Chapter-4

*In vivo validation of selected drugs in hamster pancreatic cancer model of desmoplasia*
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7.1.1 **Background and rationale**

The cross talk between PSCs and pancreatic cancer cells provides significant contribution to the malignant behaviors of cancer cells and resistance to established therapy for PC. Therefore, it is a common belief that pharmaceutically reducing the activated PSCs number in the tumor tissue either by killing (stromal ablation) or by inactivating (stromal normalization) would enhance the clinical efficacy of conventional chemotherapy.

Disulfiram (1-[diethylthiocarbamoyldisulfanyl]-N,N-diethyl-methanethioamide) a FDA approved drug has been reported to have cytotoxic effect against variety of cancer cells including PC, while sparing normal cells [158, 159]. In preclinical studies, Disulfiram (DSF) in combination with copper ions has been shown to inhibit or suppress NFκB signalling, proteasome activity, aldehyde dehydrogenase activity, and antioxidant levels in cancer cells [158-161]. To date, however, no studies have analyzed the effects of DSF on PSCs. Therefore, the major goals of this study were to establish and characterize a hamster PC model that shows the desmoplastic reactions similar to human PDAC ; and to evaluate whether pirfenidone (in presence or absence of NAC) and DSF (in presence or absence of Cu) could reduce activated PSCs growth and suppress desmoplasia *in vitro* and *in vivo*.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone), a drug approved for idiopathic pulmonary fibrosis (IPF) has shown encouraging anti-fibrotic effects in a mouse model of PC [24]. Pirfenidone decreases PSCs proliferation, invasion and migration *in vitro* and inhibits subcutaneous tumor formation in mice co-transplanted with PCCs and PSCs [24]. A recent study has shown a better patient outcome with
combined therapy of pirfenidone and N-acetylcysteine (NAC) in advanced idiopathic pulmonary fibrosis patients [162]. Moreover, NAC sensitizes human PCCs to gemcitabine by inhibiting NFκB pathway [163]; and cultivation of PSCs on extracellular matrix proteins and treatment with NAC induced deactivation of PSCs and reduced their proliferation and fibrogenic property [44]. However, till date no study has been reported that evaluate the effect of pirfenidone and NAC co-administration on desmoplastic PC. At the same time, like pirfenidone there are multiple other drugs which are approved for certain diseases other than cancer, but could have significant use in cancer therapy.

7.1.2 Methodology

7.1.2.1 Administration of DSF and/or Cu on HapT1 model of pancreatic cancer desmoplasia

To evaluate the effect of DSF and DSF+Cu, HapT1 (8x10⁴) cells were orthotopically implanted into the pancreases of hamsters. Three days after injecting cancer cells, all the tumor-bearing animals were randomly divided into four groups (n = 5 per group). Before the injection into animals, DSF (MP Biomedical) was dissolved in a solution of DMSO, olive oil, cremophor, and PBS (0.5:3:1.5:5). Each group received 200 µl i.p injection of the following treatments for six days: (i) vehicle1 group (Vec 1): DMSO-olive oil-cremophor-PBS, (ii) DSF group: DSF 50 mg/kg, (iii) vehicle2 group (Vec 2): 0.5mg/kg CuSO₄ in DMSO-olive oil-cremophor-PBS, and (iv) DSF+Cu group: DSF 50 mg/kg and 0.5mg/kg CuSO₄. At day 14, all animals were sacrificed, tumor weights measured, and gross necropsy was performed to determine the extent of metastasis. Tissue processing and various histopathological staining were done as mentioned in material method section of chapter2.
7.1.2.2 Administration of pirfenidone and NAC on HapT1 model of pancreatic cancer desmoplasia

To analyse the effects of pirfenidone or NAC alone or the combination of pirfenidone + NAC, HapT1 (8x10^4) cells were orthotopically implanted into the splenic lobe of the hamster pancreas (Figure 1A). Pirfenidone was purchased as Pirfenex tablets (Cipla Ltd), crushed using a mortar and pestle, and dissolved in sterile water to a pirfenidone concentration of 200 mg/ml. NAC (Sigma) was dissolved in sterile drinking water at a concentration of 1 g/L. Three days after the injection of cancer cells, all the tumor-bearing animals were randomly divided into four groups (n = 5 per group) and orally administered either 500 mg/kg pirfenidone (once daily) and/or 1 g/L NAC in drinking water for 21 continuous days. Control animals were provided with only sterile drinking water. At day 24, all the animals were sacrificed, tumor weights were measured, and gross necropsy was completed to determine the extent of metastasis.

7.1.2.3 Hydroxyproline assay

Frozen HapT1 tumor tissues were homogenized using mortar pestle and lyophilized. Further, collagen estimation was carried out as per the instructions provided with the hydroxyproline assay kit (Sigma; MAK008). Briefly, 5mg of dry tumor samples in 250µl of 12 M HCl was hydrolyzed at 120°C for 3 hours. The hydrolyzed samples were then briefly centrifuged and 50 µl of supernatant were aliquoted to 96-well plate in duplicate and allowed to dry in a 60°C oven. Next, 100µl of chloramine T/oxidation buffer mixture was added to each well and incubated at room temperature for 5 minutes followed by addition of 100µl of diluted 4-(Dimethylamino)benzaldehyde (DMAB) reagent and incubated for 90 minutes at 60°C. Absorbance was measured at 560 nm on Varioskan™ Flash Multimode Reader (Thermo Scientific). Collagen was quantified as µg of hydroxyproline per mg dry weight of starting material.
Tissue processing and various histological staining (H&E, Alinine blue staining, IHC) were done as mentioned in the methodology section of chapter3, statistical analysis were done as mentioned in methodology section of chapter2.
7.1.3 Results

7.1.3.1 DSF alone partially suppressed the growth of HapT1 orthotopic tumors in a syngeneic host but did not suppress desmoplastic reactions

Although the anticancer activity of DSF or DSF-Cu against PCC lines has been established previously, [158-161] its effect on cancer-associated PSCs and/or on cancer-associated fibrosis has remained unclear. Importantly, the effect of DSF on the growth of desmoplastic tumors has not yet been addressed. Therefore, the present study aimed to determine the effect of DSF on a HapT1 desmoplastic tumor model. At the end-point evaluation, we were confounded to see that animals injected with DSF-Cu demonstrated a greater tumor burden (both primary and metastatic) compared with any other group (Figure 17; data not shown). Animals injected with only DSF showed, on average, a 16% reduction in primary tumor weight and decreased metastasis (data not shown) compared with the corresponding animals treated with the vehicle (Figure 17B and C). Between the two vehicle treatment groups, there was no significant difference in the primary tumor weights. Further, the H&E stained sections showed desmoplastic reactions in all the groups (Figure 18A). Moreover, estimation of collagen deposition (through aniline blue staining) and the number of α-SMA positive cells showed no significant difference among animals of various groups (Figure 18A and B). Finally, quantification of Ki-67 positive cells showed a significant decrease and increase in the number of proliferative cells in DSF and DSF+Cu treated groups compared with their corresponding controls, respectively (Figure 18A and B).
Figure 17: Effect of DSF with or without Cu on HapT1 orthotopic tumors: Hamsters orthotopically injected with 8 x 10^4 HapT1 PCCs were injected (i.p.) with DSF, or DSF+Cu, or the respective vehicle control. A) The schematic diagram shows the treatment schedule for different groups (n = 5). B) Representative images of tumors harvested from different groups and quantification of tumor weights show smaller tumors in only DSF-treated animals compared with vehicle controls. However, animals treated with DSF+Cu have larger tumors compared with control animals. C) Quantification of tumor weights shows a significant reduction in the tumor weight only in DSF-treated tumors compared with corresponding control tumors.
Figure 18: Histopathological analysis of HapT1 orthotopic tumors treated with DSF alone or in combination with DSF+Cu: A) Histopathological analysis through H&E staining, aniline blue staining, and IHC for α-SMA shows similar levels of desmoplastic reaction in all the primary tumors, irrespective of the type of treatment. IHC for Ki67 shows the proliferative cells present in different tumor tissues B) Quantification of aniline blue and α-SMA stained sections shows similar levels of collagen deposition and abundance of activated-PSCs/myofibroblast-like cells in all groups. Quantification of Ki67-stained sections shows the relative number of proliferative cells in all the drug-treated tumors compared with their corresponding control groups (n = 5; each value presents the average of two stained sections per tumor). Data presented as mean ± SE; *p < 0.05; **p < 0.005.
7.1.3.2 Pirfenidone inhibits HapT1 tumor growth and desmoplastic reactions, and NAC enhances these inhibitory effects of pirfenidone

To investigate the effects of pirfenidone and/or NAC in an immunocompetent desmoplastic PC model, HapT1 tumor-bearing hamsters were treated with pirfenidone and/or NAC. Throughout the experiment, there were no visible stress signs or significant changes in the body weight of individual animals (data not shown). Interestingly, after 21 days of drug treatment, the animals treated with both the drugs in combination had significantly decreased tumor weights than those of animals treated with pirfenidone alone ($p = 0.0426$; Figure 20B & C) or only NAC ($p = 0.0002$; Figure 19B & C) or only vehicle/control ($p = 0.0005$; Figure 18B & C). Only pirfenidone-treated animals had significantly smaller tumors compared with controls ($p = 0.0233$ Figure 19B & C); however, only NAC treatment alone did not significantly alter the tumor weight compared to control groups (Figure 19B & C). Further, histopathological and biochemical analysis showed that pirfenidone in combination with NAC significantly reduced the number of $\alpha$-SMA-positive cells and collagen deposition compared to controls or only pirfenidone or only NAC-treated animals (Figure 20A, B & C). Quantification of Ki67-positive cells showed a significant reduction in number of proliferating cells in animals treated with both the drugs together compared to pirfenidone alone ($p = 0.0457$) or only NAC treated individually ($p = 0.0153$; Figure 20A & B).
Figure 19: Effects of pirfenidone and/or N-acetylcysteine (NAC) on the HapT1 orthotopic tumor: Hamsters orthotopically injected with $8 \times 10^4$ HapT1 PCCs were orally administered with pirfenidone (Pirf) and/or N-acetylcysteine (NAC) daily for 21 days. A) The schematic diagram shows the drug treatment schedule for different groups ($n = 5$ animals/group). B) Representative images of tumors harvested from different groups show smaller tumors in all the drug-treated animals compared with control animals. C) Quantification of tumor weights shows reduction in the tumor weight of all the drug-treated animals compared with the control animals. Pirfenidone and NAC co-administration showed a maximum reduction in tumor weight compared with the individual treatments.
Figure 20: Histopathological analysis of HapT1 orthotopic tumors treated with pirfenidone and/or N-acetylcysteine (NAC): A) Histopathological analyses through H&E staining, aniline blue staining, and IHC for α-SMA show a decrease in the extent of desmoplastic reaction in all the drug-treated animals. IHC for Ki67 shows the proliferative cells present in different tumor tissues. B) Quantification of aniline blue, α-SMA, and Ki67 stained sections shows the relative reduction in collagen deposition, the number of activated PSCs/myofibroblast-like cells, and the number of proliferative cells in all the drug-treated tumors compared with control groups, respectively (n = 5; each value presents the average of two stained sections per tumor). C) Level of hydroxyproline reflecting collagen content in tumor tissues obtained from different groups of animals (n=5/group). Data presented as mean + SE; *p<0.05; **p<0.005; ***p<0.0005.
7.1.4 Discussion

In the current study, we observed a significant cytotoxic effect of DSF or DSF+Cu against in-culture activated ha-PSCs and HapT1 cancer cells (Figure 9C, Figure 10A & 10B); however, the failure of DSF to perform in a physiologically relevant HapT1 orthotopic tumor animal model (Figure 17 and 18) strongly suggests the importance of using appropriate animal models in preclinical drug validation. The \textit{in vitro} cytotoxic effects of DSF on ha-PSCs and HapT1 cancer cells were ROS-dependent and ROS-independent, respectively (Figure 11A & B). The origin of cancer-associated fibroblast cells (CAFs) is potentially from different sources and constitutes a heterogeneous population [164]. Therefore, to rule out the possibly that DSF is ineffective against cancer-associated fibroblast cells, we isolated these cells from HapT1 orthotopic tumor tissues and treated them with DSF or DSF+Cu (Figure 21). Like PSCs, the cancer-associated fibroblast cells were susceptible to DSF or DSF+Cu treatment, and the cytotoxic effect of DSF was ROS-dependent \textit{in vitro}. Studies by others have shown that the initial level of ROS in cells is critical for DSF-induced cell death [146, 165]. By the same token, it is a well-established fact that many cells isolated from normal tissues and maintained in culture have increased levels of culture-induced ROS [166]. However, such increased basal levels of ROS are rarely or never been seen in any normal cells of humans or animals \textit{in vivo} [166]. Hence, agents like DSF that induce cell death through enhancing ROS beyond a tolerable level potentially do not show their cytotoxic effect \textit{in vivo}. A moderate cytotoxic effect of DSF on cancer cells, as evidenced by a 16% reduction in tumor weight, is potentially due to the ROS-independent cytotoxic effect of DSF on HapT1 cancer cells (Figure 17). It is possible that the failure of DSF alone to induce a highly significant antitumor effect \textit{in vivo} is due to decreased bioavailability. The failure of DSF+Cu to reduce both desmoplastic reactions and overall tumor burden can be viewed as a harbinger for the
unwanted pro-tumorigenic events that could occur due to the Cu-induced hyperactivity of DSF. In past decade, a Cu and DSF combinatorial approach has shown encouraging results in immune-compromised tumor models [158-161]. However, based on the known protumorigenic effect of Cu alone [167] and our current observation, we expect exogenous Cu would induce toxicity in DSF-treated animals.

Pirfenidone treatment significantly decreased growth of orthotopically implanted HapT1 tumors compared to controls. It also decreased intra-tumor collagen deposition, and the number of α-SMA positive cells (Figure 19 & 20). These findings corroborate previous reports of the anticancer and antifibrotic property of pirfenidone [24]. A combination of pirfenidone and NAC decreased tumor growth more than pirfenidone or NAC alone suggesting a possible therapeutic use of this combination in treating human PC. Pirfenidone and NAC are known to reduce fibrosis by inhibiting TGF-β and ROS in fibrogenic cells, respectively [168, 169]; however, the exact mechanism behind their combinatorial effect against PSCs and PCCs is yet to be investigated.

Figure 21: Effect of DSF and DSF+Cu on HapT-1 derived cancer-associated fibroblast cells (CAFs): A&B) Crystal violet staining followed by quantification clearly shows the significant cytotoxic effect of 120nM DSF and 50nM DSF with 10µM CuCl₂ on CAFs. The data presented as mean ± SE (n=3) and ***p<0.0005. C) Cell viability checked through MTT assay shows rescue of CAFs form DSF-mediated cell death after pre-incubation with 1mM NAC. The data presented as mean ± SE; **p<0.005; ***p<0.0005 (n=3).