A novel oncotherapy strategy, direct thrombin inhibitors suppress progression, dissemination and spontaneous metastasis in non-small cell lung cancer

Zhao Bing[1], Mengfang Wu[1], Zhihuang Hu[2], Tianfa Wang[1], Jinchao Yu[1], Yixin Ma[1], Qi Wang[1], Yanling Zhang[1], Di Chen[1], Tianyu Li[1], Yaran Li[3], Min Yu[1], Huijie Wang[2], and Wei Mo[1]

[1]Fudan University
[2]Fudan University Shanghai Cancer Center
[3]Fudan University School of Basic Medical Sciences

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Abstract

Background and Purpose: Cancer cachexia and cancer-associated thrombosis are potentially fatal outcomes of advanced cancer. Nevertheless, thrombin expression in NSCLC primary tumor tissues and the association between prognosis of NSCLC patients remain largely unknown. Experimental Approach: Clinical pathological analysis was performed to determine the relationship between thrombin and tumor progression. Effect of r-hirudin and DTIP on cancer progression were evaluated. Western blotting, immunohistochemistry, and immunofluorescence were used to explore the inhibition mechanism of r-hirudin and DTIP. Therapeutic effect of combination of DTIP and chemotherapy was determined. Key Results: We illustrated thrombin expression in NSCLC tissues is closely related to clinicopathological features and the prognosis of patients. Thrombin deficiency inhibited tumor progression. The novel thrombin inhibitors, r-hirudin and DTIP, inhibited cell invasion and metastasis in vitro. They inhibited tumor growth and metastasis in orthotopic lung cancer model; inhibited cells invasion and prolonged survival after injection tumor cells via tail vein; they also inhibited angiogenesis and spontaneous metastases from subcutaneously inoculated tumors. The promotional activity of thrombin in invasion and metastasis was abolished in PAR-1 deficient-NSCLC cells. r-hirudin and DTIP inhibit tumor progression through the thrombin-PAR-1-mediated RhoA and NF-ϰB signaling cascades via inhibiting the MMP9 and IL6 expression. DTIP potentiated chemotherapy-induced growth and metastatic inhibition and inhibited chemotherapy-induced resistance in mice. Conclusions and Implications: Thrombin makes a substantial contribution, together with PAR-1, to NSCLC malignancy. We concluded the anticoagulants, r-hirudin and DTIP, could be expanded for anti-tumor therap. Combination therapy of DTIP and chemotherapy might achieve a better therapeutic effect.

Abbreviations

NSCLC: non-small cell lung cancer; OS: overall survival; DTIP: Direct thrombin inhibitor peptide; r-hirudin: recombinant hirudin; PAR-1: Proteinase-activated receptors; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; gRNA: guide RNA; NF-ϰB: nuclear factor ϰB; HUVEC: human umbilical vein endothelial cells, VEGF: Vascular endothelial growth factor.

Introduction

Lung cancer is the leading cause of cancer-related deaths among humans worldwide (Kyu et al., 2018), and non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases (Yuan, Huang, Chen, Wu & Xu, 2019). Even when NSCLC is diagnosed at an operable stage and treatment with chemotherapy is applied, the incidence of recurrence and metastasis remains high with a median survival

of less than 10-12 months. Most patients initially respond to platinum or gemcitabine-based chemotherapy (Mlak et al., 2016; Pelayo Alvarez, Westeel, Cortes-Jofre & Bonfill Cosp, 2013; Wang et al., 2018), but usually relapse and acquire chemo-resistant disease, such that the 5-year survival is $< 0.5\%$ (Morabito et al., 2014). Thus, targeting tumor metastasis and drug-resistant may represent a promising strategy to treat NSCLC (Vyse & Huang, 2019).

It has long been presumed that tumors may take advantage of the hemostatic system. Many significant hemostatic abnormalities have been described in cancer patients, including disseminated intravascular coagulation, hemorrhagic events, and migratory thrombophlebitis (Soff, 2019; Tan et al., 2019). Indeed, hemostatic complications are a common cause of death in patients with cancer(Joseph S. Palumbo, 2000). Previous studies in mice have unequivocally shown that tumor cell-associated tissue factor (Yokota et al., 2014), circulating prothrombin (Horowitz et al., 2011), and several downstream thrombin procoagulant targets (i.e., platelets, fibrinogen, factor XII) strongly promote tumor cell metastatic potential (Joseph S. Palumbo, 2000; Plantureux, Crescence, Dignat-George, Panicot-Dubois & Dubois, 2018; Yokoyama, Mori & Matsuura, 2008). However, at the level of primary tumor growth, the contribution of hemostatic factors has been less clear. Thrombin is an allosteric enzyme with an elaborate structure that exerts diverse biological effects by interacting with various receptors on the surface of vascular and nonvascular cells (Lane, Philippou & Huntington, 2005). Thrombin has also been shown to contribute to tumor progression in manners both coagulation-dependent and coagulation-independent (Xue et al., 2010). A substantial amount of data supports the idea that thrombin plays important roles in tumorigenesis, contributing to inflammation, angiogenesis, and metastatic dissemination of tumor cells through its PAR-1 receptor (Battinelli, Markens, Kulenthirarajan, Machlus, Flaumenhaft & Italiano, 2014; Reddel et al., 2017; Yokota et al., 2014). Nevertheless, thrombin expression in NSCLC primary tumor tissues and the association with prognosis of NSCLC patients remain largely unknown.

PAR-1, the prototypic member of the PAR family, has been shown to respond to a highly select group of serine proteases. Cleavage of PAR-1 by thrombin initiates potent inflammatory responses, including the up-regulation of cell surface adhesion molecules, induction of hyperpermeability (Feistritzer & Riewald, 2005), and the activation of the nuclear factor ϰB (NF-ϰB) pathways (Jeffers et al., 2015).

At present, chemotherapy is an established multimodal therapy for NSCLC, however, its benefits are limited due to a low response rate or acquired tumor resistance. In the present study, Arnold et. al have shown that PAR-1 in the tumors induces the chemo-resistance of cancer. As thrombin is the prototypical PAR-1 agonist, which indicates that targeting thrombin may be particular effective in combination with routine chemotherapy.

Direct Thrombin Inhibitor Peptide (DTIP) and recombinant hirudin (r-hirudin) which are derivatives of wild-type hirudin variant 2, were developed by our group (Mo, Zhang, Chen, Wang & Song, 2009; Zhao et al., 2017). DTIP and r-hirudin bind to exosite I and to the apolar region of thrombin, while the N-terminal moiety of r-hirudin and DTIP blocks access to the thrombin active site to inhibit the activity of thrombin. r-hirudin has entered phase I clinical trials, and DTIP is a novel antithrombotic agent that could be used to prevent thrombosis without conferring an increased bleeding risk for subcutaneous injection.

Here, we investigated the protein levels of thrombin in clinical NSCLC samples, explored the relationship between thrombin expression level and clinicopathological features, prognosis of NSCLC patients. We evaluated the effects of r-hirudin and DTIP on tumor progression, dissemination and spontaneous metastasis in vitro and in vivo. We also demonstrated the presence of thrombin and PAR-1 accounts for the majority of the invasive signal. The novel findings presented here indicate the roles of r-hirudin and DTIP, anticoagulant drugs, could be expanded for anti-tumor therapy. We speculate that the use of r-hirudin and DTIP in combination would be a new breakthrough in cancer treatment.

Materials and Methods

Materials

Thrombin was obtained from Sigma Aldrich (St Louis, MO). ML161 (PAR-1 antagonist) was purchased from TargetMol (Boston, USA). The anti-NF-κB p65, anti-phospho-NF-κB p65, anti-IκBα, anti-phospho-IκBα, anti-Erk, anti-phospho-Erk, anti-STAT3, anti-phospho-STAT3, anti-Akt, anti-phospho-Akt were purchased from Cell Signaling Technology (Danvers, MA). The antibody specific for thrombin, PAR-1, and CD31 were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-MMP9, anti-IL6 and anti-GAPDH were from Proteintech (Proteintech Group, Chicago, IL). The kit for monitoring the activation of RhoA was obtained from Cytoskeleton (Denver, CO). r-hirudin and DTIP were obtained from our group, and expression and purification of r-hirudin and DTIP has been described previously (Mo, Zhang, Chen, Wang & Song, 2009; Zhao et al., 2017).

Patients and tissues

All experiments using human tissues were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Fudan University. The clinical samples of lung cancer patients were obtained from Fudan University Shanghai Cancer Center (Shanghai, China). The tissues consisted of samples from 132 patients with NSCLC, including 100 cases of adenocarcinoma (ADC), 23 cases of squamous cell carcinoma (SCC), and 9 cases of other types of NSCLC at different stages. On each tissue, healthy lung tissues were also included.

Wound healing assay

Wound healing was analysed using IBIDI Culture-Inserts (IBIDI GmbH, Martinsried, Germany). Cells were dissociated from plates using 0.05% (w/v) trypsin and seeded into culture-insert plates at a concentration of 2×10^4 cells per culture well. After 24 hours of incubation, culture inserts were removed. Photographs of the movement of cells into the scratch area were taken after 24 h using a light microscope.

Cell migration

Cell migration was measured using transwell inserts (Corning Life Sciences, Tewksbury, MA) according to manufacturer's instructions. Briefly, transwell chambers (8 mm pore size) were coated with 50 μL of diluted matrigel. Cells suspended in serum-free medium at a density of 1.5×10^5 cells/mL were seeded (0.1 ml) in the upper chambers and 0.5 mL medium containing 10% FBS was added to the lower chambers, and PBS, 10 nmol/L thrombin, 25 nmol/L r-hirudin, 50 nmol/L DTIP, 10 nmol/L thrombin + 25 nmol/L r-hirudin and 10 nmol/L thrombin + 50 nmol/L DTIP were added to both of the upper and lower chambers. After culturing for 24 hours, cells were fixed in methanol and stained with 0.1% crystal violet. The cells on the bottom of the filters were counted.

F-actin staining assay and confocal microscopy

Cells were grown on 35-mm glass-bottom dishes and fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. The cells were then blocked with 1% BSA for 20 minutes. Cells were incubated with YF-488 phalloidin (US Everbright$^{(r)}$Inc) for 30 minutes and then stained with DAPI for 5 minutes. The cells were observed under a confocal microscope.

Measuring RhoA activation

Determination of RhoA activations (RhoA-GTP) was monitored using commercially available kits. Briefly, cells were lysed using a cell lysis buffer provided by the manufacturer. Half of the lysates were saved for Western blotting quantitation of total RhoA. The remaining sample was incubated with 20 μg GST fusion protein RBD (rhotekin Rho-binding domain), at 4 $^{\circ}$C with rotation for 1 h, which is bound to the colored glutathione-sepharose beads. The RBD protein motif binds specifically to the active GTP-bound form of RhoA. Beads were washed, resuspended in loading buffer and proteins were separated on 12% SDS-PAGE followed by transferring to a PVDF membrane and Western blotting using an anti-RhoA monoclonal antibody as described by the manufacturer.

Tube formation assay

Ninety-six-well plates were precoated with 50 μL liquid Matrigel per well. After incubation at 37°C for 1 hour, HUVESs (1.5×10^4 cells/well), suspended in DMEM medium with PBS, 10 nmol/L thrombin, 25 nmol/L r-hirudin, 50 nmol/L DTIP, 10 nmol/L thrombin + 25 nmol/L r-hirudin and 10 nmol/L thrombin + 50 nmol/L DTIP and were seeded and cultured for 8 hours. Five randomly chosen fields were photographed and counted.

gRNA design and lentivirus infection

CRSIPR guides targeting *Thrombin* and *PAR-1* of human and mouse sequences were generated and cloned into LentiCRISPRv2 at BsmBI restriction sites. LentiCRISPRv2 and packing constructs were transfected into 293T cells. Virus supernatants were collected 48 h after transfection. A549 and LLC cells were infected with viral supernatants in the presence of polybrene and were then selected in growth media containing puromycin. All the cell lines used have been tested and authenticated by karyotyping. Transfection with the empty plasmid was performed which was used as negative control (NC). $A549^{THR-/-}$, $LLC^{Thr-/-}$, $A549^{PAR-1-/-}$ and $LLC^{Par-1-/-}$ cell lines were checked by PCR and sequencing.

Scoring ofimmunostained specimens

Immunostained specimens were reviewed by 2 investigators blinded to the patients' clinical status using a multihead microscope. To evaluate expression of proteins using immunohistochemistry, the intensity of proteins staining was graded by consensus on a scale from 0 to 3 (0 = negative staining; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive). The frequency of positive cells was graded by consensus on a scale from 0 to 4 (0 = less than 1%; 1 = 1% to 10%; 2 = 10% to 50%; 3 = 50% to 80%, 4 = greater than 80%). The immunohistochemical scores (HIS) were determined by staining intensity and positive cells. Based on the score of thrombin or PAR-1 in the central positive staining area in tumor section as a cutoff value, the score of thrombin or PAR-1 was classified positive (thrombin+ >2, and PAR-1 high expression >6) and negative (thrombin- [?]2, and PAR-1 low expression [?]6).

Tail vein injections

This animal study was conducted in accordance with the rules and regulations of the IACUC at the Department of Laboratory Animal Science, Fudan University (Shanghai, P.R. China). Lewis lung carcinoma (LLC) cells were collected and resuspended in serum-free medium. C57/BL6 mice were injected through the tail vein with $1x10^6$cells in 0.1 mL. Fourteen days after injection of cells, the mice were subjected to PET scanning. Mice were euthanized by pentobarbital sodium, and all lungs were dissected or further imaged by fluorescence stereomicroscopy and then were fixed in Bouin's solution, and the number of metastatic nodules on the lung surface was counted.

Subcutaneousinoculation of tumors in mice

This animal study was conducted in accordance with the rules and regulations of the IACUC at the Department of Laboratory Animal Science, Fudan University (Shanghai, P.R. China). Tumor cells at a density of $1x10^6$in 0.1mL serum-free media were injected subcutaneously into the right flank of mice. The mice were monitored daily for the development of visible tumors. Once a tumor was clearly visible, it was calipered three times each week, and the volume was estimated using the formula $V = (LW^2)/2$ where V is volume, L is longest diameter, and W is shortest diameter. Mice were euthanized by pentobarbital sodium, and the tumors were removed, some were fixed in 4% paraformaldehyde, and the other tumors were lysed for Western blotting.

Orthotopic lung cancer model in mice

Mice were anesthetized with chloral hydrate (300 mg/kg) via intraperitoneal injection and placed in the right lateral decubitus position. A total of 1×10^6 cells in 50 μL RPMI-1640 medium and 50 μL Matrigel were injected into the left lung parenchyma through the left rib cage. Mice were monitored daily. Mice were euthanized by pentobarbital sodium, and the lung tissues were removed.

Statistical Analysis

All data are expressed as mean ± SD. Unless otherwise stated, differences between the 2 groups were analyzed by unpaired t test when variances are equal, and 1-way analysis of variance followed by Newman-Keuls test were used for multiple comparisons with Prism 6 (GraphPad Inc). $P<0.05$ was considered to be statistically significant.

Results

The expression of thrombin in lung cancer is closely related toclinicopathological features and the prognosis of patients.

Thrombin has also been shown to contribute to tumor progression. However, the expression of thrombin in NSCLC tissues and the relationship between thrombin expression and clinicopathological features and the prognosis of NSCLC patients have not been reported. To confirm the presence of thrombin (prothrombin) in NSCLC, 132 patients with a pathologically confirmed diagnosis of NSCLC were analyzed. We found the expression of thrombin was significantly increased in tumors of all different types of NSCLC tissues compared with their adjacent non-tumor lung tissues (Fig. 1A-C). There was no significant difference in different subtypes of NSCLC (Fig. 1C). To further evaluate the prognostic value of thrombin for NSCLC patients, univariate and multivariate analyses were performed with the clinicopathological characteristic. As shown in Table 1, thrombin expression in tumor tissue was significantly correlated with TNM stage of NSCLC. The 5-year overall survival (OS) rates of thrombin-positive patients were significantly lower than those of thrombin-negative patients (Fig. 1D). Moreover, both the mRNA and protein levels were significantly increased in comparison to normal lung cell line (BEAS-2B) detected by Q-PCR (Fig. 1E) and western blot (Fig. 1F) in three NSCLC cell lines.

Thrombin plays an important role in the progression of lung cancer.

To observe the role of thrombin in lung cancer cells, we constructed A549$^{THR\text{-}/\text{-}}$ and LLC$^{Thr\text{-}/\text{-}}$ cells. Transwell assay results showed that thrombin depletion inhibited cell migration in A549 and Lewis cells (Fig. 2A-D). IL6 has been demonstrated to be involved in the development, progression and metastasis in several cancers (Huang et al., 2018). Matrix metalloproteinases (MMPs) affects the physical barrier of the tumor microenvironment (TME) and induces metastasis (Lyu, Xiao, Yin, Yang & He, 2019). We found that the expression levels of MMP9 and IL6 were decreased in thrombin deficient cells, and the expression of MMP9 and IL6 could be restored by adding exogenous thrombin (supplementary Fig. S1).

To assess the role of thrombin in vivo, we employed tumor models. In subcutaneous transplanted tumor model, tumor growth inhibition was observed in thrombin deficient group (Fig. 2E-G). In addition, the lung metastases were reduced in thrombin deficient group (Fig. 2H-I). In orthotopic lung tumor model, thrombin deficiency could markedly increase the survival time of mice (Fig. 2J). And the control LLC cells generated massive lung tumor burden in mice. Significantly, the depletion of thrombin led to smaller lung tumor burden (Figure 2K). HE staining of lung sections also revealed bigger tumor area in control mice as compared to the thrombin deficient group (Figure 2L). Thrombin deficiency also inhibited liver metastasis in mice. Together with the in vitro experiments, we concluded that thrombin plays an important role in the progression of lung cancer.

r-hirudin and DTIP inhibitthrombin-promotedcell migration, invasion and angiogenesis.

To further explore the role of thrombin in the lung cancer, we used exogenous thrombin to treat NSCLC cell lines in vitro. DTIP and r-hirudin which are direct thrombin inhibitors, were developed by our group. A549, Lewis (Fig. 3A) and 95D cells (supplementary Fig. S2A) incubated with 10 nmol/L thrombin displayed a remarkable promotion in the ability to migrate into the blank space compared with the normal control (NC) group. However, r-hirudin and DTIP blocked thrombin-enhanced wound-closure capability of NSCNC cells. Transwell assay results showed that thrombin-driven migration was inhibited by pre-treatment with r-hirudin and DTIP in A549, Lewis (Fig. 3B) and 95D cells (supplementary Fig. S2B). Rho GTPases are well known as regulators of actin cytoskeletal organization and cell motility. Therefore, we examined the effect of r-hirudin and DTIP on the activation of RhoA and the status of actin filament organization. Our

findings indicated that r-hirudin and DTIP could suppress the activation of RhoA in thrombin-stimulated A549 cells (Fig. 3C). As illustrated in Fig. 3D, thrombin significantly enhanced the fluorescence intensity of polymerized actin (F-actin) compared with the NS groups. However, r-hirudin and DTIP could decrease the fluorescence intensity in thrombin-stimulated cells. The appearance of membrane ruffles and the formation of lamellipodia were also decreased in r-hirudin- and DTIP-treated cells. (Fig. 3E, 3F and supplementary Fig. S2C). These data demonstrated that r-hirudin and DTIP decreased the amount of F-actin and the formation of lamellipodia in thrombin-stimulated NSCLC cells, which consequently leads to decreased cell motility and migration ability.

In previous studies, we found thrombin deficiency could reduce the expression of MMP9 and IL6. Furthermore, the expression of MMP9 and IL6 also could be increased by exogenous thrombin, which could be inhibited by r-hirudin and DTIP (Fig. 3G and supplementary Fig. S3E-G). RhoA could be activated by thrombin (Fig. 3C). CCG, an inhibitor of RhoA, could inhibit thrombin-induced expression of MMP9 and IL6. LPA, an activator of RhoA, could prevent the inhibition induced by r-hirudin and DTIP (Fig. 3G and supplementary Fig. S3E-G).

Thrombin has been reported to activate NF-κB signaling in human pleural mesothelial. The effect of r-hirudin and DTIP on the NF-κB pathway in thrombin-stimulated NSCLC cells was analyzed. The results indicated that thrombin could activate NF-κB signaling in NSCLC cells. Compared with the thrombin-treated group, r-hirudin and DTIP exhibited diminished IκBα and p65 phosphorylation, suggesting that r-hirudin and DTIP can inhibit thrombin-induced NF-κB activation. CCG could inhibit the thrombin-induced NF-κB activation, while RhoA or NF-κB activatiors (LPA and LPS) could prevent the inhibition induced by r-hirudin and DTIP (supplementary Fig. S3A-D). The results indicated that thrombin could activate NF-κB signaling via RhoA in NSCLC cells.

Studies have shown that NF-κB pathway activation upregulates the expression of cell adhesion molecules and inflammatory cytokines. We also found RhoA and NF-κB inhibitor could inhibit thrombin-induced expression of MMP9 and IL6, RhoA and NF-κB activator could prevent the inhibition induced by r-hirudin and DTIP (Fig. 3G and supplementary Fig. S3E-G), suggesting that thrombin can regulate the expression of MMP9 and IL6 via RhoA and NF-κB pathway.

Thrombin is known to promote the release VEGF and induce angiogenesis. Hence, we examined the effects of r-hirudin and DTIP on angiogenesis using a tube formation assay. After thrombin treatment, the tubule formation was promoted, r-hirudin and DTIP significantly inhibit thrombin-induced tube formation (Fig. 3H). These results demonstrated that r-hirudin and DTIP possessed an anti-angiogenic potential.

r-hirudin and DTIP exert anti-invasive andanti-metastatic abilities in a mouse lung cancer model.

Our aforementioned results suggest anti-metastatic and anti-angiogenic activity of r-hirudin and DTIP in vitro. We further confirmed the effects in vivo. In orthotopic lung tumor model, DTIP could improve the survival time of mice compared with the control group, (Fig. 4A), and mice in r-hirudin or DTIP-treated groups had smaller tumor burden (Fig. 4B), had fewer mice with liver metastases (Fig. 4C).

Murine models of experimental metastasis have been used frequently to investigate the effects of anti-haemostatic agents on cancer metastasis. Although such artificial models do not encompass the entire metastatic process, they remain useful for 'proof-of-concept'-experiments, focusing on the haematogenous phase of tumor dissemination (Mammadova-Bach et al., 2020; Sjoberg et al., 2019; Vuong et al., 2019). Gross examination of the lungs harvested from r-hirudin- or DTIP-treated mice revealed a median of 21 (n=12) or 20 (n=12) surface pulmonary metastases per animal. In contrast, the lungs harvested from normal saline treated mice (n=16) had confluent metastases that were too numerous to count and were clearly enlarged (Fig. 4D, 4E). Micro-PET scan and histologic analyses revealed scattered small foci of tumor tissue within the lungs harvested from r-hirudin- or DTIP-treated mice, while the lungs harvested from normal saline-treated mice were nearly completely effaced by tumor tissue (Fig. 4F, 4G). We found the number of mice with tumor cells colonized in the liver was largely reduced in the r-hiruin and DTIP group compared

with the normal saline group (Fig. 4I, 4J). It is important to note 78% of control mice (n=9) were dead at 24 days, with all dead at 32 days, whereas 25% of r-hirudin-treated mice (n=12) and 30% of DTIP-treated mice (n=10) were dead at 24 days (Fig. 4H).

We also examined spontaneous metastasis through subcutaneous inoculation of tumor cells in mice, which involves a more comprehensive process. Treatment of r-hirudin and DTIP for one week inhibited tumor growth slightly (Fig. 5A, 5B). Six of the 9 tumors analyzed from normal saline-treated mice showed signs of panniculus invasion, whereas only 2 of 9 tumors from r-hirudin-treated mice and 2 of 10 tumors from DTIP-treated mice had any noticeable signs of panniculus invasion (Fig. 5C, 5D).

And we also administered 1.0 mg/kg DTIP or 0.5 mg/kg r-hirudin for 21 consecutive days after one week of the injection of LLC cells. r-hirudin and DTIP significantly inhibited tumor growth (Fig. 5E-G). The number of mice with lung and liver metastases was largely reduced in r-hirudin or DTIP treated groups (Fig. 5H-K). Tumor angiogenesis was assessed using IHC analysis for CD31. The r-hirudin or DTIP treated groups showed a significant reduction of CD31-positive microvessels versus controls (Fig. 5L).

We performed immunohistochemical analysis on tumor samples to determine the expression levels of MMP9 and IL6. There is decreased expression of MMP9 and IL6 after treatment with r-hirudin and DTIP compared with normal saline-treated mice (Fig. 5M). We also examined phosphorylation levels of p65 and the key downstream signaling molecules intratumorally. We observed a marked inhibition of phospho-p65, phospho-Erk, phospho-STAT3, and phospho-Akt levels in the r-hirudin- and DTIP-treated groups (Fig. 5N).

Furthermore, we did not find increased bleeding after administration of DTIP, slight subcutaneous hemorrhage was observed after r-hirudin administration for three weeks continuously (data are not shown). These results show that DTIP, a direct thrombin inhibitor, could be extended to anti-cancer therapy.

PAR-1 is a major determinant in thrombin-promotedmetastatic of lung cancer.

Thrombin is the main activator of PAR-1 (Vu, Hung, Wheaton & Coughlin, 1991). Overexpression of PAR-1 has been detected in various types of cancers, including ovarian (Grisaru-Granovsky, Salah, Maoz, Pruss, Beller & Bar-Shavit, 2005), breast cancer (Boire, Covic, Agarwal, Jacques, Sherifl & Kuliopulos, 2005), lung cancer, prostate cancer (Black et al., 2007), and melanoma. Our previous experimental results also showed PAR-1 was highly expressed in human and mouse tumors compared with normal lung tissues (supplementary Fig. S4). However, we did not find an obvious relationship between the PAR-1 expression levels of tumors and clinical variables, such as the stage of NSCLC differentiation status and disease progression (supplementary Table 1).

To observe the role of PAR-1 in thrombin-mediated invasion and metastasis more clearly, we constructed $A549^{PAR\text{-}1\text{-}/\text{-}}$ and $LLC^{Par\text{-}1\text{-}/\text{-}}$ cells. PAR-1 depletion almost completely abrogated thrombin-promoted cell migration, and the effect of r-hirudin and DTIP on thrombin-induced migration and invasion was abolished (Fig. 6A-D). PAR-1 is a G protein coupled receptor and has been shown to induce cellular invasion through RhoA-dependent signaling. After depleting PAR-1, the activation of RhoA was inhibited and the ability of thrombin to activate RhoA was also inhibited (Fig. 6E). Similar responses were also observed via immunofluorescence staining of F-actin, as PAR-1 depletion decreased the formation of membrane ruffles (Fig. 6F). These data suggest thrombin-enhanced cell motility and migration can be completely abrogated by PAR-1 depletion in vitro. PAR-1 deficiency exhibited diminished IκBα phosphorylation and p65 phosphorylation (supplementary Fig. S5A). Importantly, thrombin-driven NF-κB activation were inhibited by pre-treatment with the specific PAR-1 inhibitor ML161 (supplementary Fig. S5A). LPA or LPS could rescue the activation of NF-κB, but thrombin could not. r-hirudin and DTIP could not inhibit NF-κB activation induced by LPA or LPS (supplementary Fig. S5B,5C), suggesting r-hirudin and DTIP inhibit thrombin-induced RhoA and NF-κB activation via PAR-1 signaling. We also found PAR-1 deficiency exhibited deceased expression of MMP9 and IL6. Besides thrombin, LPA or LPS could increase MMP9 and IL6 expression in PAR-1 deficient cells. r-hirudin and DTIP could not inhibit MMP9 and IL6 expression induced by LPA or LPS (supplementary Fig. S6), suggesting r-hirudin and DTIP inhibit thrombin-induced MMP9 and IL6 expression through RhoA and NF-κB activation via PAR-1 signaling.

To further analyze the effects of PAR-1 on lung cancer growth and metastasis, we established lung cancer model in mice using LLC cells infected by gRNA-PAR-1 lentivirus (*Par-1*$^{-/-}$ group) or LV-negative control (NC, vehicle group). In orthotopic lung tumor model, PAR-1 deficiency could markedly increase the survival rate and inhibit tumor growth in lung (Fig. 6G-I).

In the metastatic colonization model, our results confirmed PAR-1 deficient lung cancer cells lead to less lung metastatic nodes than control cells (supplementary Fig. S7A, 7B), as well as significantly decreased signal intensity in the lungs, as seen on micro-PET scans (supplementary Fig. S7C). In addition, PAR-1 deficiency could markedly increase the survival rate (supplementary Fig. S7D).

In subcutaneous tumors, PAR-1 deficient group were significantly smaller than control group (Fig. 6J, 6K). The lung metastases and liver metastases were both largely reduced in PAR-1 deficient group (supplementary Fig. S7E). In addition, based on the results of CD31 staining, we confirmed that CD31-positive microvessels decreased in the PAR-1 deficient tumors (Fig. 6L). We also found the levels of phospho-p65, phospho-Erk, phospho-STAT3, and phospho-Akt were reduced in PAR-1 deficient tumors (supplementary Fig. S7F). Together with the in vitro experiments, we concluded that PAR-1 plays an important role in the thrombin-induced progression of lung cancer.

DTIP potentiateschemotherapy-induced growth and metastasis inhibition and inhibits chemotherapeutic drug tolerance of NSCLC in mice.

Chemotherapy has been commonly prescribed in the treatment of patients with NSCLC, however, its benefits are limited due to a low response rate or acquired tumor resistance. Arnold et. al have shown that PAR-1 in the tumor induces the chemo-resistance of cancer (Queiroz et al., 2014). Meanwhile, chemotherapy such as gemcitabine, cisplatin, and paclitaxel are associated with a significant increase in the risk of arterial thromboembolic events (Zaborowska-Szmit, Krzakowski, Kowalski & Szmit, 2020). we hypothesized that DTIP could potentiate chemotherapy-induced inhibition of tumor progression.

When combination DTIP and gemcitabine, the tumor volume and tumor weight in the combination treatment group was significantly smaller than that in the groups administered DTIP alone or gemcitabine alone (Fig. 7A-C). The number of mice with lung metastases was largely reduced in the combination treatment group (Fig. 7D, 7E). We also counted the survival rates of mice in different groups. It is important to note 85% of control mice (n=7) were dead at 60 days, 57% of r-hirudin-treated mice (n=7) and 50% of gemcitabine -treated mice (n=8) were dead at 60 days, whereas, 16% combination -treated mice (n=6) were dead at 60 days (Fig. 7F). Paclitaxel could not significantly inhibit the growth of LLC in vivo. However, when combined with DTIP, the growth (Fig. 7G-I) and metastasis (Fig. 7J, 7K) of LLC were significantly inhibited. Combination of DTIP and cisplatin had a smaller tumor volume (supplementary Fig. S8A-C), but there was no significant difference in metastasis and survival time of mice compared with the group administered cisplatin alone (supplementary Fig. S8D, 8E). In addition, we evaluated the chemotherapy effects in thrombin deficient NSCLC mouse models. We found gemcitabine or paclitaxel treated mice in thrombin deficient group had smaller tumors (supplementary Fig. S9A, 9C) and longer survival time (supplementary Fig. S9B, 9D) compared with control group treated with gemcitabine or paclitaxel. These results indicated that combination therapy of DTIP and chemotherapy might achieve a better therapeutic effect.

Discussion

Metastasis has been recognized as the main cause of fatal outcomes in lung cancer patients. It is of interest that low-grade intravascular coagulation has been observed in most patients with solid tumors (Rickles, Edwards, Barb & Cronlund, 1983). Malignant tumors often exhibit hypercoagulability, which is correlated with high levels of activated thrombin (Caine, Stonelake, Lip & Kehoe, 2002). Studies have shown that exogenous thrombin is capable of enhancing tumor adhesion to platelets (Nierodzik, Kajumo & Karpatkin, 1992), endothelial cells (Klepfish, Greco & Karpatkin, 1993), and fibronectin (Chen et al., 2014) in vitro, and revealed that exogenous thrombin promotes tumor growth (Nierodzik et al., 1998). In this study we demonstrated that thrombin expression in tumor tissues was significantly correlated with metastatic potential of NSCLC, postoperative tumor recurrence, and poor prognosis of NSCLC patients. This finding

is supported by the fact that a high thrombin expression was significantly associated with the aggressive histopathological characteristics of NSCLC, such as high TNM stage. Thrombin deficiency impairs tumor progression. This indicates that thrombin may serve as an independent predictor for tumor recurrence and prognosis of NSCLC patients, which might be an explanation as to why malignant tumors often exhibit hypercoagulability.

To further explore the role of thrombin in the lung cancer, we used exogenous thrombin to treat NSCLC cell lines in vitro. we found that 10 nmol/L thrombin can promote NSCLC cell invasion, angiogenesis, and metastasis in vitro. r-hirudin and DTIP are new bivalent direct thrombin inhibitors that could effectively prevent the formation of thrombosis and embolism (Mo, Zhang, Chen, Wang & Song, 2009; Zhao et al., 2017). These results showed that r-hirudin and DTIP could inhibit thrombin-induced cell invasion, angiogenesis, and metastasis in vitro. r-hirudin and DTIP inhibit progression, dissemination and spontaneous metastasis in NSCLC mice models, thus prolonging the survival time of mice in lung cancer models. Furthermore, in our studies, we did not find increased bleeding after administration of DTIP, slight subcutaneous hemorrhage was observed after r-hirudin administration for three weeks continuously. These results show that DTIP could be extended to anti-cancer therapy.

The cleavage of the thrombin-receptor PAR-1 by thrombin exposes a new N terminus that binds to the receptor to induce trans-membrane signaling (McLaughlin, Shen, Holinstat, Brooks, Dibenedetto & Hamm, 2005). PAR-1 overexpression has been reported in malignant invasive tumor cell lines (Black et al., 2007; Queiroz et al., 2014). Our data suggested that PAR-1 is highly expressed in human NSCLC tissues, but showed no difference based on subtype and clinical stage. To further explore the role of thrombin in the PAR-1-mediated NSCLC metastasis, we used exogenous thrombin to treat PAR-1 deficient NSCLC cells in vitro, and found that the invasion and metastasis of PAR-1 deficient cell lines was inhibited in vitro and in vivo; thrombin had no effect on their invasive and metastatic.

In our study, we showed that thrombin promotes the activation of RhoA through the activation of PAR-1, thereby inducing cytoskeletal rearrangements, actin stress fiber formation, and activation of NF-$\varkappa$B. Activation of cytosolic NF-$\varkappa$B leads to expression of MMP9 and inflammatory marker IL6. r-hirudin and DTIP can bind to thrombin specifically and block the thrombin binding to PAR1, thereby inhibiting RhoA and NF-$\varkappa$B activation and reducing the expression of MMP9 and IL6 in NSCLC cells, which would be hugely beneficial for suppressing metastasis.

Chemotherapy used in NSCLC seem to cause primarily vascular complications, including venous or arterial thromboembolic events. In a nonrandomized study, the addition of low-molecular-weight heparin (LMWH) to standard gemcitabine/cisplatinum chemotherapy significantly improved survival in patients with locally advanced or metastatic pancreatic carcinoma (Icli et al., 2007). The total response rate for patients treated with LMWH was almost 60%, compared with only 12% for patients treated with chemotherapy only. In our studies, we found combination therapy of DTIP and chemotherapy results in improved anti-tumor efficacy, and DTIP could potentiate gemcitabine-induced inhibition of lung cancer and inhibit paclitaxel resistance in mice. This approach merits further evaluation in more extensive studies and in NSCLC patients.

Conclusions

In this study, we not only provide convincing evidence that thrombin plays a crucial role in NSCLC, but also an explanation as to why intravascular coagulation with generation of thrombosis has been observed in most patients with solid tumors. We conclude that thrombin plays an important role in PAR-1-mediated NSCLC invasion and metastasis through RhoA and NF-$\varkappa$B signaling cascades via promoting the expression of MMP9 and IL6. We conclude that the highly specific, potent, thrombin inhibitor DTIP inhibit tumor spontaneous seeding of the tumor systemically into the vital organs, tumor metastasis in NSCLC. We suggest treatment with DTIP should start immediately after diagnosis (before extensive tumor development) and in conjunction with chemotherapy.

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Conflict of Interest Disclosures

None of the authors has a relevant conflict of interest.

Contributions

ZB, WM, and ZY performed all of the experiments; WT, YJ, MY, WQ, CD, LT and LY participated in the research; ZB and MW designed experiments, analyzed data, and wrote the paper; YM is the supervisor of ZB; HZ and WH collected the clinical samples. All authors read and approved the final manuscript.

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Table 1. Association of thrombin expression with the clinicopathologic characteristics of 132 NSCLC patients

Figure 1 The expression of thrombin in lung cancer is closely related to clinicopathological features and the prognosis of patients.(a-c) Thrombin expression in NSCLC patients. **(a)**Paraffin sections obtained from patients with resectable NSCLC tissues were stained for thrombin. (**b)** Score of thrombin expression in adjacent non-tumor lung tissue and in NSCLCs. (**c)** Thrombin expression in different types of NSCLC. (**d)** The overall survival rates of thrombin-positive patients and thrombin-negative patients. (**e)**The mRNA level of thrombin in BEAS-2B, A549, 95D, and PC9 was determined by Q-PCR. (**f)** The expression of thrombin in BEAS-2B, A549, 95D, and PC9 was determined by western blotting analysis. Right, Summary data of western blotting were given. All the results were expressed as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS, not significant.

Figure 2Thrombin plays an important role in the progression of lung cancer. (**a)** Western blot depicting depletion of thrombin in A549 cells transfected with human thrombin gRNA compared with negative control. (**b)** Representative pictures of A549 cells migrated through the transwell are shown,

Right, quantitative analysis of invasive cells. (**c**) Western blot depicting depletion of thrombin in Lewis cells transfected with mouse thrombin gRNA compared with negative control. (**d**) Representative pictures of Lewis cells migrated through the transwell are shown, Right, quantitative analysis of invasive cells. (**e-i**)Thrombin deficient LLC cells and control cells were injected subcutaneously into the right flank of mice. (**e**) Serial calipation of tumor volume after transplantation cells into mice. **(f)** Mice were humanely euthanized, and the tumors were resected 35 days after cell injection.**(g)** The weight of resected tumors was determined. (**h**)H&E stained sections of lung tissue. (**i**) The number of metastatic nodes per lung was determined. **(j-l)** In orthotopic lung tumor model, 1 x 10^6 cells in 50 μL RPMI-1640 medium and 50 μL Matrigel were injected into the left lung parenchyma through the left rib cage. (**j**)Survival rate of mice in different groups. (**k**) Representative photograph of lungs bearing tumors (left); Gross appearance of representative lung harvested viewed under a fluorescence stereoscope (middle); H&E stained sections of lung tissue (right). (**l**) Average tumor area in the lungs was measured. **(m)**Number of mice with tumor cells in liver. All the results were expressed as mean ± SD. ANOVA followed by Dunnett's test was applied for multiple comparison. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS, not significant.

Figure 3 r-hirudin and DTIP inhibit thrombin-promoted cell migration, invasion and angiogenesis in vitro. (**a**)A549 and Lewis cells were pretreated with PBS, 10 nmol/L thrombin, 25 nmol/L r-hirudin, 50 nmol/L DTIP, 10 nmol/L thrombin + 25 nmol/L r-hirudin, 10 nmol/L thrombin + 50 nmol/L DTIP for 24 hours. The migration of r-hirudin and DTIP treated A549 and Lewis cells were assessed using wound healing assay. Right, quantitative analysis of wound area. (**b**) Transwell assay was performed to assess cell migration of A549 and Lewis cells. Right, quantitative analysis of invasive cells. (**c**) Endogenous GTP-bound form of RhoA was enriched by a pull-down assay and detected by Western blotting. Total RhoA was detected using anti-RhoA antibody. Bottom, summary data of western blotting were given. (**d**)Representative images of each group stained with phalloidin in A549 cells. (**e**)Quantification of the F-actin fluorescence intensity. (**f**)Percentages of ruffle-positive cells in different groups were calculated based on the immunofluorescence. (**g**) Expression of IL6 and MMP9 in different groups. (**h**) Effect of r-hirudin and DTIP against HUVEC tube formation on Matrigel. Left, representative photographs of five independent experiments were shown. Right, quantification of the inhibition activity of aspirin on tube formation. All the results were expressed as mean +- SD. ANOVA followed by Dunnett's test was applied for multiple comparison. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS, not significant.

Figure 4 r-hirudin and DTIP exert the ability of Anti-growth and anti-metastasis in orthotopic lung tumor model and lung cancer metastasis model.(a-c) In orthotopic lung tumor model, 1 x 10^6 cells were injected into the left lung through the left rib cage, and 1.0 mg/kg DTIP or 0.5 mg/kg r-hirudin was administered for 7 consecutive days after 3 days of the injection of LLC cells. (**a**)Survival of mice treated with saline, r-hirudin and DTIP after injection of LLC cells into the left lung. (**b**)Representative photograph of lungs (left); Gross appearance of representative lung harvested viewed under a fluorescence stereoscope (middle); H&E stained sections of lung tissue (right). (**c**) Number of NS, r-hirudin, and DTIP treated mice with liver metastasis. **(d-j)**Tail-vein injection of $1x10^6$ LLC cells was performed in mice. The LLC were engineered to express red fluorescent protein to simplify detection of tumor foci in vivo. Mice that were injected with LLC were immediately treated with saline, 1.0 mg/kg DTIP or 0.5 mg/kg r-hirudin once a day for one week. We randomly divided the mice into two groups, one was sacrificed on the 14th day to observe the metastasis of tumors, and the other group was used to analyze the survival rate of mice. (**d**)Effect of r-hirudin and DTIP on the formation of metastatic nodes on day 14. Representative photograph of metastatic nodules on lungs (upper). Gross appearance of representative lung harvested from different groups viewed under a fluorescence stereoscope (bottom). (**e**)The number of metastatic nodes per lung was determined in different groups. (**f**) Representative micro-PET images of mice 14 days after injection. (**g**)H&E stained sections of lung tissue. (**h**)Survival of mice treated with saline, r-hirudin and DTIP after injection of LLC cells via tail-vein. (**i**)H&E stained sections of liver tissue. (**j**) Number of NS, r-hirudin, and DTIP treated mice with tumor cells in liver. All the results were expressed as mean +- SD. Compared with NS group by one-way ANOVA. $*p < 0.05$.

Figure 5 r-hirudin and DTIP could inhibit the progression of tumor. LLC cells at a density of

$1x10^6$ in 0.1mL serum-free media were injected subcutaneously into the right flank of mice. (**a-d)** Mice that were injected with LLC cells were immediately treated with normal saline, 0.5 mg/kg r-hirudin or 1.0 mg/kg DTIP for one week. (**a**)The curve of tumor growth after injection of LLC cells. (**b)** In vivo imaging of each group at 4 weeks after cell injection. (**c)** H&E stained sections of tumor. (**d)** Number of NS, r-hirudin and DTIP treated mice with panniculus invasion. (**e-n)** After one week of the injection of LLC cells, mice were administered with normal saline, 1.0 mg/kg DTIP or 0.5 mg/kg r-hirudin for 21 consecutive days. (**e)** The curve of tumor growth after injection of LLC cells. (**f)** Weight of resected tumors driven from normal saline-, r-hirudin-, and DTIP-treated groups. (**g)** In vivo imaging of each group at 45 days after cell injection. (**h**)Representative photograph of metastatic nodules on lungs (upper). Gross appearance of representative lung harvested from different groups viewed under a fluorescence stereoscope (bottom) (**i)** Number of NS, r-hirudin and DTIP treated mice with lung metastasis. (**j)** Representative photograph of metastatic nodules on livers (upper). Gross appearance of representative single liver lobes harvested from different groups viewed under a fluorescence stereoscope (bottom). (**k)** Number of NS, r-hirudin and DTIP treated mice with liver metastasis. (**l)** Effect of r-hirudin and DTIP against primary tumor angiogenesis. Left, a typical photograph of immunohistochemical staining of CD31. Right, the histogram represents the number of microvessels. (**m)** Immunohistochemical analysis was performed on tumor samples to determine the expression of MMP9 and IL6 on tumors from NS, r-hirudin and DTIP treated mice. (**n)**Inhibition of phosphorylation of p65, Erk, STAT3, and Akt in tumors by r-hirudin and DTIP. Right, summary data of western blotting. All the results were expressed as mean +- SD. Compared with NS group by one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 6 PAR-1 is a major determinant in thrombin-promoted progression of lung cancer.(**a**)Western blot depicting depletion of PAR-1 in A549 cells transfected with human PAR-1 gRNA compared with negative control. (**b**)Representative pictures of A549 cells migrated through the transwell are shown, Right, quantitative analysis of invasive cells. (**c**)Western blot depicting depletion of PAR-1 in Lewis cells transfected with mouse PAR-1 gRNA compared with negative control. (**d**)Representative pictures of Lewis cells migrated through the transwell are shown, Right, quantitative analysis of invasive cells. (**e**)GTP-bound form of RhoA was enriched by a pull-down assay and detected by Western blotting. Total RhoA was detected using anti-RhoA antibody. (**f)** Representative images of each group stained with phalloidin. Right, Measurement of ruffles-positive cells infected with the indicated lentivirus. (**g-i)** 1 x 10^6PAR-1 deficient LLC cells and control cells were injected into the left lung through the left rib cage. (**g)** Survival of mice in different groups. (**h)** Representative photograph and H&E stained sections of lung tissues. (**i)** Average tumor area in total lungs. (**j-l)** Tumor cells were injected subcutaneously into the right flank of mice. (**j**)Serial calipation of tumor volume after transplantation of $LLC^{Par\text{-}1\text{-}/\text{-}}$ and LLC^{NC} cells into mice. (**k)** In vivo imaging of each group at 5 weeks after cell injection. (**l**)Representative images of anti-CD31 staining in tumor tissues. Right, the histogram represents the number of microvessels. All the results were expressed as mean +- SD. ANOVA followed by Dunnett's test was applied for multiple comparison. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS, not significant.

Figure 7Combination therapy of DTIP and chemotherapy results in improved anti-tumor efficacy.(a-f)$1x10^6$LLC cells were subcutaneously injected in the right dorsal region of mice. One week after LLC inoculation, the mice were randomly divided into four groups, normal saline, DTIP (1 mg/kg per day, s.c.), gemcitabine (120 mg/kg once a week; i.p.), and combination DTIP with gemcitabine treatment groups, and then the mice were administered normal saline or DTIP for 21 consecutive days, gemcitabine for 4 consecutive weeks. (**a)** The curve of tumor growth after injection of LLC cells. (**b**)Mice were humanely euthanized and the tumors were resected at 45 days after cell injection. (**c)** Weight of resected tumors was determined in different groups. (**d)** Representative photograph of metastatic nodules on lungs (upper). H&E stained sections of representative single lung lobes harvested (bottom). (**e**)Number of mice with liver metastasis. (**f)** Survival rate of mice in different groups. (**g-k**)$1x10^6$LLC cells were subcutaneously injected in the right dorsal region of mice. One week after LLC inoculation, the mice were randomly divided into four groups, normal saline, DTIP (1 mg/kg per day, s.c.), paclitaxel (20 mg/kg every two days; i.p.), and combination DTIP with paclitaxel treatment groups, and then the mice were administered normal saline

or DTIP for 21 consecutive days, paclitaxel for 4 consecutive weeks. (**g**) The curve of tumor growth after injection of LLC cells. (**h**) Mice were humanely euthanized and the tumors were resected at 42 days after cell injection. (**i**) Weight of resected tumors was determined in different groups.(**j**)H&E stained sections of representative single lung lobes harvested.(**k**) The number of pulmonary foci in lungs. All the results were expressed as mean +- SD. ANOVA followed by Dunnett's test was applied for multiple comparison. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS, not significant. Gemcitabine: Gem; paclitaxel: PTX

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