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ORIGINAL ARTICLE

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# Cyclopamine increases the cytotoxic effects of paclitaxel and radiation but not cisplatin and gemcitabine in Hedgehog expressing pancreatic cancer cells

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**Abstract** *Introduction*: The hedgehog signaling pathway (Hh) is frequently over expressed in pancreatic adenocarcinomas. We studied the potential cytotoxic interactions between cyclopamine, a Hh pathway inhibitor and paclitaxel, cisplatin, gemcitabine and ionizing radiation (IR). *Methods*: In vitro clonogenic survival analysis was performed with cyclopamine alone or cyclopamine in combination with paclitaxel, gemcitabine, cisplatin and IR in Hh expressing human pancreatic tumor cells and Hh non-expressing colon cancer cells. Relative cytotoxicity was assessed in combination treatment compared with exposure to single agents. Assays of apoptosis (annexin V) were performed in the presence of cyclopamine, chemotherapeutic agents, and IR. *Results*: We report that cyclopamine increased the cytotoxic effects of paclitaxel and IR in Hh expressing pancreatic carcinoma cells. These effects were not observed in Hh non-expressing cells. Cyclopamine did not significantly increase killing by cisplatin or gemcitabine in Hh expressing pancreatic cancer cells. *Conclusions*: These data suggest strategies to combine Hh inhibitors with radiotherapy and chemotherapeutic agents, specifically paclitaxel and related compounds in the treatment of pancreatic cancer.

**Keywords** Cyclopamine · Paclitaxel · Hedgehog · Pancreatic cancer

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## Introduction

Pancreatic cancer is a common malignancy with an extremely poor prognosis reflected by the equivalence of annual incidence and mortality rates [1]. Although surgery has been the main treatment modality for pancreatic cancer, very few patients are long term survivors. Consequently, the addition of adjuvant and neoadjuvant treatment has been investigated. For example, a small randomized trial from the Gastrointestinal Tumor Study Group demonstrated a modest survival benefit from 5-fluorouracil (5-FU) based chemotherapy and radiation therapy after pancreatic resection [2]. By contrast, a recent randomized trial by the European Study Group for Pancreatic Cancer found a significant 5-year survival benefit in post-operative treatment with chemotherapy but not combined radiotherapy and chemotherapy [3]. Reports of neoadjuvant therapy for pancreatic cancer are currently limited to non-randomized, single institution experiences [4, 5]. Results from these studies suggest that patients who respond to pre-operative chemo-radiotherapy may benefit in terms of survival. Radiotherapy combined with chemotherapy is the current treatment for locally advanced pancreatic cancer; however, the results are unsatisfactory with median survivals ranging from 6 to 10 months [2, 6]. Collectively, these data indicate the need for new therapeutic approaches to this disease.

The hedgehog signaling pathway (Hh) is important in tissue growth and differentiation and plays an important role in embryogenesis as well as adult tissue homeostasis. Hedgehog protein gradients are essential for ventral/dorsal patterning in vertebrate central nervous systems, and normal development in a variety of tissues including integument, musculoskeletal, gastrointestinal, and urogenital systems. Secreted Hh protein binds the Patched (Ptc) receptor, thereby disinhibiting the transmembrane receptor protein Smoothened (Smo). These events allow Hh pathway activation in

part by activating the downstream transcription factor Gli. Activation of Hh signaling has been demonstrated in pancreatic cancer through overexpression of pathway elements Hh, Ptc, and Gli [7]. For example, Thayer et al. [8] reported a transgenic model of early pancreatic cancer where Hh overexpression is accompanied by K-ras and Her-2/*neu* mutations in pancreatic intraepithelial neoplasia, ultimately progressing to invasive adenocarcinoma. Aberrant Hh signaling has also been described in breast, esophagus, gastric, basal cell carcinoma, medulloblastoma, and prostate cancers.

Cyclopamine is a steroidal alkaloid derived from the Lilly plant that inhibits Hh signaling via direct interaction with the protein Smoothened [9]. It has been recently reported that prostate cancer xenografts undergo complete regression after high dose cyclopamine treatment [10]. Cyclopamine has also demonstrated significant anti-tumor effects in human tumor xenografts of medulloblastoma [11]. In pancreatic cancer cell lines which over-express Hh pathway signaling, cyclopamine induced apoptosis, while other Hh non-expressing pancreas cell lines were resistant [7]. In the same study, cyclopamine treatment inhibited growth of human pancreatic cancer xenografts. These data suggest targeting the hedgehog pathway is a promising approach for the treatment of pancreatic cancer. Although several studies have shown a cytotoxic effect of cyclopamine on various tumor cells that over express hedgehog pathway proteins, the potential use of cyclopamine as a single agent for treatment of pancreatic cancer is limited by heterogeneity of tumor populations, differential tumor cell sensitivity, limited drug availability, and high production costs [12].

We investigated whether cyclopamine enhanced the cytotoxic effects of agents commonly used in cancer therapy. These cytotoxins include gemcitabine, cisplatin, paclitaxel and ionizing radiation (IR). We demonstrate a greater than expected cytotoxic effect from either agent alone when cyclopamine is combined with paclitaxel in Hh over-expressing pancreatic cancer cells compared with no interactive killing when cyclopamine is combined with cisplatin or gemcitabine. When cyclopamine is combined with IR, additive killing is demonstrated. Additionally cyclopamine inhibited X-ray potentially lethal damage repair in tumor cells. These findings suggest a strategy to combine cyclopamine with paclitaxel and/or IR to enhance the therapeutic ratio and potentially improve the treatment of pancreatic cancer.

## Materials and methods

### Cell culture and cell lines

MiaPaCa-2, BxPC-3, and HCT116 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). MiaPaCa-2 cells were grown in DMEM high glucose; supplemented with L-glutamine, 10% fetal bovine serum (FBS), and penicillin/streptomycin 1%. BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics. HCT116 cell were maintained in MEM medium supplemented with 10% FBS and L -glutamine.

### Colony formation assay

Two hundred and fifty to 1,000 cells were plated in 60 mm dishes. At 24 h cells were irradiated (3.5 Gy), and cyclopamine 2–10 μmol (Toronto Research Chemicals) was added to culture media, or combination of both. For chemotherapeutic agents, the drug was added to culture media at appropriate concentration 24 h after plating. Cultures were incubated for 10–14 days. After incubation, cells were fixed and stained with 0.25% crystal violet, and colonies containing more than 50 cells were counted. Plating efficiency was normalized compared to control.

### Cell-cycle analysis

Cyclopamine 4 μM was added to culture media of synchronized MiaPaCa-2 cells in exponential growth phase followed by 3.5 Gy. Cells were washed and fixed in ethanol at 24 h intervals, treated with RNase, followed by propidium iodide staining for 30 min on ice. Cells were subsequently analyzed by FACScan (Becton Dickinson, San Diego, CA) with the use of CellQuest software for cell cycle distribution.

### Annexin V-PE assay

Cyclopamine 4 μM was added to culture media of exponentially growing cells with or without chemotherapeutic agents and irradiated at 12 h. Cells were trypsinized after 24, 48 or 72 h. Annexin levels were measured (Annexin V-PE staining kit, BD biosciences/Pharmingen) for $0.5\times10^6$ freshly detached cells. The presence of membrane permeabilization was monitored by 7-AAD (7-amino-actinomycinD) staining per manufacturer's protocol. Cells were subsequently analyzed by FACScan (Becton Dickinson, San Diego, CA) with the use of CellQuest software. The percentage of apoptotic cells was calculated by scoring for cells positive for either annexin alone (early apoptotic) or both annexin and 7-AAD (late apoptotic). All experiments were done in triplicate.

### Potentially lethal damage repair

Cells ($1\times10^6$) were plated in 60 mm dishes and after 24 h, irradiated (3.5 Gy) in the presence or absence of cyclopamine, 4 μM. At 2 h intervals, cells were trypsinized and plated in multiple dilutions to assess survival by colony formation.

## Results and discussion

We tested whether cyclopamine is cytotoxic to hedgehog expressing pancreatic tumor cells, MiaPaCa-2 and BxPC3, compared with human colon cancer cells, HCT 116, which do not express hedgehog [13, 14]. Figure 1 shows the effect of cyclopamine on survival as measured by colony formation. At 10 μM cyclopamine MiaPaCa-2 and BxPc3 demonstrated 7 and 11% survival whereas HCT 116 demonstrated 74% survival. Hh expressing MiaPaCa-2, BxPC3, and non-Hh expressing HCT-116 had 29, 33, and 92% survival, respectively, with 4 μM cyclopamine (not shown). These data illustrate that cyclopamine is preferentially cytotoxic to Hh expressing pancreatic tumor cells compared with non-Hh expressing cells. In order to examine whether cyclopamine toxicity could be mediated by cell cycle disturbance, we performed flow cytometry studies. Cyclopamine at 4 μM demonstrated no effects on cell cycle (data not shown) at 24 and 72 h post treatment.

We next investigated the potential cytotoxic interaction between cyclopamine and chemotherapeutic agents in MiaPaCa-2 cells. Figure 2 demonstrates the effect of paclitaxel, cisplatin and gemcitabine, alone or in combination with 2 or 4 μM cyclopamine on survival as measured by colony formation. Cyclopamine 4 μM demonstrated a survival of 29% (Fig. 2a) and paclitaxel 3.5 nM demonstrated survival of 91% (Fig. 2b). The combination paclitaxel and cyclopamine yielded a survival of 7% ($P<0.001$). Cisplatin alone, 0.8 μM gave 17% survival, and the combination of cyclopamine and cisplatin 11% survival ($P=0.56$). Gemcitabine demonstrated 35% survival and in combination with cyclopamine, 41% survival. Considered together these data suggest a potentially greater than additive effect between paclitaxel and cyclopamine, and no cytotoxic interaction between cisplatin and cyclopamine or gemcitabine and cyclopamine.

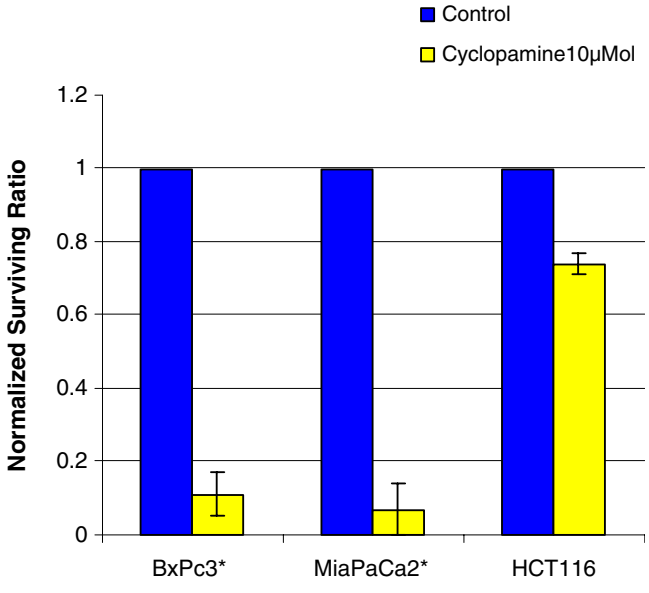


**Fig. 1** Clonogenic assay in pancreatic cancer cell lines (BxPc3 and MiaPaCa-2) versus HCT116 colon carcinoma cell line following exposure to 10 μM cyclopamine (*$P<0.001$)

To test whether an increase in apoptosis accounted for the interactive killing between paclitaxel and cyclopamine, we exposed MiaPaCa-2 cells to paclitaxel 1.7 nM, or a combination of paclitaxel and cyclopamine 4 μM for 24 h. Apoptosis was measured by annexin V staining (Fig. 3). Paclitaxel alone induced 64.9% apoptosis whereas the combination of paclitaxel and cyclopamine demonstrated 83.5% apoptosis (compared to 18.2% in the cyclopamine group). These data suggest that some of the interactive killing between paclitaxel and cyclopamine is due in part to an increase in apoptosis.

We next studied the effects of cyclopamine and IR (Fig. 4); 3.5 Gy yielded 28, 66, and 24% survival in MiaPaCa-2, BxPC3, and HCT116, respectively. Cyclopamine 4 μM and irradiation demonstrated 4% survival in MiaPaCa-2, 7% survival in BxPC3, and 35% survival in HCT116 cells. We next studied whether the combination of cyclopamine and IR enhanced radiation killing by increasing apoptosis as measured by annexin V assay. Figure 5 illustrates the effect of cyclopamine or a combination of cyclopamine 4 μM and 3.5 Gy. Cyclopamine induced apoptosis in 18.15, 29.8, and 32.9% of cells at 24, 48, and 72 h. The baseline apoptotic rate in the control group was 16.9%. Apoptosis following 3.5 Gy radiation was 34.6, 29.5 and 31.2% at 24, 48, and 72 h, respectively. Apoptosis following exposure to a combination of radiation and cyclopamine was not significantly different from radiation alone (32.11, 28.5, and 32.3% at 24, 48, and 72 h, respectively). In order to determine whether non-apoptotic cell death accounts for cytotoxicity from cyclopamine, analysis of 7-amino-actinomycin D (7AAD) staining was performed. Seventeen percent of cells were positive for 7AAD at 48 h following cyclopamine treatment, 23% were positive for 7AAD at 48 h, and 18% were positive in the combined cyclopamine and irradiation groups. Since apoptosis accounted for 30% of the killing in the combined cyclopamine plus radiation group, and 7AAD positive cells accounted for 18% (yet 95% of cells did not form colonies), we conclude that an increase in either necrosis or apoptosis at later time points accounted for the increased killing. These data considered together demonstrate that cyclopamine has an additive cytotoxic effect when combined with irradiation in hedgehog expressing tumor cells not accounted for by an increase in apoptosis. In cells that do not express the hedgehog pathway, cyclopamine had no effect on survival following irradiation.

Many cytotoxic chemotherapeutic agents rely on DNA damage for effective tumor cell killing. Although the connection between aberrant Hedgehog activation and development of certain human cancers is highly correlated, no conclusive data is available regarding intersection of DNA repair mechanisms and the Hedgehog pathway. We investigated the possible interaction of the

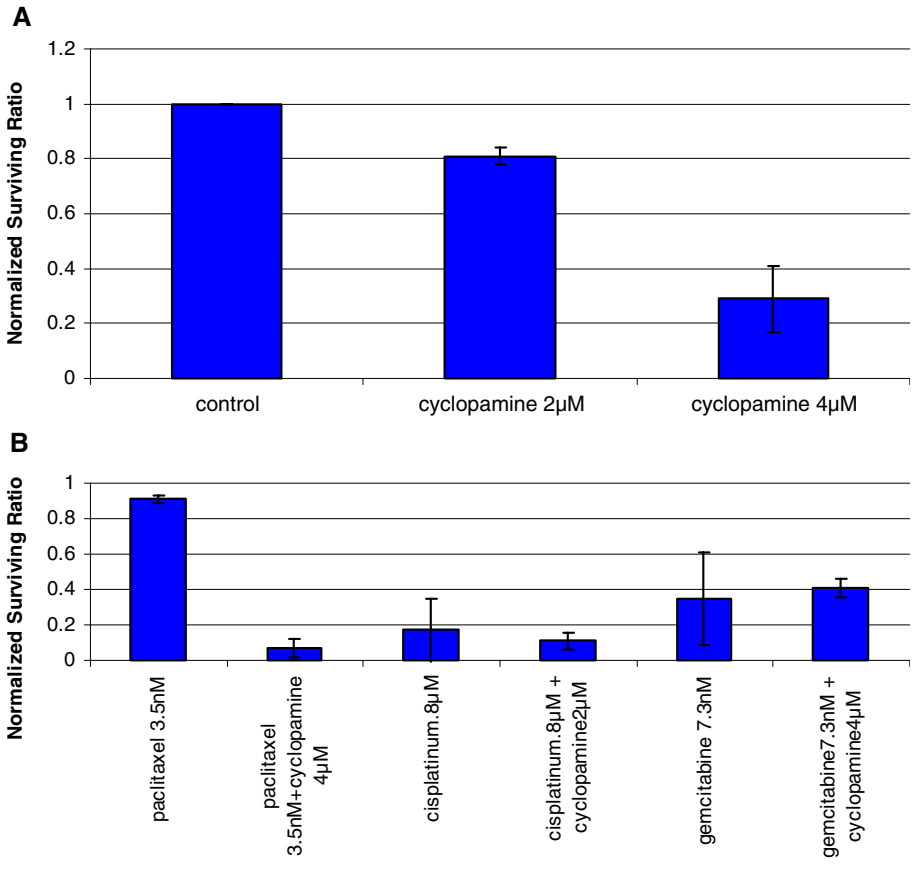


**Fig. 2** Colony formation (MiaPaCa-2) following exposure to cyclopamine (**a**), or combination cyclopamine with paclitaxel, cisplatin, or gemcitabine (**b**)

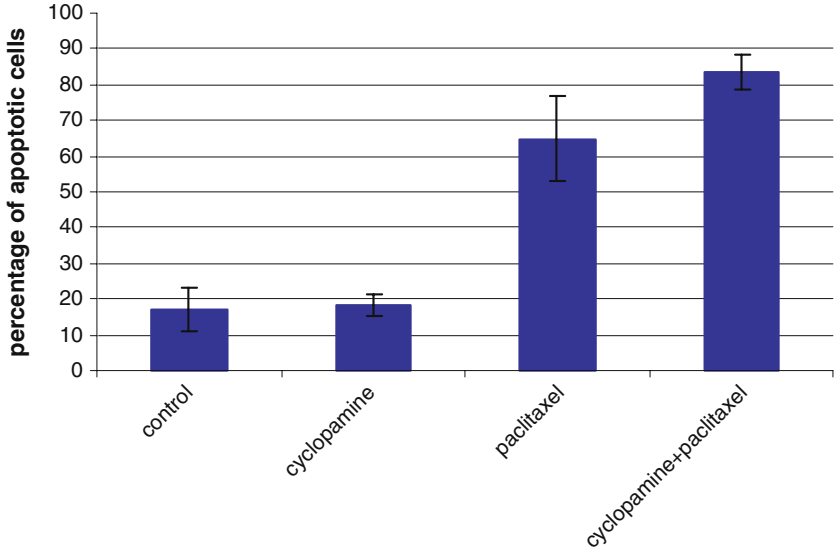


**Fig. 3** Percentage of apoptotic cells (MiaPaCa-2) following exposure to cyclopamine 4 μM, paclitaxel 1.7 nM, or combination of both for 24 h measured by Annexin V staining

Hedgehog pathway with repair of potentially lethal damage (PLDR) of DNA. PLDR is defined as an increase in cell survival following delay in subculture from confluent tumor cells following radiation. This assay has been reported to correlate with clinical radiosensitivity [15–17]. Figure 6 demonstrates that after irradiation, the surviving fraction increases over time, indicative of the occurrence of PLDR. Our results demonstrate a 20% decrease in surviving fraction (37–16%, $P<0.001$) 24 h after radiation with addition of cyclopamine. This result suggests that cyclopamine inhibits PLDR, and that a potential interaction between DNA repair mechanisms and the Hedgehog pathway exists.

Paclitaxel is a cytotoxic agent that stabilizes microtubules. Although no data is available regarding effects of cyclopamine on the microtubule cytoskeleton, several reports have described putative roles for the Hedgehog pathway in this compartment. In *Drosophila* the kinesin

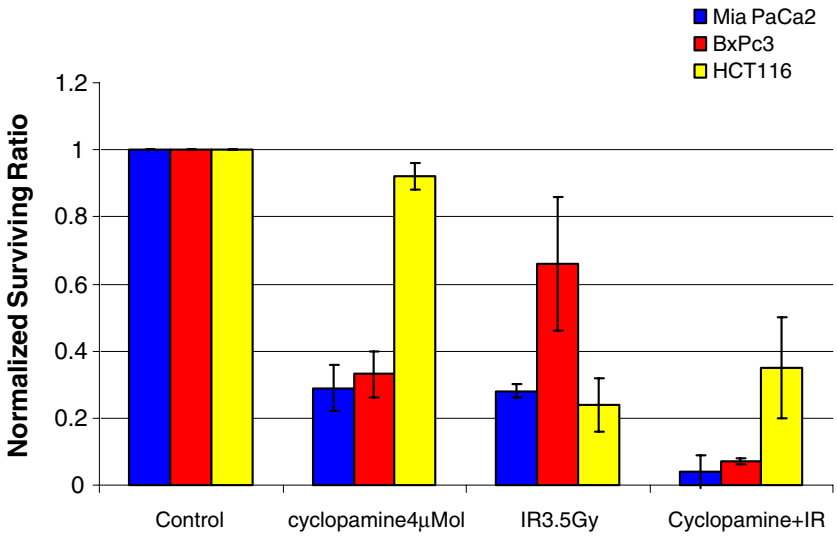


**Fig. 4** Normalized surviving fraction in clonogenic assay in two pancreatic cell lines (MiaPaCa-2 and Bxpc3) and one colon cancer cell line (HCT116) following exposure to cyclopamine 4 μM, radiation, or both. ($P < 0.05$ for cyclopamine plus irradiation vs. irradiation alone in MiaPaCa-2 and BxPc3 cell lines)

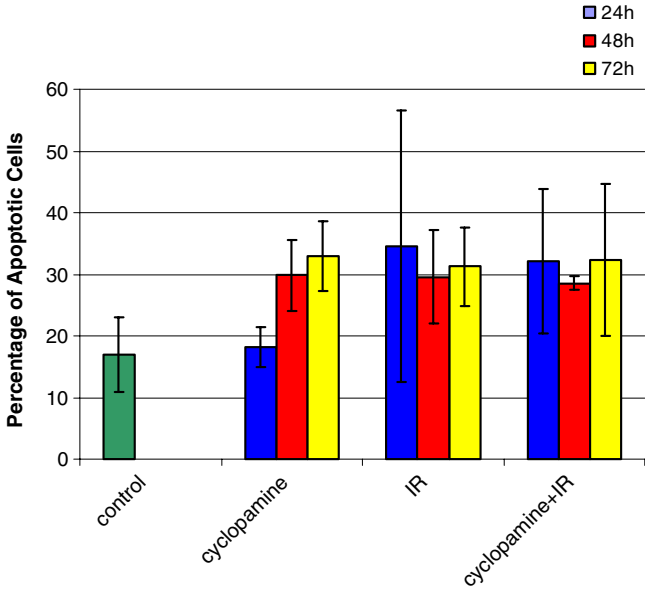


**Fig. 5** Percentage of apoptotic cells (MiaPaCa-2) following exposure to cyclopamine 4 μM, 3.5 Gy radiation, or both in 24, 48, or 72 h measured by Annexin V staining

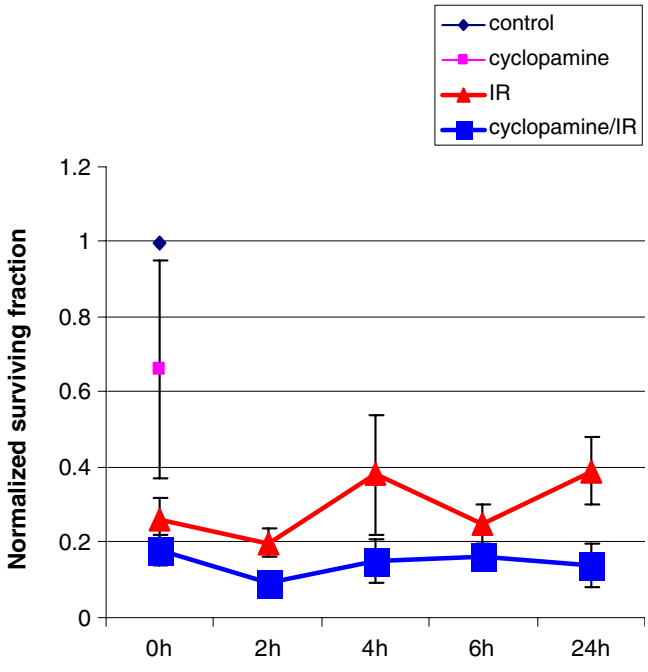


**Fig. 6** Assay for potentially lethal damage repair (PLDR) (MiaPaCa-2) after 3.5 Gy with or without cyclopamine 4 μM

motor-like protein Costal2 sequesters a multi-protein complex on microtubules in the absence of Hedgehog. Cells treated with Hedgehog demonstrate inhibition of this microtubule binding [18]. A later study identified an interaction between this same complex and the membrane protein Smoothened, activated by Hedgehog binding to its receptor, Patched [19]. It is possible that the Hedgehog pathway may in fact mediate an important signal transduction pathway where microtubule and/or motor protein dynamics are an essential step for downstream gene regulation of cytokinesis or cell death pathways. Recently intraflagellar transport (IFT) proteins and the motor protein Kinesin-II, components of microtubule-based transport systems, have been demonstrated to be required for Hh signaling by regulating both the positive and negative transcriptional activities of Gli proteins [20]. We speculate that involvement of the microtubule cytoskeleton in nuclear localization of Gli protein may be required for hedgehog signaling and may explain the interactive tumor killing in Hh expressing pancreatic cancer cells demonstrated by combined paclitaxel and cyclopamine. IR and cyclopamine demonstrated additive, interactive cytotoxic effect. Gemcitabine and cisplatin, however, appear to have no significant contribution to the cytotoxic effects of cyclopamine.

These data suggest that although gemcitabine is employed in pancreatic cancer treatment strategies, efforts directed at combining cytotoxins with cyclopamine should focus on paclitaxel.

In summary our data suggest that cyclopamine is a potentially useful agent to improve results in pancreatic cancer when combined with paclitaxel or radiation therapy. We speculate that paclitaxel and cyclopamine, compared with radiation and cyclopamine, interact through different pathways since an increase in tumor cell apoptosis mediates some of the interactive cytotoxic effects with paclitaxel and cyclopamine, but not radiation and cyclopamine.

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