Targeting developmental regulators of zebrafish exocrine pancreas as a therapeutic approach in human pancreatic cancer

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Summary

Histone deacetylases (HDACs) and RNA polymerase III (POLR3) play vital roles in fundamental cellular processes, and deregulation of these enzymes has been implicated in malignant transformation. Hdacs and Polr3 are required for exocrine pancreatic epithelial proliferation during morphogenesis in zebrafish. We aim to test the hypothesis that Hdacs and Polr3 cooperatively control exocrine pancreatic growth, and combined inhibition of HDACs and POLR3 produces enhanced growth suppression in pancreatic cancer. In zebrafish larvae, combination of a Hdac inhibitor (Trichostatin A) and an inhibitor of Polr3 (ML-60218) synergistically prohibited the expansion of exocrine pancreas. In human pancreatic adenocarcinoma cells, combination of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and ML-60218 produced augmented suppression of colony formation and proliferation, and induction of cell cycle arrest and apoptotic cell death. The enhanced cytotoxicity was associated with supra-additive upregulation of the pro-apoptotic regulator BAX and the cyclin-dependent kinase inhibitor p21CDKN1A. tRNAs have been shown to have pro-proliferative and anti-apoptotic roles, and SAHA-stimulated expression of tRNAs was reversed by ML-60218. These findings demonstrate that chemically targeting developmental regulators of exocrine pancreas can be translated into an approach with potential impact on therapeutic response in pancreatic cancer, and suggest that counteracting the pro-malignant side effect of HDAC inhibitors can enhance their anti-tumor activity.

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Introduction

Pancreatic adenocarcinoma, the most common form of cancer in exocrine pancreas, is among the most lethal human malignancies (Siegel et al., 2011). Accumulating evidence indicates that the signaling pathways governing organogenesis are often deregulated in tumors, such as those of the pancreas (Yee and Pack, 2005; Koorsstra et al., 2008). Zebrafish has been proven as a powerful model to study genetic regulation of vertebrate development and oncogenesis, and it is expected to facilitate drug discovery for treatment of various human diseases, including pancreatic cancer (Yee, 2010). The established models including wild-type (WT), germ-line mutant and transgenic zebrafish have been utilized to identify the genetic pathways and their interactions that control exocrine pancreatic development and cancer (Yee, 2010; Yee et al., 2011). Genetic or chemical modulation of the developmental regulators of exocrine pancreas, such as histone deacetylases (HDACs) and RNA polymerase III (POLR3), may offer new opportunities for improving therapeutic response in this generally incurable disease.

HDACs and histone acetyltransferases regulate gene transcription by controlling the acetylation status of nucleosomal histones as well as non-histone proteins (Haberland et al., 2009). HDACs play regulatory roles in various cellular processes that are fundamental to embryological development (Haberland et al., 2009; Brunmeir et al., 2009). Hdac1 is required for epithelial proliferation and normal growth of exocrine pancreas during morphogenesis in zebrafish (Noel et al., 2008; Zhou et al., 2011). HDACs play contributory roles in cellular proliferation and survival, as inhibition of HDACs induces cell cycle arrest, differentiation, and death in various cancers including pancreatic adenocarcinoma (Lane and Chabner, 2009). However, loss-of-function in HDACs exerts both stimulatory and inhibitory effects on gene transcription, and some of them may be opposing to their anti-tumor actions (Zupkovitz et al., 2006). Thus, the therapeutic potential of HDAC inhibitors in various malignancies including
that of exocrine pancreas may be enhanced when used in combination with small molecules that counteract their pro-malignant side effects such as an increase in POLR3-mediated transcripts.

POLR3 mediates transcription of non-coding RNAs, which are involved in a variety of cellular functions including protein biosynthesis and gene regulation (White, 2008). Deregulation of the POLR3 transcription complex plays a central role in mediating the actions of tumor suppressors, oncogenes, and transforming viruses (Marshall and White, 2008). In spite of the fundamental role of POLR3 in cellular functions, the zebrafish polr3b\{'\}null mutation, which affects the second largest subunit of Polr3, selectively disrupts development of exocrine pancreas and intestine with impaired transcription of tRNA genes (Yee et al., 2005; Yee et al., 2007; Yee, 2010). These findings suggest that inhibition of POLR3 may preferentially perturb cell cycle progression of rapidly proliferating cells in cancers, given that POLR3 transcripts are elevated in malignant cells and over-expression of tRNA has been implicated in malignant transformation (Marshall and White, 2008). The small molecule ML-60218 was developed as a potent and selective inhibitor of Polr3-mediated transcription in eukaryotes (Wu et al., 2003). It will be enticing to test if ML-60218 used in combination with HDAC inhibitors can augment the growth-suppressive effect of HDAC inhibitors in tumors including that of exocrine pancreas, by counteracting their pro-malignant side effect of stimulating POLR3-mediated transcription.

The objective of this study is to test our hypothesis that combined inhibition of HDACs and POLR3 cooperatively suppresses the growth of exocrine pancreas during morphogenesis and in cancer. We present evidence that the HDAC inhibitor, trichostatin A (TSA) that reversibly inhibits classes I and II HDACs (Yoshida et al., 1995; Marks et al., 2001), in combination with ML-60218, synergistically arrested the growth of exocrine pancreas in zebrafish larvae by blocking cell cycle progression and up-regulating expression of the cyclin-dependent kinase (cdk) inhibitors. These effects are recapitulated in human pancreatic adenocarcinoma cells, in which combination of the clinical HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), and ML-60218 produced supra-additive suppression of cellular proliferation and induction of apoptotic cell death. These enhanced cytotoxic effects are associated with ML-60218-augmented SAHA-upregulated expression of BAX and p21^{CDKN1A} as well as ML-60218-repressed SAHA-stimulated expression of tRNAs. Results of this study indicate that chemical targeting of the epigenetic and transcriptional regulators of development in zebrafish exocrine pancreas can be potentially translated into a therapeutic approach in human pancreatic cancer.

**Results**

**Hdac are required for growth and morphogenesis in zebrafish exocrine pancreases**

Our recent study indicates a crucial role of Hdac1 in exocrine pancreatic epithelial proliferation (Zhou et al., 2011). Here, we determined the role of Hdacs in the developing exocrine pancreas by treating WT zebrafish larvae with TSA between 48 and 72 hours post-fertilization (h.p.f.) when the pancreatic epithelia maximally proliferate during this period (Yee et al., 2007). First, TSA at various concentrations was added at 48 h.p.f., and acetylation of histones H3 and H4 was analyzed at 72 h.p.f. At a concentration of 165 nM, TSA induced maximal level of acetylated histone H3 and near-maximal level of acetylated histone H4 (Fig. 1). The effect of TSA on exocrine pancreas was then determined by incubating WT zebrafish larvae with 165 nM TSA for 24 hours. The TSA-treated larvae appeared grossly normal. They developed exocrine pancreas of reduced size, and acinar morphogenesis was disrupted (Fig. 2A). While TSA significantly reduced the number of pancreatic epithelia (4’-diamidino-2-phenylindole or DAPI containing nuclei) by 34%, the proliferative rate as determined by the proportion of epithelia in S-phase (5-bromo-2-deoxyuridine or BrdU containing nuclei) was not significantly decreased (Fig. 2B). The effect of TSA on exocrine pancreas was associated with increased levels of acetylated histones H3 and H4 (Fig. 2C). Therefore, Hdacs are required for normal growth and morphogenesis of exocrine pancreas through regulating the acetylation status of histones in zebrafish.

Combination of inhibitors of Hdacs and Polr3 arrests expansion of exocrine pancreas during morphogenesis

Considering the growth requirement of Hdacs (Figs 1, 2) and our previous findings of Polr3 in the proliferation of pancreatic epithelia (Yee et al., 2007), we hypothesize that Hdacs and Polr3 cooperatively regulate the growth of exocrine pancreas. To test this hypothesis, we examined the effects of a combination of TSA
and the Polr3 inhibitor ML-60218 on the growth of exocrine pancreas during morphogenesis. First, we determined the optimal concentrations of TSA or ML-60218 by incubating WT zebrafish larvae at various concentrations of each small molecule and observing for signs of toxicity and lethality. The zebrafish larvae incubated in the medium containing TSA at a maximal concentration of 330 nM appeared grossly normal. The larvae treated with ML-60218 at a concentration of 110 \( \mu M \) or below appeared normal without precipitation of ML-60218 (supplementary material Table S1). Thus, TSA at 165 nM and ML-60218 at 110 \( \mu M \) were used for the subsequent experiments.

Addition of TSA and ML-60218 at 48 h.p.f. to WT zebrafish larvae for 24 hours completely arrested expansion of the exocrine pancreas, such that the size of the exocrine pancreas remained the same as that in untreated WT larvae at 48 h.p.f. (Fig. 3A; Yee et al., 2005). Neither TSA nor ML-60218 at the concentrations being used alone caused any apparent reduction of organ size. No apparent effect was observed by treatment with either TSA + dimethyl sulfoxide (DMSO, the solvent used to dissolve TSA and ML-60218) or DMSO alone (N. S. Yee, unpublished). The same effects of these small molecules were observed in WT zebrafish larvae of various strains including AB, TLF, and WIK (N. S. Yee, unpublished).

To determine the cellular basis of the growth-suppressive effect of the combination of inhibitors on exocrine pancreas, the rate of epithelial proliferation was assessed. The combination of TSA and ML-60218 caused a significant reduction in the proportion of S-phase (BrdU+) nuclei by 23% of that in control (Fig. 3B). Neither TSA nor ML-60218 significantly reduced the proportion of BrdU+ nuclei. Moreover, TSA + ML-60218 supra-additively reduced the number of DAPI positive nuclei by 48% (\( P<0.01 \)). Using morphometric analysis, the combination of TSA + ML-60218 significantly reduced cell growth by 22%, whereas neither TSA nor ML-60218 alone caused any significant impairment of cell growth (Fig. 3B). No increase in apoptotic cell death was observed in the exocrine pancreas of the larvae treated with TSA + ML-60218 using Apoptag \(^*\) (N. S. Yee, unpublished). These results indicate that the combination of TSA and ML-60218 produces enhanced suppression of exocrine pancreatic growth by inhibiting epithelial proliferation with impaired progression from G1 to S phase of the cell cycle and reduced cell growth.

To explore the mechanism by which the combination of TSA and ML-60218 perturbed cellular proliferation, the acetylation status of histones H3 and H4 and expression of p21\(^{cdkn1a}\) and p27\(^{cdkn1b}\) as molecular markers of progression of cell cycle from G1 to S phase were assessed by immunoblotting and real-time polymerase chain reaction (PCR), respectively. TSA caused a slight accumulation of acetylated histones H3 and H4, and combination with ML-60218 enhanced this effect by 2-fold and
70%, respectively (Fig. 3C). Consistent with the effects on acetylated histones, the combination of TSA and ML-60218 significantly up-regulated the levels of both \( p21^{cdkn1a} \) and \( p27^{cdkn1b} \) mRNA by about 1.3-fold over control, and either TSA or ML-60218 alone caused relatively little changes (Fig. 3D). No significant alteration of total tRNA and 5s rRNA levels was detected among the experimental groups and controls as revealed by electrophoretic separation of total RNA extracted.
from whole larvae in each group (N. S. Yee, unpublished). Taken together, the combination of TSA and ML-60218 causes growth arrest of exocrine pancreas by suppressing pancreatic epithelial proliferation that is associated with enhanced accumulation of acetylated histones and up-regulated expression of p21<sup>p19<sub>1A</sub></sup> and p2<sup>p19<sub>1b</sub></sup>.

### Combination of SAHA and ML-60218 produces enhanced suppression of proliferation in human pancreatic adenocarcinoma by impairing cell cycle progression and inducing apoptosis

To translate the findings of the zebrafish studies, we examined the effects of the clinically used HDAC inhibitor SAHA in combination with ML-60218 in the human pancreatic adenocarcinoma cell lines PANC-1 and BxPC-3. These cells have been shown to carry mutations in K-RAS and p53 (Berrozpe et al., 1994; Moore et al., 2001), and they are resistant to the cytotoxic effects of the chemotherapeutic agent gemcitabine at clinically used concentrations (1 μM and 10 μM) and even up to 50 μM (supplementary material Table S2A). SAHA is a synthetic inhibitor of classes I and II HDACs. It was used at 5 μM in this study based on the dose–response in cellular proliferation of PANC-1 and BxPC-3 (Chun et al., 2009). ML-60218 was used at 100 μM, which is approximately the same concentration used in the experiments with zebrafish (Fig. 3). In a soft agar assay that mimics in vivo conditions of tumor growth, the combination of SAHA and ML-60218 produced enhanced reduction of anchorage-independent colony formation (Fig. 4A). Neither SAHA nor ML-60218 alone significantly inhibited PANC-1 colony formation; but when combined, they reduced PANC-1 colony formation by 47%. In BxPC-3, SAHA reduced colony formation by 35%, ML-60218 had no effect, and SAHA + ML-60218 reduced colony formation by 70%, which was significantly different compared to SAHA alone. DMSO did not significantly affect colony formation either alone or in combination with SAHA (N. S. Yee, unpublished).

To gain insights into the effects on colony formation, cellular morphology was analyzed by phase contrast microscopy (Fig. 4B). In both PANC-1 and BxPC-3, the combination of SAHA and ML-60218 produced morphologic changes including cell enlargement and flattening, irregular shapes with cytoplasmic projections, accumulation of cytoplasmic vacuoles, and nuclear fragmentation. These morphologic changes suggest that the enhanced suppression of colony formation might involve induction of cytodifferentiation, apoptotic cell death, and senescence-like cytostasis.

To investigate the cellular mechanisms underlying the suppression of colony formation, the effects of SAHA and ML-60218 on cellular proliferation were analyzed using the MTS assay. The combination of SAHA and ML-60218 produced enhanced inhibition of cellular proliferation (Fig. 5A). In PANC-1, neither SAHA nor ML-60218 had any effect, but the combination significantly reduced proliferation by 23%. In BxPC-3, SAHA reduced proliferation by 49%, ML-60218 had no effect, and the combination of SAHA and ML-60218 reduced proliferation by 53%. To determine the cytotoxicity of SAHA and ML-60218 in normal pancreatic epithelia, the immortalized human pancreatic ductal epithelia H6c7 were treated with the inhibitors and analyzed for proliferation. Similar to their effects on PANC-1 and BxPC-3, the combination of SAHA and ML-60218 produced enhanced cytotoxicity in H6c7 cells (supplementary material Table S2B). However, the proliferation-suppressing effect of the combination of SAHA and ML-60218 in H6c7 cells (reduced by 54%) (supplementary material Table S2B) is less than that of gemcitabine at 1 μM and 10 μM (reduced by 64% and 82%, respectively) (supplementary material Table S2A).

Next, the effect on cell cycle progression was analyzed by flow cytometric determination of DNA content (Fig. 5B). In PANC-1 and BxPC-3, SAHA increased the proportion of cells in G<sub>0</sub>/G<sub>1</sub> (28%), and decreased the proportion of cells in the S phase (28% and 24%, respectively). ML-60218 alone produced no or little effect. The combination of SAHA and ML-60218 caused a further accumulation of cells in G<sub>0</sub>/G<sub>1</sub> (additional 3% and 5% in PANC-1 and BxPC-3, respectively) as compared to SAHA alone. The effect on cell death was then determined by flow cytometric detection of Annexin-V binding. Compared to control PANC-1, SAHA caused 13% more cells to undergo apoptosis, ML-60218 had no effect, and SAHA + ML-60218 induced apoptosis in an additional 21% of cells (Fig. 5C). In H6c7 cells, the combination of SAHA and ML-60218 produced a smaller proportion of apoptotic cells than gemcitabine (3-fold and 4-fold, respectively) relative to control (supplementary material Table S2C). Moreover, the combination of SAHA and ML-60218 produced fewer dead cells than gemcitabine (no increase and 4-fold, respectively) as compared with no treatment (supplementary material Table S2C).

To further explore the mechanisms by which SAHA and ML-60218 induced apoptosis, expression of the anti-apoptotic protein survivin was evaluated by confocal microscopy. In the untreated or DMSO-treated BxPC-3 cells, survivin was present diffusely throughout the cells. Either SAHA or the combination of SAHA and ML-60218 caused survivin to be expressed almost exclusively in the nuclei (Fig. 5D), and this effect presumably abolishes the anti-apoptotic action of survivin by accelerating its degradation in the nucleus (Connell et al., 2008a,b).

These results show that SAHA induces cytotoxicity in pancreatic adenocarcinoma by causing cell cycle arrest and apoptotic cell death, and these effects are enhanced by ML-60218. The combination of SAHA and ML-60218 also produces enhanced cytotoxicity in the pancreatic ductal epithelia, but the extent of cytotoxicity is less than that produced by gemcitabine.

### SAHA and ML-60218 cooperatively up-regulate expression of BAX and p2<sup>p19<sub>1A</sub></sup> protein

In an attempt to gain insights into the mechanism underlying the enhanced actions of the combination of SAHA and ML-60218, we analyzed histone acetylation by immunoblotting (Fig. 6A). SAHA alone increased the levels of acetylated histones H3 and H4 in PANC-1 by 40% and 110%, respectively. In BxPC-3, SAHA increased acetylated histones H3 and H4 by 20% and 4.3-fold, respectively. The combination of SAHA and ML-60218 produced similar effects as SAHA alone in both cell lines. The increased level of acetylated histones is consistent with the established action of SAHA and expected to modulate gene expression.

To further characterize the growth-suppressive actions of SAHA and ML-60218, the level of the pro-apoptotic regulator BAX was analyzed by immunoblotting (Fig. 6B). Consistent with the results of the flow cytometric detection of apoptosis, the combination of SAHA and ML-60218 produced enhanced expression of BAX by 2.1-fold and 6.2-fold over control in PANC-1 and BxPC-3, respectively. Either SAHA or ML-60218...
alone produced relatively little or no increase in the level of BAX. Expression of the cyclin-dependent kinase inhibitor p21CDKN1A was then examined. In PANC-1 and BxPC-3, SAHA caused elevation of the p21 CDKN1A protein levels by 60% and 80%, respectively, and these were further increased by 1.6-fold and 5-fold over control, respectively, when SAHA was combined with ML-60218.

These results indicate that the combination of SAHA and ML-60218 suppresses cellular proliferation by impairing cell cycle progression from G1 to S phase with up-regulation of p21^{CDKN1A} and by inducing apoptosis through the BAX-mediated mitochondrial pathway with nuclear localization of survivin.

**ML-60218 reverses SAHA-stimulated tRNA expression**

We attempted to understand the mechanism that mediates the enhanced cytotoxicity of the combination of SAHA and ML-60218 by examining their effects on tRNA levels. It has been shown that histone acetylation influences chromatin structure and...
organization that play a role in protein–DNA interactions and regulate expression of POLR3-transcribed genes (Lee et al., 1993; Boyd et al., 2000; Noma et al., 2006). The activity of POLR3 transcription complex is up-regulated in most of the examined pancreatic adenocarcinoma cell lines (Table 1), and over-expression of tRNA has been implicated in malignant transformation (Marshall and White, 2008). We hypothesize that inhibition of HDACs by SAHA leads to up-regulated expression of POLR3-mediated transcripts including tRNAs, which in turn may antagonize its anti-tumor activity. To test this hypothesis, we quantified tRNAs in the pancreatic cancer cells treated with SAHA alone or in combination with ML-60218 using real-time PCR. SAHA significantly increased the levels of tRNA_{Met} in both PANC-1 and BxPC-3 by 71% and 85%, respectively, and increased tRNA_{Ser} in PANC-1 and BxPC-3 by 2.2-fold and 1.9-fold, respectively (Fig. 7A). In control cells, ML-60218 alone had slight effect on the levels of tRNA_{Met} and tRNA_{Ser}. When added to SAHA-treated cells, ML-60218 significantly reduced SAHA-stimulated expression of tRNA_{Met} and tRNA_{Ser} by 55% and 64%, respectively in PANC-1, and by 43% and 77%, respectively, in BxPC-3.

Therefore, SAHA inhibits HDACs and increases acetylation of histones H3 and H4, resulting in up-regulated expression of the pro-apoptotic regulator (BAX), the CDK inhibitor (p21 CDKN1A), and tRNAs (tRNA_{Met} and tRNA_{Ser}). At a concentration that it does not significantly affect tRNA level, ML-60218 reverses SAHA-induced up-regulation of tRNAs, and this may contribute to the enhanced cell cycle arrest and apoptosis in pancreatic adenocarcinoma by the combination of SAHA and ML-60218, as illustrated in Fig. 7B.

Discussion
HDACs and POLR3 play critical roles in the signaling mechanisms that control embryonic and neoplastic development. It was unclear if HDACs and POLR3 coordinately regulate growth of exocrine pancreas. Whether POLR3 could be utilized as a therapeutic target or to enhance the anti-tumor activities of HDAC inhibitors was unexplored. In this
study, we show that the combination of HDAC inhibitors and the POLR3 inhibitor ML-60218 produces supra-additive suppression of epithelial proliferation with cell cycle arrest in the developing exocrine pancreas of zebrafish as well as in the gemcitabine-resistant human pancreatic adenocarcinoma cell lines. Our data provide a proof of principle for enhancement of the anti-tumor activity of HDAC inhibitors potentially by counteracting their "pro-oncogenic" side effects, as ML-60218 represses SAHA-induced up-regulated expression of tRNAs. The precise mechanisms underlying the enhanced growth-suppressive activity of either SAHA or TSA in combination with ML-60218 remain to be determined. Results of this study demonstrate direct translation of the zebrafish studies into a therapeutic approach in human pancreatic adenocarcinoma by combinatorial

Table 1. Relative levels of tRNA\textsuperscript{Met} and tRNA\textsuperscript{Ser} in human pancreatic adenocarcinoma cell lines. Quantification of tRNA\textsuperscript{Met} and tRNA\textsuperscript{Ser} in pancreatic adenocarcinoma cell lines and the pancreatic ductal epithelia H6c7 by real-time PCR. Each value represents the mean relative tRNA level ± s.e.m. in the cell line as compared with that in the H6c7 cells. This experiment was repeated 3 times. Each sample of real-time PCR was performed in triplicates. Note that POLR3-mediated transcription has been shown to be cell cycle-dependent (White et al., 1995; Scott et al., 2001), and each tRNA level represents the outcome of an asynchronous population of cells. *P<0.05, statistically significant. †P<0.1, trend of statistical significance. NS, not statistically significant.

|          | BxPC-3 | Capan-1 | HPAF-II | MIA PaCa-2 | Panc 02.03 | PANC-1 | PL45 | H6c7 |
|----------|--------|---------|---------|------------|------------|--------|------|------|
| tRNA\textsuperscript{Met} | 2.13   | 6.16    | 4.34    | 1.02       | 4.92       | 3.06   | 1.69 | 1.00 |
| ±1.00    | ±0.66  | ±3.63   | ±0.48   | ±0.35      | ±0.32      | ±0.86  |      |      |
| †P<0.1   | *P<0.01| NS      | NS      | *P<0.01   | *P<0.01   | NS     |      |      |
| tRNA\textsuperscript{Ser} | 1.81   | 1.87    | 0.64    | 1.44       | 0.88       | 1.47   | 1.15 | 1.00 |
| ±0.50    | ±0.34  | ±0.31   | ±0.80   | ±0.31      | ±0.13      | ±0.86  |      |      |
| †P<0.1   | *P<0.05| NS      | NS      | NS         | *P<0.01   | NS     |      |      |
Fig. 7. ML-60218-mediated suppression of SAHA-induced up-regulation of tRNA as a potential mechanism that contributes to the enhanced anti-cancer actions in pancreatic adenocarcinoma. (A) The tRNA^{Met} and tRNA^{Ser} levels in PANC-1 and BxPC-3 cells treated for 48 hours as indicated are represented as % of control (untreated or DMSO). Each value is the mean ± s.e.m. of three independent experiments, triplicate samples for each PCR, each PCR performed twice, and expressed as % relative to controls. Statistical analysis was performed comparing each experimental group with control, except where indicated. *P < 0.05, statistically significant. #P < 0.1, trend of statistical significance. NS, not statistically significant. (B) A working model that summarizes the enhanced cytotoxic actions of the combination of ML-60218 and either TSA or SAHA. i, inhibitor; AcH3, acetylated histone H3; AcH4, acetylated histone H4.
targeting of an epigenetic regulator and a transcriptional mediator of exocrine pancreatic development.

Mechanisms underlying enhanced cytotoxicity of the combination of inhibitors of HDACs and POLR3 in exocrine pancreas

HDAC inhibitors exert their anti-tumor activities by inducing apoptosis, cell cycle arrest, differentiation, senescence, as well as by modulating immune responses and altering angiogenesis (Frew et al., 2009). Our data indicate that ML-60218 enhances the ability of HDAC inhibitors to induce apoptosis and cell cycle arrest. These cellular effects correlate with the ability of ML-60218 to augment SAHA-induced up-regulated expression of the pro-apoptotic protein BAX, and the CDK inhibitor p21CDKN1A. The mechanisms that underlie the enhanced growth-suppressive action of HDAC inhibitors and ML-60218 may at least partially involve reversal of HDAC inhibitors-stimulated, up-regulated expression of tRNA as illustrated in the working model (Fig. 7B). Of note, ML-60218 was used at a relatively non-toxic concentration, such that it did not produce any gross abnormality in the zebrafish larvae, and it had no appreciable effect on tRNA levels in the pancreatic cancer cells.

Inhibition of HDACs is expected to stimulate transcription of a variety of genes including those encoding tRNAs, as shown in this study. In agreement with these findings, HDAC1 has been shown to repress POLR3-mediated transcripts such as 5S tRNA (Lee et al., 1993). Moreover, transcriptional profiling of zebrafish larvae with germ-line loss-of-function mutation in polr3 subunits (Zhou et al., 2011). We have previously shown that the zebrafish loss-of-function mutation, polr3bslimjim, caused impaired activity of Polr3 and reduced levels of tRNAs, and led to diminished epithelial proliferation in exocrine pancreas (Yee et al., 2005; Yee et al., 2007; Yee, 2010). This is in agreement with the established role of POLR3-mediated transcripts such as tRNAs in protein translation required for fundamental processes including cell division and growth (White, 2008). Consistent with the fact that cancer cells exhibit uncontrolled proliferation, the POLR3 transcriptional complex level and/or activity are increased in various human cancers (White, 2008) and pancreatic adenocarcinoma cells (this study). We hypothesize that ML-60218-enhanced cytotoxicity of SAHA or TSA occurs at least in part by counteracting the HDAC inhibitors-induced up-regulation of POLR3 activity resulting from histone hyperacetylation. Our finding that ML-60218 abolishes SAHA-stimulated expression of Polr3-mediated transcripts including tRNA Met and tRNA Ser provides support for this mechanism. Our hypothesis is further supported by recent reports that over-expression of tRNA can lead to malignant transformation (Marshall et al., 2008; Johnson et al., 2008) and suppression of apoptosis (Mei et al., 2010). Whether other Polr3-transcribed RNAs, such as 5S tRNA, 7SL RNA and microRNAs, are involved in the enhanced cytotoxicity of ML-60128 and HDAC inhibitors remains to be determined. In future studies, we aim to test this hypothesis by directly modulating tRNAs and other POLR3-transcribed RNAs and determining the effect on SAHA-induced cytotoxicity and its enhancement by ML-60218.

Overlapping mechanisms that regulate HDACs and POLR3 may also contribute to the enhanced growth-suppressive effects of the combination of inhibitors of HDACs and POLR3. Several lines of evidence indicate that both HDAC1 and the POLR3 transcription complex share common regulatory pathways that involve retinoblastoma (RB) and p53 (Robertson et al., 2000; Rayman et al., 2002; Lagger et al., 2003; White et al., 1996; Cairns and White, 1998). Both RB and p53 exert their inhibitory actions through specific interaction with POLR3-specific transcription factor IIIB (TFIIB) that is required for recruiting TFIIC and transcribing tRNAs (Larminie et al., 1997; Chu et al., 1997; Sutcliffe et al., 1999; Crighton et al., 2003; reviewed by Marshall and White, 2008). Moreover, the complex containing HDAC1, DNMT1, and RB has been shown to interact with TFIIB (Robertson et al., 2000). We hypothesize that functional interactions between HDACs and the POLR3-containing transcription complex coordinately regulate cellular proliferation and survival. Further investigation of the molecular nature of such interactions is expected to help elucidate the underlying mechanisms underlying the enhanced cytotoxicity of the inhibitors of HDACs and POLR3. In addition, modulation of the established pathways of proliferation and survival such as RAS/MEK/ERK and PI3K/AKT signaling as well as functional analysis of the transcriptomic and proteomic profiles of the cells treated with SAHA, ML-60218, or the combination of SAHA and ML-60218, will help understand the mechanisms underlying their enhanced cytotoxic actions.

Conclusion and Prospective

We present evidence supporting the use of zebrafish as a translational platform for therapy in human pancreatic cancer by chemically targeting developmental regulators of exocrine pancreas. Our results demonstrate that the combination of small molecule inhibitors of HDACs and POLR3 produces synergistic suppression of exocrine pancreatic growth in the developing zebrafish and enhanced impairment of proliferation in human pancreatic adenocarcinoma cells. The augmented anti-proliferative and pro-apoptotic effects may be partially attributed to ML-60218 reversal of SAHA-induced increase in tRNA levels, though the precise mechanisms remain to be determined. SAHA is well-tolerated clinically, yet the safety of using ML-60218 alone and in combination with SAHA in humans is unknown. Ongoing studies using the zebrafish and mouse models of pancreatic adenocarcinoma aim to determine the in vivo efficacy and potential toxicity of the combination of SAHA and ML-60218 and generate insights into the mechanisms for overcoming therapeutic resistance by modulating HDACs and RNA metabolism. However, our studies provide further support for utility of zebrafish as a biological system to identify the pathways and mechanisms that regulate the growth of exocrine pancreas with the goal of improving treatment response in patients with pancreatic adenocarcinoma and producing a beneficial impact on their survival.

Materials and Methods

Zebrafish stock

WT zebrafish of AB, TLF, or WIK strains were obtained from the Zebrafish International Resource Center (Portland, Oregon, USA). Zebrafish husbandry was conducted as described (Westerfield, 2007). To facilitate visualization of exocrine pancreas in the larvae, 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich ©, St. Louis, Missouri, U.S.A.) was added to embryo medium (E3) at 0.003% to inhibit skin pigmentation as indicated.

Small molecules and drugs

TSA (Upstate Biotechnology/Millipore™, Waltham, Massachusetts, U.S.A.) was dissolved in DMSO (Sigma-Aldrich ©) to prepare a stock solution of 3.3 mM (1 mg/ml), and stored in aliquots at 4°C. A working solution of TSA was made by diluting the stock solution with E3 medium to 33 μM (10 μg/ml) and stored at 4°C.
The diluted TSA solutions were added to E3 medium for treatment of larvae. ML-60218, 165 nM TSA + 110 nM ML-60218, 5 µM SAHA + 0.5% DMSO or control (DMSO or untreated) for 48 hours. As previously described (Chun et al., 2009), the cells were incubated with propidium iodide, and the DNA content of the cells was analyzed using a FACScan™ flow cytometer (Becton, Dickinson and Company) by guest on February 9, 2021http://bio.biologists.org/ Downloaded from

Soft agar colony assay
A two-layer, soft agar gel was used to assess the ability of SAHA and ML-60218 to inhibit colony formation of PANC-1 and BxPC-3 in vitro as described (Chun et al., 2009). In this study, the cells were treated with SAHA, ML-60218, SAHA + ML-60218, SAHA + DMSO, or DMSO as described above. The experiment was performed three times with each treatment in triplicate samples.

Cell morphology
In each well of 6 well cell culture cluster (costar®, Corning Inc.) 5 x 10^5 Panc-1 or BxPC-3 cells / 4 ml medium were seeded and allowed to adhere overnight. The cells were then incubated with SAHA, ML-60218, SAHA + ML-60218, or control (DMSO or untreated) for 24 hours. Images were acquired under an inverted light microscope with phase contrast (Olympus IX71).

Flow cytometric analysis of cell cycle
PANC-1 and BxPC-3 cells were seeded at 5 x 10^5 cells in 4 ml medium per well of a 6 well cell culture cluster (costar®, Corning Inc.) and incubated overnight. The cells were starved from serum for 24 hours and then incubated in culture medium containing SAHA, ML-60218, SAHA + ML-60218, or control (DMSO or untreated) for 48 hours. As previously described (Chun et al., 2009), the cells were incubated with propidium iodide, and the DNA content of the cells was analyzed using a FACScan™ flow cytometer (Becton, Dickinson and Company) by guest on February 9, 2021http://bio.biologists.org/ Downloaded from

Flow cytometric analysis of cell death
PANC-1 cells seeded at 5 x 10^4 in 3 ml medium per well of a 6 well cell culture cluster (costar®, Corning Inc.) were treated with SAHA, ML-60218, and SAHA + ML-60218, or control (DMSO or untreated) for 48 hours. The cells were incubated with (1:20) diluted recombinant human Annexin V conjugated with fluorescein isothiocyanate (FITC) (Invitrogen™/Life Technologies, Grand Island, New York, U.S.A.), and then propidium iodide (Invitrogen™/Life Technologies) at a final concentration of 1 µg/ml at room temperature. Analysis was conducted within 1 hour on the FACScan™ flow cytometer (Becton, Dickinson and Company) by guest on February 9, 2021http://bio.biologists.org/ Downloaded from

Immunofluorescent detection of survivin
BxPC-3 cells were seeded at 2 x 10^5 in 1 ml medium per chamber of a 2-chambered glass slide (LAB TEK®, Nunc™, Thermo Fisher Scientific, Rochester, New York, U.S.A.), treated with SAHA, ML-60218, and SAHA + ML-60218, or for 48 hours with 1% paraformaldehyde in phosphate buffered saline (pH 7.4), and incubated with 500 µl of (1:100) diluted monoclonal mouse anti-survivin antibodies (Cell Signaling Technology, Inc., Danvers, Massachusetts, U.S.A.), washed with Tris-buffered saline, and then incubated with 500 µl of (1:100) diluted goat anti-mouse Immunoglobulin G conjugated to Alexa Fluor® 488 nm (Invitrogen™/Life Technologies). Phalloidin conjugated to Alexa Fluor® 546 nm (Invitrogen™/Life Technologies) was used at 1:100 dilution to detect actin and visualize the cytoplasm. The expression pattern of survivin was examined and imaged under the MRC-1024 confocal laser scanning microscope (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.).

Immunoblot analysis
Total protein was extracted from zebrafish larvae and human cell lines and then analyzed by immunoblotting. For zebrafish, the larvae at 72 h.p.f. were homogenized in a lysis buffer containing 63 mM Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3.5% sodium dodecyl sulfate (SDS), and protease inhibitors (Roche Diagnostics, Indianapolis, Indiana, U.S.A.), at a concentration of 2°C for 1 hour, and supernatant was collected after centrifugation. For PANC-1 and BxPC-3, the cells were grown to approximately 70% confluence in 100-mm tissue culture dishes, and then treated for 48 hours with SAHA, ML-60218, SAHA + ML-60218, or DMSO, and the cells were fixed with 1% paraformaldehyde, then treated with glycine, washed with phosphate-buffered saline, and lysed using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The lysates of either zebrafish or human cells were then sonicated on ice for 30 seconds, centrifuged at 2 x 10^4 g for 10 minutes at 4°C, and the protein concentration of the supernatant was quantified using the BCA Protein Assay Kit (Bio-Rad). Immunoblot analysis was essentially performed as described (Yee et al., 2010). Primary antibodies include rabbit anti-human acetylated histone H3 (1:5,000), rabbit anti-human acetylated histone H4 (1:5,000), rabbit anti-human histone H3 (1:25,000), rabbit anti-human histone H4 (1:25,000) (Upstate Biotechnology/ Millipore®), goat anti-human BAX (Santa Cruz Biotechnology Inc., Santa Cruz, California, U.S.A.).

The present study demonstrates that the expression of survivin is increased in response to HDAC inhibitors, and that the inhibition of HDACs may lead to the induction of apoptosis in pancreatic cancer cells. The results suggest that HDAC inhibitors may be potential therapeutic agents for the treatment of pancreatic cancer.
California, U.S.A.; 1,100), rabbit anti-human p21

(Invitrogen

Lifetechologies) RNeasy

Mini Kit (Qiagen, Inc., Valencia, California, U.S.A.) according to the manufacturer’s instructions. To generate cDNA, the RNA was reverse-transcribed with SuperScript III Reverse Transcriptase and Random Primers (Invitrogen

Lifetechologies) according to the manufacturer’s protocol. The cDNA was amplified with gene-specific primer pairs located on two adjacent exons to achieve a high level of specificity and to avoid detection of genomic DNA. Primers were designed to have similar GC contents and annealing temperature using the Primer3 program (http://flogo.

wumit.edu). The primers were synthesized by International DNA Technology, Inc. (Corvallis, Iowa, U.S.A.). Transcript levels were determined by using TaqMan

Universal PCR Master Mix (Applied Biosystems®, Foster City, California, U.S.A.) and the TaqMan® Gene Expression Detection System (Applied Biosystems®) as essentially described (Yee et al., 2010). The conditions were 2 minutes at 50 ˚C, 10 minutes at 95˚C followed by 40 cycles of 15 seconds at 95˚C, and 1 minute at 60˚C followed by a final step of 10 minutes at 72˚C.

The sequences of the primers used for the analysis of zebrafish p21

CDKN1A (Abcam®, Cambridge, Massachusetts, U.S.A., 1:25,000), and rabbit anti-human actin (Sigma®, 1:2000). Secondary antibodies include horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, 1:5,000) or donkey anti-goat IgG (Santa Cruz Biotechnology, Inc., 1:5,000). The intensity and area of the protein bands were quantified using Adobe

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7. The levels of acetylated histones H3 and H4 were compared to those of total histones H3 and H4, respectively; those of BAX and p21

CDKN1A were compared with actin.

Quantitative real-time PCR

Total RNA was isolated from zebrafish larvae or human cell lines using TRIzol

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