 127]. The re-activation of the Hh signaling pathway has been implicated in the continued growth and stem cell characteristics of PC cells [63, 90, 108, 109]. Inhibition of the Hh signaling pathway has been shown to decrease proliferation, increase apoptosis, and decrease stem cell characteristics of PC cells [27-42, 46-52].

Phytochemicals in garlic have also demonstrated an increase in autophagy and apoptosis in several cancer cell types including PC and represent significant potential for cancer chemoprevention and/or complementary chemotherapy [43, 46]. Garlic oil at a dose of 10µM - 100µM for 24 hours was shown to inhibit cell proliferation and induce G2/M cell cycle arrest in PC cell lines AsPC-1, MiaPaCa-2, and PANC-1, measured by MTT assay and flow cytometry, respectively [45]. Another study showed similar results with a 55% decreased cell viability in tumorigenic PC cells (Capan-2) and 30% decreased cell viability in normal pancreatic epithelial (H6C7) cells following 24-hour exposure to 100µM DATS measured by MTT assay [44]. DATS exposure (100µM for 24 hours) also induced G2/M cell cycle arrest as measured by flow cytometry [44]. Following DATS treatment there was also a dose-related decrease in the expression of cell cycle regulatory proteins such as Cyclin D1 [44]. This suggests that DATS as well as potentially other constituents of garlic oil have the ability to inhibit PC development, proliferation, or metastasis. This also implies potential synergistic effects of multiple OSCs in the treatment or chemoprevention of PC.

DATS has also been utilized in some animal studies *in vivo* and found to be well tolerated, chemo-protective, and exhibit anti-cancer effects [59]. DATS has been shown to inhibit cancer growth *in vivo* in several cancer types including prostate, colon, breast, gastric, lung, skin, glioblastoma, and leukemia [59]. The anti-cancer effects of DATS are largely attributed to the activation of apoptotic pathways and G2/M cell cycle arrest [59, 73]. In one study DATS (30 mg/kg orally for 7 days) was shown to exert a protective role

in ethanol-associated oxidative stress which led to induced liver injury in male Kun–Ming mice [128]. DATS (30-40 mg/kg injection for 24 days) has also been shown to ameliorate cisplatin-induced oxidative injury while also inhibiting cancer cell growth and proliferation in lung carcinoma xenograft BAL b/c nude mice [129].

Our lab has previously shown that DATS inhibits cell growth, colony formation ability, and modulates Hh signaling in PC cells [125]. This research showed that DATS induces a dose-dependent reduction in Gli-1 protein and mRNA, and an increase in Shh protein levels in AsPC-1 cells, as well as a decrease in colony formation ability in BxPC-3 and Mia PaCa-2 cells [125]. In the present study the impact of DATS upon core Hh signaling proteins was expanded upon to include additional targets and cell lines. This study evaluated the impact of DATS treatment on Hh signaling proteins Gli-1, Gli-3, SMO, and Shh in AsPC-1, BxPC-3, and MIA PaCa-2 cell lines.

3.2 Materials and Methods

3.2.1 Reagents

Diallyl trisulfide (#D3202) was purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) (#BP321100) and phosphate buffered saline (PBS) pH 7.4 (#10010023) were purchased from Fischer Scientific (Waltham, MA). Paraformaldehyde 4%, glycine, cytoseal 60, and triton X-100 were purchased from VWR International (VWR) (Radnor, PA). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (St. Louis, MO) and 4’,6-diamidino-2-phenylindole (DAPI) (#4083) was purchased from Cell

Signaling (Danvers, MA). Primary antibodies Gli-1 (#3538 for AsPC-1 and BxPC-3, #2534 for MIA PaCa-2) and Shh (#2207) were purchased from Cell Signaling, and Gli-3 (#NBP2-29627) was purchased form Novus Biologicals (Littleton, CO). SMO (#SAB1412475) and β-actin (#A5441) were purchased from Sigma Aldrich. SMO (#PA5113312) and Gli-3 (#PA5-28029) for immunofluorescence were purchased from Invitrogen (Carlsbad, CA). Secondary antibodies, anti-rabbit (#95017-556) and anti-mouse (#95017-332) were purchased from VWR. Secondary antibodies, anti-goat (#PA1-29617) and Alexa Fluor 488 goat anti-rabbit (#2018207) were purchased from Invitrogen.

3.2.2 Cell Culture

Human pancreatic cancer cell lines MIA PaCa-2, AsPC-1, and BxPC-3 were purchased from American Type Culture Collection (ATCC) (Manassas, VA). MIA PaCa-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) purchased from VWR and supplemented with 10% fetal bovine serum (FBS) (ATCC) and 2.5% horse serum (VWR). AsPC-1 and BxPC-3 cells were grown in RPMI 1640 (ATCC) and supplemented with 10% FBS (ATCC). All media was additionally supplemented with 1% penicillin streptomycin solution purchased from Corning (Corning, NY) which contains a mix of the antibiotics penicillin (10,000 IU) and streptomycin (10,000µg/mL). Cells were maintained at 37° C in a 5% CO_2 atmosphere. Cells were sub-cultured with the use of trypsin 0.05% EDTA (Fisher Scientific).

3.2.3 Western Blotting

Cells ($1x10^6$) were plated onto 100-mm plates and allowed to incubate for 24 hours. Cells were treated with vehicle (0.55% v/v DMSO) or DATS (20, 40, 60, 80, 100μM/L) for 24 hours. Cells were collected by cell scraping, pelleted at 2500 RPM (1006 RCF) for 10 minutes, and stored at -80° C. Cell pellets were lysed with buffer containing 96% 1X RIPA buffer (Cell Signaling), 1% phosphatase inhibitor cocktail 2 (100x) (Sigma-Aldrich), 1% phosphatase inhibitor cocktail 3 (100x) (Sigma-Aldrich), and 2% protease inhibitor cocktail (50x) (Sigma-Aldrich). Cell lysate was collected, and protein concentration determined by Bradford Assay at 595nm using a Multiskan (accuSkan) FC microplate reader (Fisher Scientific). Protein samples were prepared for Western blotting with the addition of buffer containing 95% laemmli lysis buffer (Sigma-Aldrich) and 5% 2-mercaptoethanol (Bio-Rad, Philadelphia, PA). Protein samples were then boiled for 5 minutes before storage at -20° C. Western blotting was performed with 10% sodium-dodecyl sulfate polyacrylamide gels (#1610172, 10% TGX FastCast Starter Kit, Bio-Rad) and run under standard conditions (100-130V for 1.5-2 hours). Gels were transferred to nitrocellulose PVDF membranes (#1704156, Trans-Blot Turbo Mini-PVDF, Bio-Rad) with a Trans-Blot Turbo transfer system (Bio-Rad) running at standard conditions (1.3A, 25V, 10 minutes). Following blocking for 2 hours at room temperature (RT) in 5% (w/v) milk (or BSA for Gli-1 in AsPC-1 or BxPC-3) in tween tris-buffered saline (TTBS), membranes were incubated in primary antibody 24 hours or 48 hours (Gli-1, SMO in AsPC-1) at 4° C. Membranes were washed three times for 5-minute intervals at

RT in TTBS. Membranes were then incubated in secondary antibody for 1 hour at RT before being washed three times for 15-minute intervals at RT in TTBS. Following incubation in Super Signal West Femto Maximum Sensitivity Substrate (ECL) (#PI34095, Thermo-Scientific, Waltham, MA) proteins were visualized in a ChemiDoc imaging system (Bio-Rad). Restore Western Blot Stripping Buffer (#PI21059, VWR) was used on select membranes for re-blotting additional targets (SMO). Densitometric analysis of Western blotting membranes was performed with Image Lab software (Bio-Rad).

3.2.4 Immunofluorescence Microscopy

Cells (50,000) were plated onto 22mm sterile coverslips and allowed to incubate for 24 hours. Cells were treated with vehicle (0.55% v/v DMSO) or DATS (60, 80, 100µM/L) for 24 hours. Growth medium was removed, and cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT. Coverslips were washed three times in PBS before permeabilization with 0.5% Triton X-100 in PBS for 10min at RT. Coverslips were again washed three times in PBS before blocking with 0.5% BSA, 0.15% glycine in PBS for 1 hour at RT with gentle rocking. Primary antibody in blocking buffer at a dilution of 1:100 was added and allowed to incubate at 4 °C overnight with gentle rocking. Primary antibody was removed, and coverslips were washed three times in PBS. Secondary antibody in blocking buffer was added at a dilution of 1:1000 and allowed to incubate for 1 hour at RT and protected from light exposure. Secondary antibody was removed, and coverslips were washed three times in PBS before being counterstained with DAPI (0.5µg/mL) for 3min at RT and protected from light exposure. Coverslips were washed

three times in PBS before being mounted onto slides with Cytoseal 60. Slides imaged with a Leica TCS SP8 confocal microscope and LSX software at a magnification of 63X.

3.2.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The isolation of RNA from frozen cell pellets was performed as previously decribed by our lab [125]. In short, RNA was extracted from frozen cell pellets (-80°C) using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA) and PureLink DNase Kit (Invitrogen). cDNA was synthesized using the BioRad iScript cDNA Synthesis Kit (Hercules, CA). Primers for Gli-3 (Forward 5'-GGG GAC AAA GAT GAA AGC AA-3', Reverse 5'-GCT TTG AAC GGT TTC TGC TC-3') [130] and GAPDH (Forward 5'- ATG GGG AAG GTG AAG GTC G -3', Reverse 5'- GGG GTC ATT GAT GGC AAC AAT A -3') [131] was purchased from Integrated DNA Technologies (Coralville, IA). Quantitative real-time PCR was performed using a CFX96 Touch Real-Time PCR detection system (BioRad) with LightCycler 480 SYBR Green I Master hot start reaction mix (Roche, Basel, Switzerland). Target sequences were amplified with initial denaturing at 95°C for 10 minutes, followed by 45 cycles of a 3-step amplification consisting of 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 30 seconds [125]. Fold change was calculated relative to the control GAPDH, using the $2^{-\Delta\Delta Ct}$ method.

3.2.6 Statistical Analysis

All numerical data are presented as mean ± standard error of the mean. Statistical significance was determined by one-way ANOVA followed by Dunnett's

multiple comparisons test. Statistical analysis was performed using Graph Pad Prism Version 8.4.3 software. Protein band quantification and normalization to Actin (housekeeping protein) was performed in Image Lab software (Bio-Rad). A *P*-value of less than 0.05 was considered statistically significant.

3.3 Results

To investigate the impact of DATS upon the Hh signaling pathway three human pancreatic cancer cell lines (AsPC-1, BxPC-3, and MIA PaCa-2) were evaluated. Cells were treated with vehicle (0.55% v/v DMSO) or DATS (20, 40, 60, 80, 100µmol/L) for 24 hours. Analysis of Gli-1, Gli-3, Shh, and SMO protein level in response to 24-hour DATS treatment was done by Western blotting.

As Gli-1 is the primary and constitutive activator of the Hh signaling pathway it was the first molecular target investigated. Gli-1 protein expression was significantly reduced in a dose-dependent manner by up to 83% following 24 hours of 60 µmol/L DATS treatment in AsPC-1 cells relative to vehicle (n=3) (Figure 3.1 A). This result was similar with prior findings from our lab [125]. Gli-1 protein expression in BxPC-3 cells was evaluated at higher doses (up to 100 µmol/L DATS treatment for 24 hours) which showed a decrease in Gli-1 protein at 100 µmol/L DATS by 49% (n=3) relative to control (Figure 3.1 B). There was no significant effect on Gli-1 protein expression following DATS treatment in MIA PaCa-2 cells (n=3) up to 60 µmol/L DATS treatment for 24 hours (Figure 3.1 C).

The next molecular target investigated was the pathway activator and repressor Gli-3. The antibody used for Gli-3 recognized a common epitope present in the N terminus of Gli-3 which allowed for the visualization of the full form (Gli-3FL) and the truncated repressor form (Gli-3R) by Western blotting and immunofluorescence. Gli-3R protein expression was significantly reduced in a dose-dependent manner by up to 59% following 24 hours of 60 µmol/L DATS treatment in BxPC-3 cells relative to vehicle (n=3) by Western blotting (Figure 3.2 A). Gli-3FL in BxPC-3 cells showed a similar dose-related trend following DATS treatment but did not reach statistical significance (Figure 3.2 A). Confocal microscopy immunofluorescence for Gli-3 showed a decrease in Gli-3 fluorescent signal at a dose of 80 µmol/L DATS treatment for 24 hours (n=3) (Figure 3.2 C). Gli-3FL was further evaluated by qRT-PCR analysis at a dose of 80 µmol/L DATS treatment for 24 hours. qRT-PCR analysis showed no significant effect ($P<0.5$) in Gli-3FL mRNA expression (n=3) (Figure 3.2B).

The full-length form Gli-3FL and the transcriptional repressor Gli-3R were not significantly impacted in AsPC-1 cells (n=3) up to 100 µmol/L DATS treatment for 24 hours (Figure 3.3 A). Gli-3FL and Gli-3R protein expression was not significantly impacted in MIA PaCa-2 cells (n=3) (Figure 3.3 C) following 24 hours of 60 µmol/L DATS treatment.

Protein levels of the N-terminally truncated and physiologically active form of Shh increased by up to 101% following 24 hours of 60 µmol/L DATS treatment in AsPC-1 cells relative to vehicle (n=3) (Figure 3.4 A). This was in agreement with previous data from our lab that showed DATS treatment increases Shh protein level in AsPC-1 cells

[125]. Levels of Shh protein were not significantly impacted in BxPC-3 or MIA PaCa-2 cells (n=3) following 24 hours of 60 µmol/L DATS treatment relative to vehicle (Figure 3.4 B - Figure 3.4 C).

Protein levels of the G protein-coupled receptor SMO (40 kDa) were not significantly impacted in AsPC-1, BxPC-3 (n=3), or MIA PaCa-2 cells (n=2) following 24 hours of 60 µmol/L DATS treatment by Western blotting (Figure 3.5 A, C, E) or immunofluorescence microscopy (Figure 3.5 B, D, F). However, the antibody used for SMO showed effect on several other bands (~76 kDa) which require further investigation.

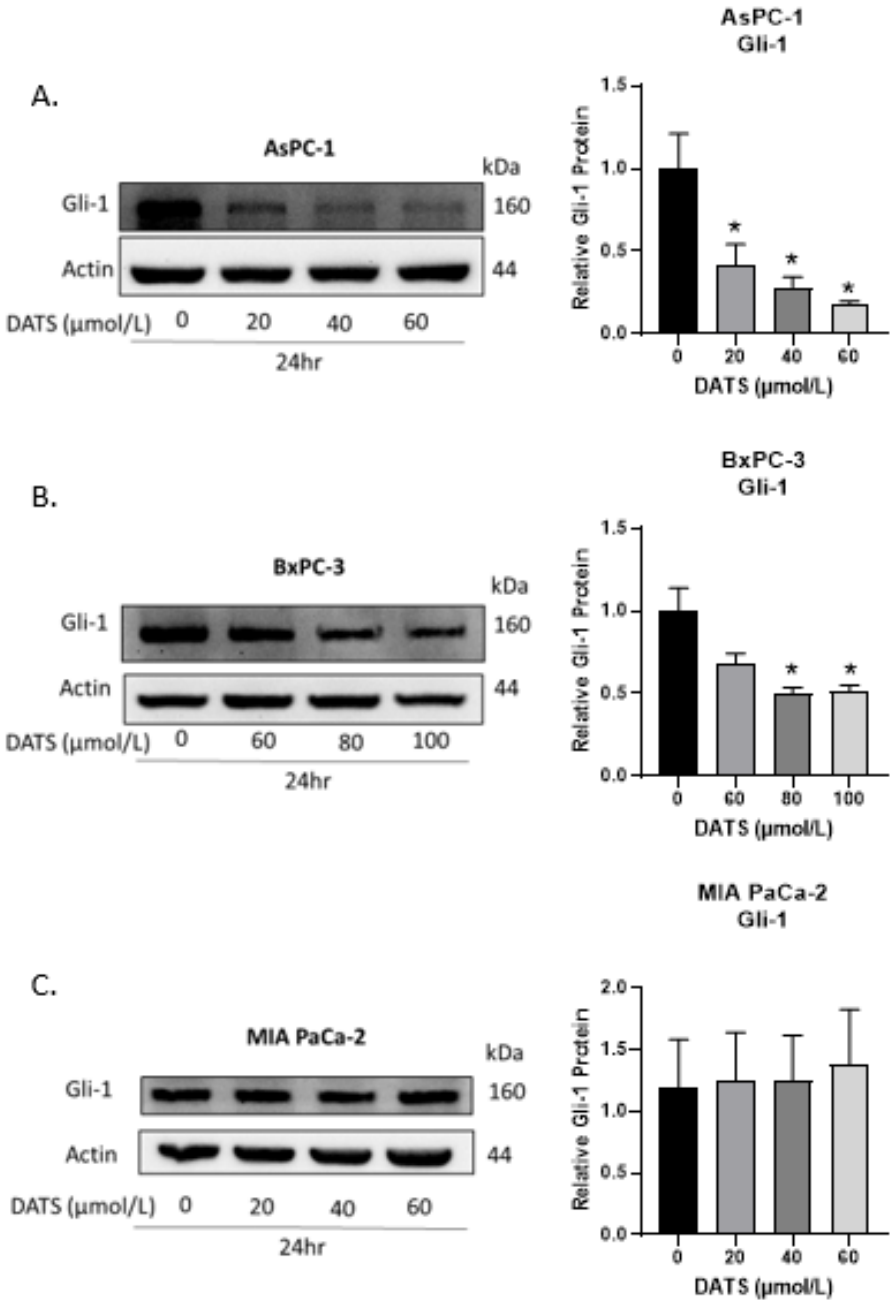

Figure 3.1 *DATS treatment impact upon levels of Gli-1.* (A) (B) (C) Representative experiment of Western blotting for Gli-1 in AsPC-1, BxPC-3, and MIA PaCa-2 cells, respectively, following DATS treatment for 24 hours. Densitometric analysis of Western blots, columns represent mean, bars represent SEM of three independent experiments. *, significant, $P < 0.05$, by one-way ANOVA followed by Dunnett's multiple comparisons test.

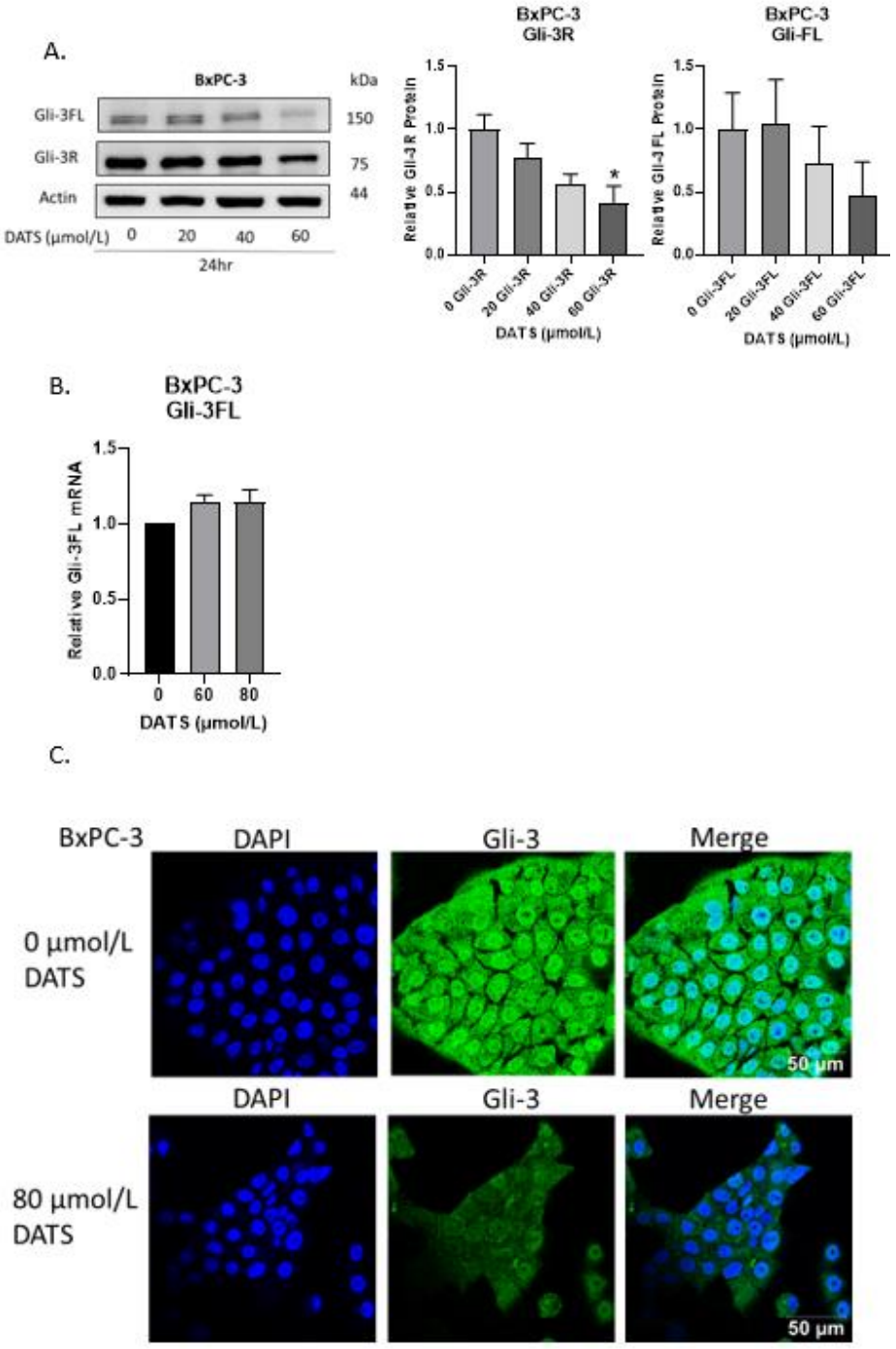

Figure 3.2 *DATS treatment impact upon levels of Gli-3 in BxPC-3.* (A) Representative experiment of Western blotting for Gli-3FL and Gli-3R in BxPC-3 following DATS treatment for 24 hours. (B) qRT-PCR analysis of Gli-3FL mRNA (n=3). Columns represent mean, bars represent SEM of three independent experiments. *, $P < 0.05$, significant by one-way ANOVA followed by Dunnett's multiple comparisons test. (C) Immunocytochemistry of DAPI (blue) and Gli-3 (green) in BxPC-3 cells following DATS treatment for 24 hours (n=3).

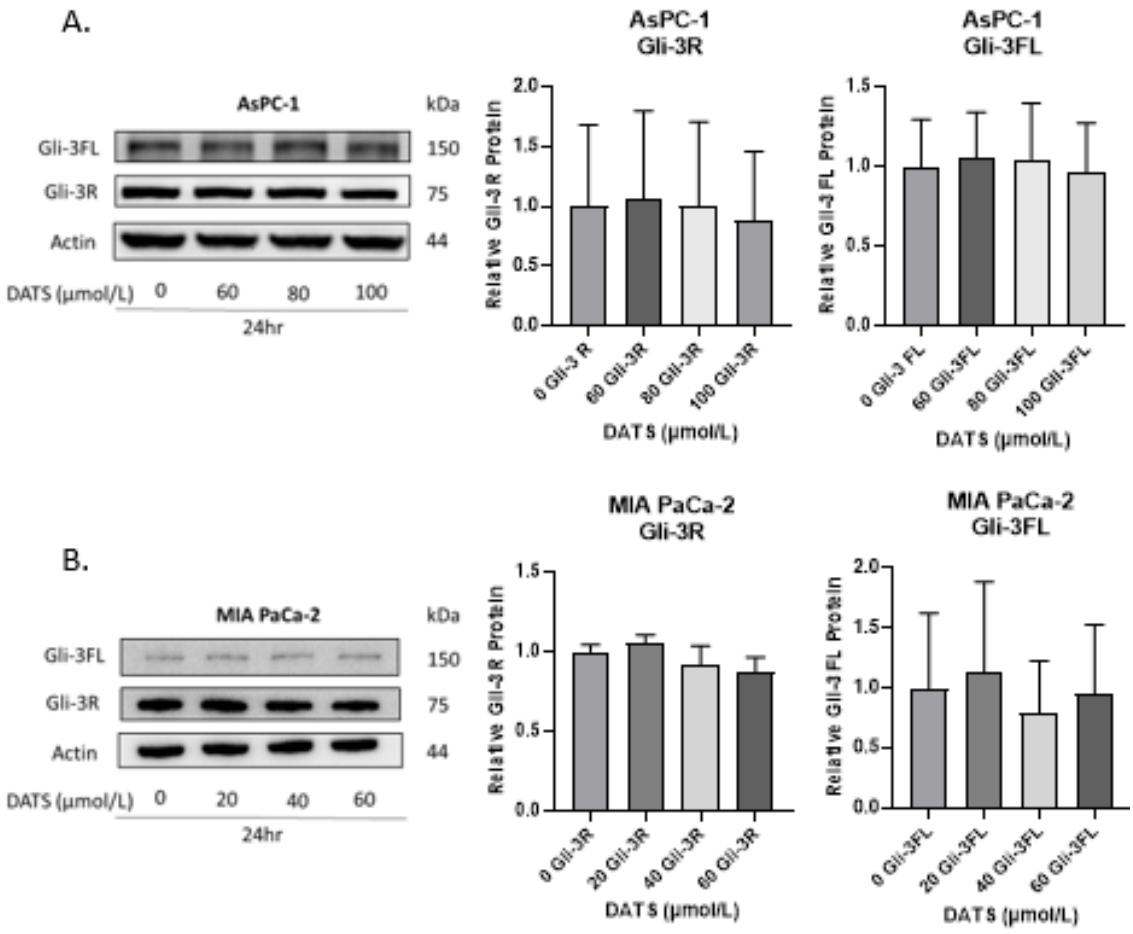

Figure 3.3 *DATS treatment impact upon levels of Gli-3FL and Gli-3R protein in AsPC-1 and MIA PaCa-2.* (A) (B) Representative experiment of Western blotting for Gli-3FL and Gli-3R in AsPC-1, and MIA PaCa-2 cells, respectively, following DATS treatment for 24 hours. Densitometric analysis of Western blots, columns represent mean, bars represent SEM of three independent experiments. *, $P < 0.05$, significant by one-way ANOVA followed by Dunnett's multiple comparisons test.

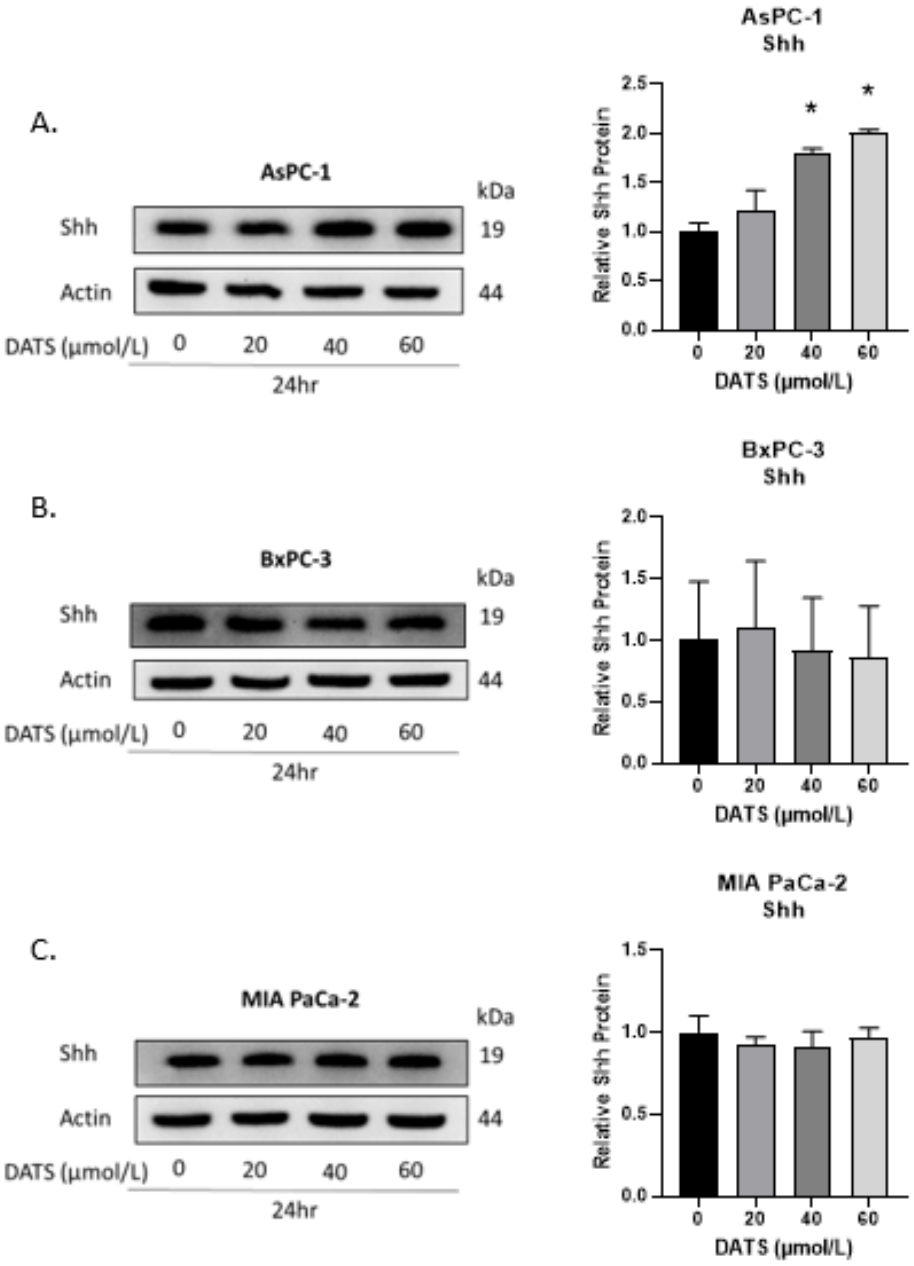

Figure 3.4 *DATS treatment impact upon levels of Shh.* (A) (B) (C) Representative experiment of Western blotting for Shh in AsPC-1, BxPC-3, and MIA PaCa-2 cells, respectively, following DATS treatment for 24 hours. Densitometric analysis of Western blots, columns represent mean, bars represent SEM of three independent experiments. *, $P < 0.05$, significant by one-way ANOVA followed by Dunnett's multiple comparisons test.

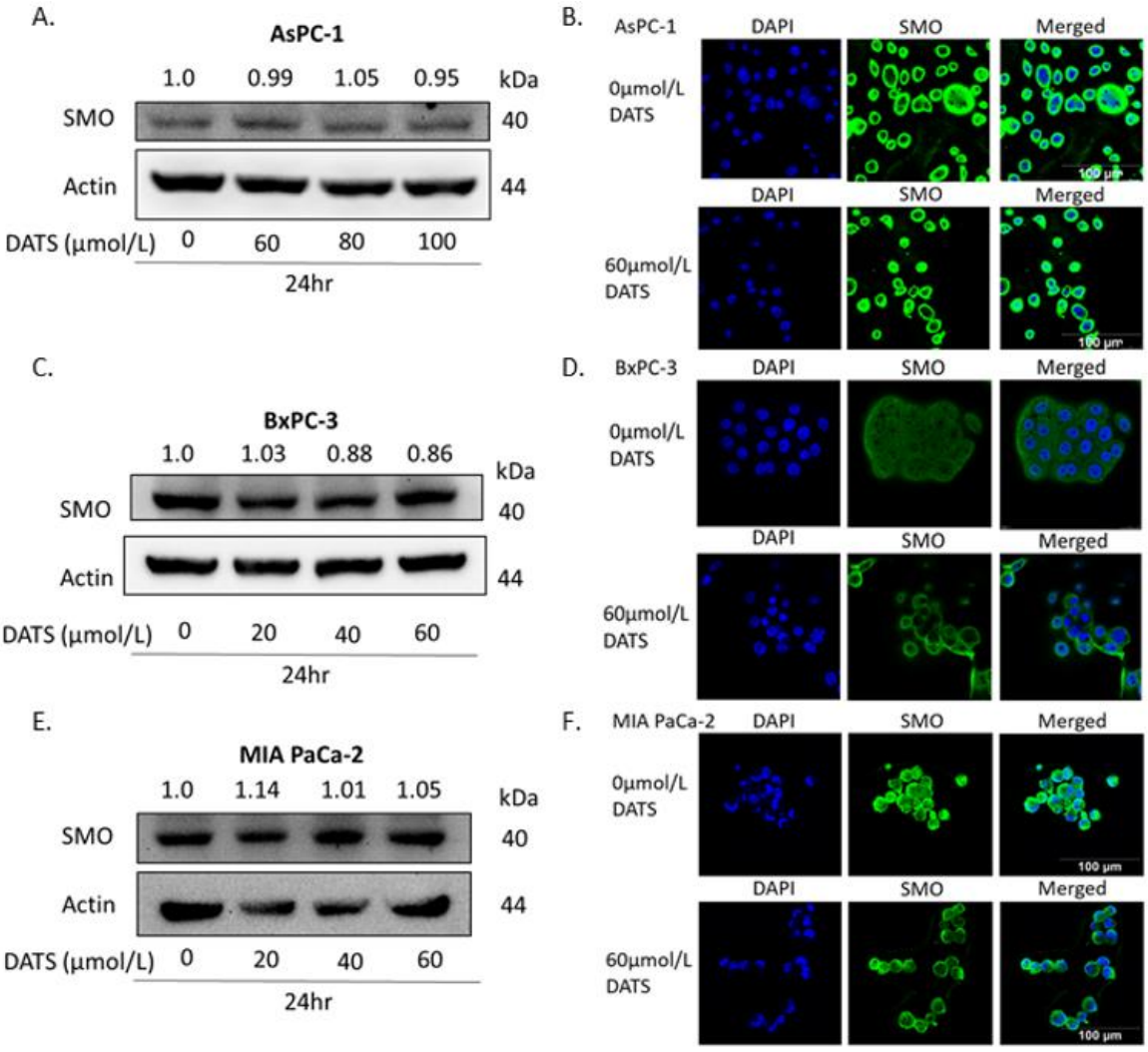

Figure 3.5 *DATS treatment impact upon levels of SMO.* (A) (C) (E) Representative experiment of Western blotting for SMO in AsPC-1, BxPC-3, and MIA PaCa-2 cells, respectively, following DATS treatment for 24 hours. Densitometric analysis of Western blots, values represent mean of three independent experiments. (B) (D) (F) Immunocytochemistry of DAPI (blue) and SMO (green) in AsPC-1, BxPC-3, and MIA PaCa-2 cells, respectively, following 24 hours of DATS treatment.

3.4 Discussion

The objective of the present study was to characterize the impact of DATS upon the Hh signaling pathway in pancreatic cancer cells. Previous research from our lab showed that DATS treatment reduces protein and mRNA levels of Gli-1 and increases Shh protein levels in AsPC-1 cells [125]. The present study showed that DATS treatment induces a significant inhibition of Gli-1 protein in both AsPC-1 and BxPC-3 cells. This suggests that Hh signaling is inhibited in these two cell lines. As more molecular targets were evaluated, however, a more complex picture of Hh signaling modulation following 24-hour DATS treatment began to emerge. In AsPC-1 cells there is a decrease in Gli-1 following DATS treatment, but there is a statistically significant increase in Shh as well as no observable change in Gli-3, and SMO. DATS treatment of BxPC-3 cells led to Hh signaling inhibition, as shown by a decrease in Gli-1 and Gli-3. MIA PaCa-2 cells showed no significant modulation in Hh signaling at the tested concentration of DATS. These data suggest that canonical Hh signaling may be inhibited in AsPC-1 and BxPC-3 cells, however, modulation of core Hh signaling proteins in AsPC-1, BxPC-3, and MIA PaCa-2 cells may be due in part to non-canonical means and/or cross-talk with other cell signaling pathways.

Hh signaling can be regulated through both canonical and non-canonical means [109]. In canonical Hh signaling a ligand is secreted and binds to the transmembrane protein receptor PTCH1 which relieves inhibition of SMO (a positive regulator of Hh signaling and proto-oncogene) [10]. Alternatively, the pathway can also be

activated/regulated through non-canonical Hh signaling by bypassing the ligand-receptor signaling axis [109, 132, 133]. Non-canonical Hh signaling is poorly characterized but has been reported to be regulated by several pathways including KRAS, PKC, transforming growth factor β (TGFβ), and Tumor Necrosis Factor Alpha (TNF-α) [109, 134-139]. For example, in one study utilizing large scale multi-cancer analysis showed a strong correlation between increased TGFβ ligands, but not Hh ligands with the increased protein expression of Gli-1 and Gli-2 [140]. Studies have implicated the existence of at least two distinct mechanisms of non-canonical Hh signaling [10, 132, 139, 140]. Type I non-canonical Hh signaling whereby PTCH1 and/or PTCH2 functions independently of SMO and Type II non-canonical Hh signaling which functions through SMO independently of Gli-1 transcription factors [132, 141, 142].

PC cell lines (AsPC-1, BxPC-3, and MIA PaCa-2) possess a range of various mutations, including mutations in genes associated with non-canonical Hh signaling. Both AsPC-1 and MIA PaCa-2 cells contain activating mutations in KRAS, while BxPC-3 does not [143, 144]. BxPC-3 contains a mutation in the tumor suppressor gene SMAD4, while MIA PaCa-2 does not [143]. The status of SMAD4 in AsPC-1 is currently unknown with inconsistent results reported [143]. While not directly implicated in non-canonical Hh signaling, cyclooxygenase-2 (COX-2) is associated with angiogenesis, which in turn influences tumor growth and metastatic spread of various cancers including PC [145]. COX-2 expression has been detected in BxPC-3 cells but not in AsPC-1 or MIA PaCa-2 cells [143]. As a result of the presence or absence of these various genetic mutations,

these three cell lines represent several of the currently identified/implicated pathways involved in non-canonical Hh signaling (KRAS, TGFβ-1, and PKC through TGFβ-1 upregulation) [133, 143, 144, 146]. This suggests that the differential response to DATS exposure in AsPC-1, BxPC-3, and MIA PaCa-2 may be the result of the mutational status of these genes.

The present study is one of the few to evaluate Gli-3 protein expression in a study investigating a dietary agent's impact on Hh signaling and the only study to investigate this effect with an OSC. Our results showed a differential impact of treatment upon the expression of Gli-3 protein (Figure 3.2 and 3.3). Studies investigating Hh signaling predominantly focus on the pathway transducers Gli-1 and Gli-2 while excluding Gli-3. While Gli-3 is not portrayed as playing a significant role in Hh gene induction in the canonical Hh pathway, growing evidence has implicated a multi-functional role of Gli-3 in Hh signaling induction and inhibition, cell survival, and sensitization to SMO antagonists [147, 148]. In one study, siRNA mediated Gli-3 knockdown appeared to sensitize PC cells (PANC-1) to cyclopamine (a SMO antagonist) [148]. This suggests that Gli-3 is an all too often overlooked indicator of Hh signaling status. The present study showed differential modulation of Gli-3 among the cell lines exposed to DATS. This was determined by Western blotting analysis which showed a decrease in Gli-3FL and Gli-3R only in BxPC-3 cells, which was further confirmed by immunofluorescence microscopy (Figure 3.2).

DATS treatment induced inhibition of Gli-1 protein in AsPC-1 and BxPC-3 cells despite a dose related increase in Shh in AsPC-1 and no change in Shh in BxPC-3 cells. This apparent insensitivity to Shh may be related to disassembly of the primary cilia following DATS exposure. Canonical Hh signaling requires an intact primary cilium for Shh stimulation of the pathway. Deciliation prevents mechanosensitive Hh signaling [149-151]. Formation of the primary cilium occurs during G0 and G1 and is re-absorbed during G2 to M phase transition. Both formation and disassembly of the primary cilium is mediated, in part, by the action of a tubulin deacetylase and histone deacetylase 6 (HDAC6) [150-152]. Deciliation in mammalian cells occurs predominantly through whole cilium shedding in a matter of seconds [153]. The relevance to Shh is that mere moments after a cell initiates disassembly of the primary cilium, it may become entirely insensitive to Shh stimulation. DATS and other components of garlic oil has been shown to induce G2/M cell cycle arrest (a state in which there is no primary cilium present) in multiple forms of cancer [41, 59, 67]. DATS has also been shown to inhibit HDAC activity in various cancers which may potentially prevent formation of the primary cilium [67, 150, 154]. In one study, DATS at a dose of 20μM for 12 hours was shown to induce G2/M arrest via microtubule network disruption in HCT-15 colon cancer cells [155]. Taken together, this suggests that DATS may reduce mechanosensitivity to Shh stimulation through preventing formation of the primary cilium and/or locking cells in an unciliated state.

Chapter 4. Conclusion

While many forms of cancer have decreased in incidence, and increased in treatment options and early detection technologies, PC has remained relatively stagnant [1, 2, 4]. The lack of early detection technologies and predominant late-stage diagnosis of PC highlights the importance of identifying dietary nutraceuticals with chemopreventive potential. Consumption of such nutraceuticals has the potential to inhibit PC cell growth and stem cell characteristics. The present study discussed the currently identified dietary nutraceuticals which have been shown to inhibit the Hh signaling pathway in PC cells. The present study also investigated the *Allium* derived organosulfur compound DATS, and its effect upon the Hh signaling pathway in PC cells.

Hh signaling in vertebrates is poorly characterized and what constitutes inhibition of this pathway lacks a coherent consensus. While some studies have claimed an increase in PTCH1 is indicative of Hh signaling inhibition, other studies purport the opposite to be true [28, 31, 36-38, 40]. The present study further corroborates the conclusion that ascertaining the status of Hh signaling (active or inactive) is complex. While AsPC-1 and BxPC-3 showed inhibition of the Hh signaling pathway through a reduction in Gli-1, the expression of other components of the pathway (namely Gli-3) were differentially impacted. This highlights the potential value of including often overlooked Hh signaling components such as PTCH2 and Gli-3.

A differential modulation of Hh proteins was shown with a significant downregulation of Gli-3 in BxPC-3, but no significant effect in AsPC-1 or MIA PaCa-2

cells. To better understand the implications of these results future research could investigate the relationship between the mutation status of suspected non-canonical Hh signaling pathways (KRAS, Cox-2, SMAD4, etc.) and differential response of Hh signaling inhibition in PC. Such studies could further elucidate Hh signaling in general, why differential impact on Hh signaling is observed between cell lines, as well as furthering the understanding of PC drug resistance and Hh signaling re-activation following SMO inhibition. Future research could also investigate the status of the primary cilium following DATS exposure and how the status of the primary cilium correlates with DATS induced G2/M cell cycle arrest. Such a study could reveal the mechanism behind the apparent insensitivity to Shh stimulation as well as potentially elucidate the mechanism of action of DATS. Lastly, increasing the dose from 60 µmol/L DATS to 100 µmol/L DATS for 24 hours showed a significant reduction in Gli-1 protein expression in BxPC-3 cells. This suggests that an increased dose and/or treatment time is indicated for molecular targets which showed no effect at a dose of 60 µmol/L DATS for 24 hours. Such a study could determine if the cell lines which have shown increased resistance to DATS induced Hh signaling modulation are susceptible to Hh signaling inhibition at higher doses or if these cells respond differently to DATS treatment.

In conclusion, the Hh signaling pathway has been implicated as a causative pathway in PC cell growth, homeostasis, chemoresistance, and metastasis [4,6,7,9]. Several dietary agents have been shown to inhibit Hh signaling in PC cells. Epidemiological evidence suggests increased *Allium* vegetable consumption is

associated with the lowest risk for developing PC [61]. The present study showed that DATS treatment induced significant inhibition of core Hh signaling proteins in both AsPC-1 and BxPC-3 cells suggesting inhibition of the Hh pathway. This study supports further research on DATS as a potential chemopreventive agent capable of inhibiting Hh signaling in PC cells.

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