D-dopachrome tautomerase is over-expressed in pancreatic ductal adenocarcinoma and acts cooperatively with macrophage migration inhibitory factor to promote cancer growth

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Previous studies have established the important role of MIF in the development of pancreatic ductal adenocarcinoma (PDAC) for both therapeutic and diagnostic perspectives, but little is known about the expression and function of D-dopachrome tautomerase (DDT), a functional homolog of MIF, in PDAC. In the present study, we demonstrated that DDT was over-expressed in PDAC tissues in a pattern correlated with MIF. In the pancreatic cancer cell lines, PANC-1, BXPC-3 and ASPC-1, both DDT and MIF were expressed and co-localized with each other in the endosomal compartments and plasma membrane. Knockdown of DDT and MIF in PANC-1 cells cooperatively inhibited ERK1/2 and AKT phosphorylation, increased p53 expression, and reduced cell proliferation, invasion and tumor formation. These effects were rescued by the re-expression of MIF or DDT, but not by the forced expression of the tautomerase-deficient mutants of DDT and MIF, P1G-DDT and P1G-MIF. Finally, we observed that 4-iodo-6-phenylpyrimidine (4-IPP), a covalent tautomerase inhibitor of both DDT and MIF, attenuated PANC-1 cell proliferation and colony formation in vitro and tumor growth in vivo. Thus, targeting the tautomerase sites of both MIF and DDT may offer more efficient therapeutic benefits to PDAC patients.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related mortality in the world, with a 5-year survival rate below 1%.1–3 The tumor has a propensity to metastasize even when small, and the cancer cells are generally resistant to chemotherapy and/or radiotherapy. These features may contribute to >3/4 of patients diagnosed with this disease cannot be offered curative treatment and, therefore, may determine the high mortality rate among patients with PDAC. An improved understanding of the mechanisms that contribute to PDAC development is therefore urgently needed.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that is released from activated macrophages and other immune cells.4,5 MIF binds to its cell surface receptor CD74, but signaling additionally requires the recruitment of co-receptors such as CD44 or CXCR2 and CXCR4.6,7 MIF is directly associated with the growth of several types of carcinomas, 8–11 and anti-MIF therapy with immunoglobulins and antisense oligonucleotides has been shown to have anti-tumorogenic effects.12,13 Recent studies have shown that MIF is over-expressed in PDAC,14 and is involved in PDAC progression by inducing epithelial to mesenchymal transition and enhancing tumor aggressiveness.15 A recent study showed that MIF was over-expressed in PDAC-derived exosomes, and played a role in liver pre-metastatic niche formation.16 Inhibition of MIF leads to cell cycle arrest and apoptosis in pancreatic cancer cells.17 Though the underlying mechanisms remain to be investigated, data from other cancer studies suggest that MIF promotes tumor growth through (i) activation of PI3K/Akt pathway thereby enhancing cell proliferation;18,19 (ii) inhibition of p53-dependent apoptosis20 and (iii) inhibition of the antitumor immune response.21

Several studies have shown that d-dopachrome tautomerase (DDT, also referred to as MIF-2), belongs to the MIF family, which utilizes a proline at the N terminus as an enzymatic base and have evolved from microbes to mammals.22–25 DDT has also been shown to bind to the MIF receptor CD74, activate

Key words: D-dopachrome tautomerase, macrophage migration inhibitory factor, pancreatic cancer, proliferation, tumor growth, invasion

Abbreviations: DDT: D-dopachrome tautomerase; DMEM: Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; ERK1/2: extracellular signal-regulated kinase 1/2; MIF: macrophage migration inhibitory factor; PDAC: pancreatic ductal adenocarcinoma; RNAi: RNA interference; 4-IPP: 4-iodo-6-phenylpyrimidine

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What’s new?
The cytokine MIF, released by macrophages, helps pancreatic cancer gain a foothold, and therapies that inhibit MIF are already in use. Less is known about DDT, a homolog of MIF that activates the same pro-inflammatory pathways that MIF does. These authors investigated whether DDT might give MIF a boost. First, they showed that pancreatic cancer cells do overexpress DDT. Removing DDT and MIF from pancreatic cancer cells slowed cell proliferation and tumor formation. Finally, they slowed tumor growth in mice using a tautomerase inhibitor of DDT and MIF. The two enzymes, it seems, work together to spur cancer growth, and this tautomerase inhibitor could lead to new therapeutic strategies.

the ERK1/2 MAP kinase signaling cascade and recapitulates many of the inflammatory functions of MIF. DDT and MIF are constitutively present in the cytosol and are exported from cells during infection or cellular stress, to activate receptors and initiate various biological responses. Recent studies have indicated that these two proteins have cooperative effects, including detrimental effects in cancer. These activities are decreased when DDT and/or MIF are reduced by siRNA. Combined loss of MIF and DDT leads to dramatically reduced cell cycle progression, anchorage independence, focus formation and increased programmed cell death when compared to individual silencing of MIF or DDT. These findings suggest that inhibiting both DDT and MIF may prove superior for improving therapeutic efficacy in diseases associated with both proteins.

Although the pro-tumorigenic function of MIF in PDAC has recently been established, the role of DDT in PDAC remains unexplored. In this study, we sought to investigate whether DDT functions cooperatively with MIF in survival signaling. We demonstrated an over-expression of DDT, which was correlated with the over-expression of MIF, in human PDAC tissues. Both proteins were expressed and co-localized with each other in endosomal compartments and plasma membrane in PDAC cell lines. In PANC-1 cells, silencing of both MIF and DDT synergistically decreased the phosphorylation of extracellular signal-regulated kinase and AKT, and increased the expression of p53, the latter was reversed by the expression of the tautomerase-deficient mutant (P1G). The high efficient knockdown lentiviral constructs for MIF (pGIPZ-shMIF-1, shMIF-2) and DDT (pGIPZ-shDDT-1 and pGIPZ-shDDT-2) were obtained from Open Biosystems (Rockford, IL). The third lentiviral construct for MIF or DDT knockdown (shMIF-3 and shDDT-3, respectively) was generated by using the shRNA design software from Dharmaco siDESIGN Center (www.dharmacon.com/sidesign/) as described previously. The oligos were 5’-TAATAGTTGATGTAGACCCGG–3’ for shMIF-3 and 5’–GCCAGGAAGCTTATTAT–3’ for shDDT-3. A scramble shRNA construct (pGIPZ-shGFP) was used as control. Lentiviral particles were produced by transfecting 293T cells according to the manufacturer’s protocol. Viral supernatants was collected after 72 hr following transfection, and the particles were concentrated by using LentiXTM Concentrator over night at 4°C (Clontech, Mountain View), the aliquots were stored at −80°C. The titer of the concentrated particles were measured before using. The PANC-1 cells (5 × 10⁵ cells/well) seeded in 6-well culture plates were then transduced with MIF or DDT shRNA-1, shRNA-2 and shRNA-3 lentviruses. Stably transduced cells were selected by culturing in the presence of blasticidin. The transduced cells were screened in western blot analysis to determine the levels of MIF or DDT expression. Transduction with the lentiviral shMIF-3 or shDDT-3 affect MIF or DDT expression levels in a much lower efficiency (see Fig. 3a). Therefore, cells transduced with this lentiviral construct were used as a control in some experiments.

Material and Methods
Pancreatic cancer cell culture
The pancreatic cancer cell lines, PANC-1, ASPC-1 and BXPC-3, were purchased from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in 5% CO₂ atmosphere at 37°C.

DDT and MIF knockdown
The high efficient knockdown lentiviral constructs for MIF (pGIPZ-shMIF-1, shMIF-2) and DDT (pGIPZ-shDDT-1 and pGIPZ-shDDT-2) were obtained from Open Biosystems (Rockford, IL). The third lentiviral construct for MIF or DDT knockdown (shMIF-3 and shDDT-3, respectively) was generated by using the shRNA design software from Dharmaco siDESIGN Center (www.dharmacon.com/sidesign/) as described previously. The oligos were 5’-TAATAGTTGATGTAGACCCGG–3’ for shMIF-3 and 5’–GCCAGGAAGCTTATTAT–3’ for shDDT-3. A scramble shRNA construct (pGIPZ-shGFP) was used as control. Lentiviral particles were produced by transfecting 293T cells according to the manufacturer’s protocol. Viral supernatants was collected after 72 hr following transfection, and the particles were concentrated by using LentiXTM Concentrator over night at 4°C (Clontech, Mountain View), the aliquots were stored at −80°C. The titers of the concentrated particles were measured before using. The PANC-1 cells (5 × 10⁵ cells/well) seeded in 6-well culture plates were then transduced with MIF or DDT shRNA-1, shRNA-2 and shRNA-3 lentviruses. Stably transduced cells were selected by culturing in the presence of blasticidin. The transduced cells were screened in western blot analysis to determine the levels of MIF or DDT expression. Transduction with the lentiviral shMIF-3 or shDDT-3 affect MIF or DDT expression levels in a much lower efficiency (see Fig. 3a). Therefore, cells transduced with this lentiviral construct were used as a control in some experiments.

Generation of HEK293 cells stably expressing MIF or DDT
HEK293 cells growing in 100 mm dishes were transfected with the pcDNA3.1 plasmids containing full-length human MIF or DDT. Stable HEK293 cells containing the recombinant MIF or DDT were established after selections with appropriate antibiotics.

Generation of recombinant adenoviral particles
Recombinant adenoviral vectors (pAd/CMV/V5-DEST, Invitrogen, Carlsbad, CA) containing full-length human MIF, full-length human DDT and the tautomerase deficient mutant forms of MIF (P1G-MIF) and DDT (P1G-MIF) generated using Q5® Site-Directed Mutagenesis Kit (New England Biolabs), were prepared as previously described. Briefly, these adenoviral vectors were propagated in HEK293 cells (Invitrogen, Carlsbad, CA) using the Stratagene MBS Mammalian Transfection Kit. After incubation in 37°C for 7
d, the transfected cells were harvested and subjected to four freeze/thaw cycles. After centrifugation of the lysates at 12,000 × g for 10 min at 4°C, the supernatant was transferred to a fresh screw-cap mini-centrifuge tube and stored at −80°C. A further amplification of the recombinant adenoviruses was performed using the same procedure, and the cell lysates were centrifuged on cesium chloride step gradients at 60000 × g at 4°C for 2 hr to separate viruses from defective particles and empty capsids. Viruses, dialyzed against PBS, were aliquoted in a buffer containing 10 mmol/l Tris, pH 7.4, 10 mmol/l MgCl2 and 10% v/v glycerol and stored at −80°C.

**Western blotting**

Cells were lysed in ice-cold lysis buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The protein extracts were denatured in a boiling water bath for 5 min and then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes which were probed with primary antibodies overnight at 4°C, followed by incubation with Horseradish Peroxidase (HRP)-conjugated secondary antibodies at room temperature for 60 min. Target proteins were detected with a chemiluminescence kit. Primary antibodies used include those against MIF, p-ERK1/2, ERK2, p-AKT, AKT (BD Biosciences, Franklin Lakes, NJ), DDT (Cell-Signaling Technology, Danvers, MA), p53 and β-actin (Sigma, St. Louis, MO).

**Immunohistochemistry**

PDAC tissue microarrays were purchased from the National Engineering Center for BioChips in Shanghai, China. The study was approved by the institutional Ethics Committee, and the written consent was obtained from all participants. Tumor tissue microarrays comprising 64 pathologically confirmed PDAC tumor sections were created as described previously (14). Microarrays were stained with an anti-MIF antibody (catalog no. sc-20121, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-DDT antibody (catalog no. sc-86406, Santa Cruz Biotechnology) at a 1:100 dilution with no antigen retrieval and biotin/avidin amplification following standard procedures. Staining intensity was scored as 0 (negative), 1 (weakly positive), 2 (strongly positive) and 3 (very strongly positive). The percentages of cells stained were scored into four categories: 0 (0%), 1 (1–40%), 2 (41–60%) and 3 (61–100%). The final staining scores, calculated by staining intensity x percentage of stained cells, were demonstrated as one of four categories: negative, low, moderate or high staining (numerically scored as 0–3). Averages scores were computed for staining of each sample.

**Immunofluorescence and confocal microscopy**

Cells growing on coverslips, cells were fixed with 4% paraformaldehyde (PFA) and were blocked with 5% normal donkey serum containing 0.1% Triton-X100 in PBS for 1 hr at room temperature. Cells were incubated with a rabbit polyclonal anti-MIF or anti-DDT antibody, or with an antibody mixture containing a rabbit anti-MIF (Abcam) and a mouse monoclonal anti-DDT (Abcam) at 4°C overnight. After washing three times with PBS containing 0.1% Triton-X100, the cells were incubated with a Alexa Fluor 594-conjugated donkey anti-rabbit IgG, or with a mixture of Alexa Fluor 594-conjugated donkey anti-mouse IgG (Life Technologies) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies) for 2 hr at room temperature, and washed three times with PBS containing 0.1% Triton-X100, incubated with 1 µg/ml DAPI (Sigma Aldrich) for 10 min. Confocal microscopy was performed on an LSM-710 laser scanning microscope (Zeiss, Oberkochen, Germany) with a 40 × 1.3 numerical aperture oil immersion lens. All digital images were captured at the same settings. Final images were processed using Adobe PhotoShop software.

**Enzyme-linked immunoabsorbent assay**

Enzyme-linked immunoabsorbent assay (ELISA) kits for detection of secreted DDT (ABIN419429) and MIF (DMF00B) were purchased from antibodies-online and R&D Systems, respectively, and used according to the manufacturer’s instructions.

**Invasion assay**

Cell invasion was quantified by the number of cells that migrated directionally through a Matrigel (25 µg/ml)-coated 8 µm-pore polyvinylpyrrolidone-free polycarbonate filter (Poretics, Livermore, CA) in Boyden Chambers. Filters were plated into contact with the lower chamber containing 200 µl of serum-free medium or of 10% FCS contained medium (control). Briefly, cells (1 × 106 cells/ml) were resuspended in serum-free DMEM and were then added to the top of each chamber and allowed to migrate through coated filters for 24 hr. Nonmigrated cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached on the lower membrane surfaces were stained with Diff-Quik (Baxer Scientific, McGaw Park, IL). Cells were counted at a 400× magnification in standard microscopy and the mean number of cells per field in five random fields was recorded. Triplicate filters were used and the experiments were repeated three times.

**Cell proliferation assay**

PANC-1 cells were plated in a 96-well plate (2,000 cells/well), which was incubated with DMEM for different days 1–12 days at 37°C in an atmosphere of 5% CO2 in air. Thereafter, 20 µl of Cell Counting Kit 8 solution was added to each well of the plate and incubated for an additional 4 hr in the same condition. Then, absorbance at 450 nm was measured using a microplate reader, wherein the absorbance value indicated the proliferative capacity.

**Colony formation assays**

PANC-1 cells (1.5 × 104) were suspended in 1 ml of 0.4% sea plaque agarose containing10% fetal bovine serum, then plated on top of 1 ml of semisolid 0.8% agarose in 35 mm plates and cultured for 2 weeks. The colonies were stained
with 1% crystal violet for 30 sec after fixation with 4% paraformaldehyde for 5 min. The number of colonies, defined as >50 cells/colony were counted. Three independent experiments were performed.

Tumor growth assay in mice
We purchased 6–8-week-old female BALB/c nude mice from Beijing Vital River Laboratories (VRL) and mice were quarantined for 2 weeks. For tumor growth assay, mice
(n = 8 per group) were subcutaneously injected with 1 × 10^7 PANC-1 cells infected with lentiviral control shRNA (shCT), shMIF, shDDT or both shMIF and shDDT. To evaluate the effect of small molecule tautomerase inhibitor on tumor growth, mice (n = 8 per group) were subcutaneously injected with 1 × 10^7 PANC-1 cells, followed by treatment (i.p.) with 4-IPP (80 mg/kg in corn oil) or vehicle control (corn oil). Two weeks after injection, tumor volume was measured every 2 days by the use of calipers and calculated as follows: V (mm³) = 0.5 × a × b², where a and b represent the long diameter and perpendicular short diameter (mm) of the tumor, respectively. At the end of experiments, mice were killed and tumors were excised and weighed.

**Statistical analysis**

For *in vitro* experiments, values were expressed as mean ± SEM. Comparison between two groups was done using the Student t test. All experiments were repeated three times. For *in vivo* studies, tumor volumes were calculated as mean ± SEM. Wilcoxon rank sum test and Kruskal–Wallis test were used to compare treatment differences.

### Results

**DDT was over-expressed in PDAC tissues in a pattern correlated with that of MIF**

To evaluate the clinical relevance of MIF and DDT in PDAC patients, we analyzed PDAC microarrays with MIF and DDT antibodies, respectively. The PDAC microarrays that we used contain 64 samples. A sample of cancer tissue and a corresponding noncancerous tissue from each patient were included. Approximately 94% (60 of 64) tumor samples showed positive DDT staining. 28% (18 of 64) of the samples demonstrated high staining, 42% (27 of 64) demonstrated moderate staining, 23% demonstrated low staining, and only 6% were negative (4 of 64) (Fig. 1b). Similar results for MIF staining were observed: positive staining in 94% (60 of 64), high staining in 30% (19 of 64), moderate staining in 45% (29 of 64), low staining in 19% and negative staining in 6% (4 of 64). In contrast, associated “normal” sections for some tumors also existed on the array and generally displayed staining in the low range of the scale. Both DDT and MIF staining showed cytoplasmic and membrane localization. To assess whether the expression levels of DDT and MIF demonstrate a correlation, the staining results were quantified (low = 1, moderate = 2, high = 3), multiple sections of the same tumors
were averaged, and Spearman’s correlation coefficient \( \rho \) was used to measure the association between DDT and MIF expression on the arrays. A positive correlation (\( R^2 = 0.346; \ p = 0.0001 \)) was observed (Fig. 1c). Using HEK293 cells stably expressing human MIF or DDT, we attempted to verify the specificity and selectivity of MIF and DDT antibodies used for the above immunohistochemistry. As shown in Figure 1d, the rabbit polyclonal anti-MIF antibody specifically stained the cells overexpressing MIF without cross-reaction with the DDT-expressing cells, whereas the rabbit polyclonal anti-DDT antibody specifically stained the cells overexpressing DDT without cross-reaction with the MIF-expressing cells.
We also examined the expression of MIF and DDT in the human PDAC cell lines, PANC-1, BXPC-3 and ASPC-1 cells. Confocal microscopy showed that both MIF and DDT were expressed in all these three cell lines, and co-localized with each other in the endosomal compartments and plasma membrane (Fig. 2a). ELISA assay showed that all the three cell lines secreted significant amounts of both MIF and DDT (Figs. 2b–2d).

**Knockdown of MIF and DDT synergistically inhibited ERK1/2 and AKT phosphorylation, increased p53 expression and attenuated proliferation and invasion of PANC-1 cells**

To obtain MIF or DDT knockdown PANC-1 cell lines, tumor cells were transduced with MIF or DDT shRNA lentiviral constructs (shMIF-1–3 and shDDT-1–3, respectively). After drug selection and single-cell cloning, the resulting clones were screened for the subsequent functional assays. Transduction of PANC-1 cells with the lentiviral shRNA-1 and shRNA-2 for MIF (shMIF-1 and shMIF-2) or DDT (shDDT-1 and shDDT-2) produced cloned cells that were severely deficient in MIF and DDT production, respectively (Fig. 3a). In contrast, transduction of PANC-1 cells with the shRNA-3 for MIF (shMIF-3) or DDT (shDDT-3) resulted in a much lower efficient knockdown of MIF or DDT (Fig. 3a). Whereas the high efficient knockdown of MIF or DDT with the specific lentiviral shRNA-1 and shRNA-2 resulted in a robust and significant inhibition of proliferation in the PANC-1 cells (Fig. 3b), the lower efficient knockdown of MIF or DDT with the specific shRNA-3 inhibited cell proliferation to a much less extent (Fig. 3b). Clearly, the extent of MIF or DDT knockdown is correlated with the extent of inhibition of cell proliferation.

To further exploring the underlying mechanisms, PANC-1 cells were infected with the lentiviral shRNA-1 or shRNA-2 specifically for DDT and MIF, respectively, and pro-growth signaling was assessed. Both DDT- and MIF-deficient cells showed decreased ERK and AKT phosphorylation and a concurrent increase in p53, which regulates the cell cycle and hence functions as a tumor suppressor (Fig. 3c). To examine whether DDT and MIF act cooperatively in the pro-growth signaling in PDAC growth, we next performed dual knockdown of DDT and MIF experiment in PANC-1 cells. In this experiment, a mixture of shMIF-1 and shMIF-2 (1:1 ratio) or a mixture of shDDT-1 and shDDT-2 (1:1 ratio), referred to as shMIF and shDDT, respectively, was used to knockdown the specific proteins. Verification of individual and dual shRNA knockdown of DDT and MIF expression in PANC-1 cells was obtained by Western blot analysis (Fig. 3d). Assessment of Akt and ERK phosphorylation indicated that dual knockdown of DDT and MIF showed a more significant decrease in phosphorylation of ERK and AKT than the individual knockdown of DDT or MIF (Fig. 3d). We also observed that dual knockdown of MIF and DDT resulted in more significant increase in p53 expression compared to the single knockdown of MIF or DDT (Fig. 3d).

The additive inhibitory effect of dual knockdown of MIF and DDT on the pro-growth signaling as described above suggests that knockdown of DDT and MIF may synergistically inhibit PDAC growth. To this end, we assessed the growth characteristics of PANC-1 cells that lack DDT and/or MIF expression by proliferation assays. Individual knockdown of DDT or DDT reduced monolayer cell proliferation by roughly 50% (Fig. 3e). Intriguingly, dual knockdown of MIF and DDT resulted in a more significant inhibition of cell proliferation (Fig. 3f). We also examined the effect of dual knockdown of MIF and DDT on PDAC growth using clonogenic survival assay. Whereas individual knockdown of MIF or DDT reduced clonogenic survival by roughly 50%, a more significant reduction was observed when both MIF and DDT were knocked down (Figs. 3g,3h). The reduction in colonies occurred in both the number as well as the sizes of the colonies.

Figure 3. Knockdown of DDT and MIF synergistically inhibits PANC-1 cell growth and invasion. (a) Western blot analysis of MIF and DDT in PANC-1 cells infected with different lentiviral particles bearing control shRNA (shCT), MIF shRNAs (shMIF-1–3) and DDT shRNAs (shDDT-1–3). Shown are representative results from three independent experiments. (b) The proliferation assay of PANC-1 cells infected with different lentiviral particles bearing control shCT, shMIF-1–3 and shDDT-1-3. Data are mean ± SD from three independent experiments. (c) Western blot analysis of control (shCT), DDT knockdown (shDDT-1 and shDDT-2) and MIF knockdown (shMIF-1 and shMIF-2) PANC-1 cell lysates probed with MIF, DDT, phospho-ERK1/2 (p-ERK1/2), total ERK2 (ERK2), phospho-Akt (p-Akt), total Akt (Akt), p53 and β-actin antibodies, respectively. Shown are representative results from three independent experiments. (d) Western blot analysis of control (shCT/shCT), DDT knockdown (shCT/shDDT) and MIF knockdown (shCT/shMIF) and dual knockdown (shCT/shDDT/shMIF) PANC-1 cell lysates probed with MIF, DDT, phospho-ERK1/2 (p-ERK1/2), total ERK2 (ERK2), phospho-Akt (p-Akt), total Akt (Akt), p53 and β-actin antibodies, respectively. Shown are representative results from three independent experiments. (e) Cell proliferation assay of control, DDT knockdown and MIF knockdown PANC-1 cells. Data are mean ± SD from three independent experiments. *p < 0.05 compared with shDDTs and shMIFs versus shCT at all points measured. (f) Cell proliferation assay of control, DDT knockdown, MIF knockdown and dual knockdown PANC-1 cells. Data are mean ± SD from three independent experiments. *p < 0.05 for both shDDT and shMIFs versus shCT at all points measured. (g) Colony formation of control, DDT knockdown, MIF knockdown and dual knockdown PANC-1 cells. Data are mean ± SD from three independent experiments. *p < 0.05 compared with shDDTs and shMIFs versus shCT. p < 0.05 compared with shDDT or shMIF. (h) Invasion of control, DDT knockdown, MIF knockdown and dual knockdown PANC-1 cells. Scale bar = 10 μm (j) Quantification of the invasion of control, DDT knockdown, MIF knockdown or dual knockdown PANC-1 cells. Shown are representative results from three independent experiments. *p < 0.05, **p < 0.01 for both shDDT and shMIF versus shCT. p < 0.05 compared with shDDT or shMIF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
PDAC is characterized by perineural invasion, which is one of the important causes of local recurrence. To examine whether DDT acts in concert with MIF to promote PDAC invasion, PANC-1 cells were infected with DDT shRNA (a mixture of shDDT-1 and shDDT-2, 1:1 ratio), MIF shRNA (a mixture of shMIF-1 and shMIF-2, 1:1 ratio) or in combination, and an invasion assay was performed. We observed that knockdown of either MIF or DDT resulted in a significant decrease in cell invasion, whereas dual knockdown of DDT and MIF resulted in a more significant inhibition of cell invasion (Figs. 3i, 3j).

To expand our observations to in vivo tumor model, PANC-1 cells infected with DDT shRNA (a mixture of shDDT-1 and shDDT-2, 1:1 ratio), MIF shRNA (a mixture of shMIF-1 and shMIF-2, 1:1 ratio) or in combination were also injected subcutaneously into the flanks of nude mice and followed over a period of 4 weeks to monitor tumor growth. Control PANC-1 cells generated 1.0 cm³ tumors in just over 4 weeks and were sacrificed because of the large tumor burden. In contrast, individual knockdown of DDT or MIF resulted in a significant decrease in tumor growth rate. Intriguingly, dual knockdown of DDT and MIF resulted in a more significant decrease in tumor growth rate (Fig. 4). Thus, DDT and MIF play a synergistic role in promoting PDAC growth.

Adenoviral re-expression of wildtype MIF or DDT, but not the forced expression of their P1G mutant forms, rescued PANC-1 tumorigenic phenotype

Both DDT and MIF possess an initial proline residue (Pro-1) in the N-terminal domain, which determines their tautomerase activity. A previous tautomerase-null MIF gene knock-in study showed that replacing Pro-1 with glycine (P1G) interfered with MIF-dependent growth regulation. We hypothesize that in the DDT- or MIF-knockdown PANC-1 cells, adenovirus-mediated re-expression of wildtype MIF or DDT, rather than the forced expression of their tautomerase-deficient mutants (P1G-MIF and P1G-DDT), should rescue PANC-1 tumorigenic phenotypes. To this end, the combined MIF/DDT lentiviral shRNA infected cells were adenovirally infected with wildtype or mutant MIF, DDT or both. Verification of the re-expression of DDT or MIF in PANC-1 cells was obtained by Western blot analysis (Fig. 5a). Intriguingly, the expression of p53, which was elevated by the dual knockdown of DDT and MIF, was reversed in MIF- or DDT-rescued cells, but was not reversed by the forced expression of P1G-MIF- and/or P1G-DDT (Fig. 5a). Clonogenic survival assay demonstrated that whereas re-expression of MIF or DDT rescued the defect of clonogenic survival in PANC-1 cells with dual DDT and MIF knockdown, forced expression of P1G-MIF or P1G-DDT did not show rescue effect (Fig. 5b). Similarly, the defect in cell proliferation induced by dual knockdown of DDT and MIF was rescued by re-expression of DDT or MIF, but not by the forced expression of P1G-DDT or P1G-MIF (Fig. 5c).

To further investigate the role of DDT and MIF tautomerase activity in their tumorigenic role in vivo, PANC-1 cells co-infected with lentiviral DDT and MIF shRNAs were adenovirally co-infected with wildtype DDT and MIF or tautomerase-deficient mutant forms of DDT and MIF (P1G-DDT and P1G-MIF), before being injected subcutaneously into the flanks of nude mice and followed over a period of 4 weeks to monitor tumor growth. As expected, defect of tumor growth induced by the dual knockdown of DDT and MIF was rescued by re-expression of wildtype DDT and MIF, but not by the forced expression of P1G-DDT and P1G-MIF (Fig. 5d). Thus, the tautomerase activity is required for DDT or MIF to promote PDAC growth.

The tautomerase inhibitor of both MIF and DDT reduced PANC-1 growth in vitro and in vivo

Although the tautomerase site of DDT and MIF are biophysically very different, 4-ido-6-phenylpyrimidine (4-IPP) forms a covalent bond with Pro-1 of both proteins and inhibits both DDT and MIF. We attempted to examine whether 4-IPP could inhibit PANC-1 growth. PANC-1 cells were grown in the culture medium containing 50 μM 4-IPP or vehicle as control, and proliferation and colony formation were assessed. As shown in Figures 6a and 6b, treatment with 4-IPP significantly reduced PANC-1 proliferation and colony formation.

To examine whether treatment with 4-IPP attenuates PANC-1 growth in vivo, BALB/c nude mice subcutaneously injected with PANC-1 cells were treated i.p. with 4-IPP (80 mg/kg in corn oil) or vehicle control (corn oil) for 4 weeks, and tumor formation was measured. Compared to

Figure 4. DDT and MIF knockdown synergistically impairs growth of PANC-1 cells in vivo. Xenograft tumor assay of PANC-1 cells with control (n = 8), DDT knockdown (n = 10), MIF knockdown (n = 8) and dual knockdown (n = 8). Data are mean ± SD. **, p < 0.01, ***, p < 0.001 for both shDDT and shMIF versus shCT, p < 0.05 compared with shDDT or shMIF.
the vehicle-treated mice which showed tumors of considerable size, mice treated with 4-IPP showed markedly reduced tumor formation within 28 days (Fig. 6c). By evaluating the expression of MIF or DDT expression in the tumor tissues, we observed that 4-IPP treatment did not significantly change the expression of either MIF or DDT (Figs. 6d, 6e).

Discussion

Previous studies have established the important role of MIF in the development of PDAC for both therapeutic and diagnostic perspectives,14–17 but little is known about DDT, especially its biological functions, in PDAC. In this study, we demonstrate for the first time that DDT had correlated expression pattern with MIF in PDAC and have significantly overlapping functions with MIF in promoting PDAC development. Individual knockdown of MIF or DDT resulted in decreased phosphorylation of ERK1/2 and AKT, increased expression of p53, decreased cell proliferation, decreased colony survival and decreased tumor growth in xenograft tumors. Importantly, dual knockdown of DDT and MIF in PANC-1 cells resulted in more significant inhibition of ERK1/2 and AKT phosphorylation, cell proliferation, colony formation, invasion and tumor growth in vivo then the individual knockdown of MIF or DDT. Finally, we provided evidence that the tautomerase activity of MIF and DDT were responsible for their tumor-promoting effect, and in fact, a dual tautomerase inhibitor of MIF and DDT, 4-IPP, significantly decreased PANC-1 cells proliferation and colony formation in vitro and tumor formation in vivo. These findings suggest that simultaneous targeting of MIF and DDT may have important, and previously unrecognized, therapeutic benefits for PDAC patients.

DDT and MIF were not only expressed in PDAC tissues in a correlated pattern but also co-localized with each other in the endosomal compartments and in the plasma membrane in all three PDAC cell lines tested. It is known that
MIF is endocytosed, and MIF-stimulated AKT signaling is reduced by endocytosis inhibitors, indicating that MIF signaling is at least in part due to endosomal signaling mechanisms. Though mechanisms underlying DDT signaling remain to be investigated, the co-localization of DDT with MIF in the endosomal compartments and the cooperative effect of MIF and DDT in PDAC signaling suggest that DDT and MIF share the same signaling pathways. In nonsmall cell lung cancer, the cooperative effects of MIF and DDT are dependent upon the cell surface receptor, CD74. Although not shown here, most of the PDAC tissues and all the PDAC cell lines used in this study express CD74 at moderately high levels, consistent with prior studies demonstrating elevated CD74 expression in a large percentage (~85%) of human colorectal adenomas. Thus, it is likely that both DDT and MIF bind to CD74 to initiate pro-growth signaling pathways in PDAC cells.

Since p53 is the strongest tumor suppressor gene, which can regulate cell cycle arrest, apoptosis and senescence, re-activation of p53 and its pathway seem to be very plausible target for cancer therapy. MIF was first identified as a negative regulator of p53, a tumor suppressor gene and a key regulator of the cell proliferation, by Hudson and colleagues using a functional p53 library screening assay. Several studies have since validated MIF as being an important endogenous regulator of p53 expression and activity in a variety of contexts.

Figure 6. Inhibition of PANC-1 growth by 4-IPP. (a) Cell proliferation assay of vehicle (control) or 4-IPP treated PANC-1 cells. Data are mean ± SD. *, p < 0.05, **, p < 0.01 compared with control. (b) Colony formation of vehicle (control) or 4-IPP treated PANC-1 cells. Data are mean ± SD. **, p < 0.01 compared with control. (c) Xenograft tumor assay of PANC-1 cells with vehicle treatment (n = 8) or 4-IPP treatment (n = 8). Data are mean ± SD. *, p < 0.05 compared with vehicle treatment. (d) Representative Western blot analysis of MIF and DDT in xenograft tumors treated with vehicle (n = 8) or 4-IPP (n = 8). (e) Quantification of MIF and DDT immunoblots in xenograft tumors treated with vehicle (n = 8) or 4-IPP (n = 8). Data are mean ± SD.
biological processes. A number of mechanistic pathways have been proposed for MIF-dependent p53 antagonism including: bioactive lipid metabolism; regulation of the COP9 signalosome subunit 5 (CSN5); direct, physical interaction with p53; indirect interaction with M-23-H1 and redox maintenance. Here, we demonstrate that inhibition of DDT and MIF cooperatively enhanced p53 expression in PANC-1 cells, consistent with the previous observation in human lung adenocarcinoma cell lines, suggesting that DDT and MIF synergistically promote PDAC development through at least in part inhibiting p53. However, we cannot exclude other potential mechanisms employed by MIF and DDT to promote PDAC growth. Coleman et al. reported that MIF and DDT play a synergistic role in inducing the expression of CXCL8 and VEGF in nonsmall cell lung cancer cells, most likely through activating CD74 and the downstream c-Jun-N-terminal Kinase (JNK) signaling, whereas Brock et al. reported that MIF and DDT acting through CD74 antagonize AMPK activation by maintaining glucose uptake, ATP production and redox balance. Whether these pathways are involved in the PDAC should be investigated in future.

MIF and DDT belong to the tautomerase family of proteins, which utilize a proline at the N terminus as an enzymatic base and have evolved from microbes to mammals. Cells expressing a P1→G mutant form of MIF (P1G-MIF) exhibit reduced binding affinity to the receptors CD74 and CXCR2 and impaired ability to induce ERK1/2 activation, associated with reduced proliferative capacity. In the present study, we showed that the increased p53 expression by the dual knockdown of MIF and DDT was reversed by re-expression of MIF or DDT, but not by the expression of P1G-MIF or P1G-DDT, suggesting that the Pro-1 residue is required for both DDT and MIF to inhibit p53 in PANC-1 cells. However, this does not necessary indicate that the tautomerase activity of DDT and MIF is indispensable for the roles described above, since a previous study using a P1G-MIF knock-in mutant to conclude that Pro-1 is important in protein–protein interactions, and the reduction in this biological activity is not due loss of catalytic function.

Finally, we demonstrate that the small molecule compound 4-IPP, which inhibits the tautomerase activities of both MIF and DDT, significantly blocked PDAC cell growth in nude mice bearing PANC-1 cells. In line with our results, previous studies on lung cancer cells and squamous carcinoma cells also showed an inhibitory effect of 4-IPP on the cancer cell motility and growth. Although the tautomerase site of DDT and its homologue MIF are biophysically very different, 4-IPP forms a covalent bond with Pro-1 of both proteins, thereby ameliorating their biological functions. Thus, it is feasible that targeting distinct tautomerase sites of DDT and MIF with a single molecule to inhibit PDAC growth.

It should be noted that as pro-inflammatory cytokines, the roles for DDT and MIF in tumor immunology and inflammation are important considerations not described in this study. In the murine melanoma model, macrophage-derived MIF participates in macrophage alternative activation, which contributes to tumor growth, and MIF deficiency promotes tumor-associated macrophages to secret pro-inflammatory cytokines, and reduces anti-inflammatory, immunosuppressive and pro-angiogenic gene products, thereby reducing tumor outgrowth. In the murine breast cancer cell line 4T1, MIF promotes tumor growth and metastasis by inducing myeloid-derived suppressor cells in the tumor microenvironment. A recent study demonstrated that MIF was highly expressed in PDAC-derived exosomes, and its blockade prevented liver premetastatic niche formation by blocking the secretion of transforming growth factor β from Kupffer cells and blocking the upregulation of fibronectin production by hepatic stellate cells. Whether DDT acts in concert with MIF in these functions to promote cancer development remains to be investigated.

In summary, our study demonstrated that DDT was over-expressed in PDAC tissues and cell lines in a pattern correlated with MIF, and knockdown of DDT and MIF in PANC-1 cells cooperatively inhibited cell proliferation, invasion and tumor formation. The tautomerase activities of both MIF and DDT are required for their negative regulatory role in p53 and their tumor-promoting functions. Thus, targeted inhibition of both MIF and DDT should offer more robust therapeutic benefits to PDAC and probably other cancer patients.

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