Small-animal SPECT/CT imaging of cancer xenografts and pulmonary fibrosis using a $^{99m}$Tc-labeled integrin αvβ6-targeting cyclic peptide with improved in vivo stability

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Abstract

Integrin αvβ6 is expressed at an undetectable level in normal tissues, but is remarkably upregulated during many pathological processes, especially in cancer and fibrosis. Noninvasive imaging of integrin αvβ6 expression using a radiotracer with favorable in vivo pharmacokinetics would facilitate disease diagnosis and therapy monitoring. Through disulfide-cyclized method, we synthesized in this study, a new integrin αvβ6-targeted cyclic peptide (denoted as cHK), and radiolabeled it with $^{99m}$Tc. The ability of the resulting radiotracer $^{99m}$Tc-HYNIC-cHK to detect integrin αvβ6 expression in pancreatic cancer xenografts and idiopathic pulmonary fibrosis was evaluated using small-animal single-photon emission...
Computational tomography (SPECT)/computed tomography (CT). $^{99m}$Tc–HYNIC–cHK showed significantly improved in vivo metabolic stability compared to the linear peptide-based radiotracer $^{99m}$Tc–HYNIC–HK. $^{99m}$Tc–HYNIC–cHK exhibited similar biodistribution properties to $^{99m}$Tc–HYNIC–HK, but the tumor-to-muscle ratio was significantly increased (2.99 ± 0.87 vs. 1.82 ± 0.27, P < 0.05). High-contrast images of integrin $\alpha v \beta 6$-positive tumors and bleomycin-induced fibrotic lungs were obtained by SPECT/CT imaging using $^{99m}$Tc–HYNIC–cHK. Overall, our studies demonstrate that $^{99m}$Tc–HYNIC–cHK is a promising SPECT radiotracer for the noninvasive imaging of integrin $\alpha v \beta 6$ in living subjects.

**Keywords** Integrin $\alpha v \beta 6$, Pancreatic cancer, Pulmonary fibrosis, Molecular imaging, Peptide cyclization, Single-photon emission computed tomography (SPECT)

**INTRODUCTION**

Integrin $\alpha v \beta 6$, a member of the integrin family, is present at undetectable levels in adult differentiated tissues, but is overexpressed during embryogenesis, tumorigenesis, and tissue injury (Breuss et al. 1993; Desgrozelle and Cheresh 2010; Peng et al. 2016). Increased expression of integrin $\alpha v \beta 6$ usually correlates with more aggressive disease and poor prognosis (Bates and Mercurio 2005; Elayadi et al. 2007; Lee et al. 2006), and the upregulation of integrin $\alpha v \beta 6$ expression in a wide variety of cancers is associated with increased tumor cells migration, invasion, and metastasis (Bates 2005; Bates and Mercurio 2005). In addition to cancer, de novo or increased expression of integrin $\alpha v \beta 6$ has also been observed in pathological process of fibrosis (Patsenker et al. 2010; Penning 2010; Peng et al. 2016). Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrotic lung disease with a poor prognosis (Gribbin et al. 2006; Navaratnam et al. 2011; Wells 2013). Integrin $\alpha v \beta 6$-mediated transforming growth factor (TGF)-$\beta$ activation has been implicated in multiple models of lung fibrosis, and the upregulated expression of integrin $\alpha v \beta 6$ has also been found in patients with IPF (Horan et al. 2008; Xu et al. 2009). Importantly, the expression of integrin $\alpha v \beta 6$ is temporally and spatially associated with the course of the fibrosis progression (Puthawala et al. 2008).

Due to the critical role of integrin $\alpha v \beta 6$ in tumorigenesis and fibrogenesis, it has emerged as an appealing target for diagnostic imaging, prognosis evaluation, and therapeutic responses monitoring (Agarwal 2014; Cantor et al. 2015; Saini et al. 2015; Yang et al. 2015). Therefore, noninvasive and quantitative imaging of integrin $\alpha v \beta 6$ expression by molecular imaging techniques would be of great potential for better management of these diseases. Previous studies have focused on the development of positron emission tomography (PET) and single-photon emission computed tomography (SPECT) radiotracers for in vivo imaging of integrin $\alpha v \beta 6$ expression (Hackel et al. 2013; Hausner et al. 2007, 2008, 2009a, b, 2013; Hu et al. 2014; John et al. 2013; Kimura et al. 2012; Li et al. 2011; Liu et al. 2014b; Man et al. 2013; Nonthefer et al. 2009; Saha et al. 2010; Satpati et al. 2014; Singh et al. 2014; Ueda et al. 2014; Zhu et al. 2014). Although promising, most of these radiotracers are based on linear peptides, which have poor in vivo metabolic stability and suboptimal pharmacokinetics. For example, we observed recently that a $^{99m}$Tc-labeled linear peptide (RGDLATRLQA-QEDGQVGRK, the HK peptide) completely degraded in vivo within 30 min after injection, leading to a very low tumor uptake and tumor-to-nontumor ratios (Liu et al. 2014b).

Peptide cyclization has been reported to be a powerful strategy to improve the stability of peptides by means of adopted resistance to enzymatic degradation (Besser et al. 2000; Bogdanowich-Knipp et al. 1999a, b; Gilon et al. 1991; Pakkala et al. 2007; Roxin and Zheng 2012; Shi et al. 2016). To overcome the limitations of $^{99m}$Tc-labeled HK peptide, in this study, we selected the first seven amino acid residues of the integrin $\alpha v \beta 6$-targeting HK peptide and added a lysine residue at C-terminal in order to conjugate the chelator. We then cyclized it by adding a cysteine residue at N- and C-terminals, respectively, to generate a new peptide (CRGDLATLQAL(2-sulfonatobenzaldehyde) hydrazono) nicotinate (HYNIC)-NHS and then radiolabeled with $^{99m}$Tc. The resulting radiotracer $^{99m}$Tc–HYNIC–cHK was evaluated in vivo as a SPECT radiotracer for imaging of integrin $\alpha v \beta 6$ expression in both cancer and IPF mouse models.

**RESULTS**

**Chemistry and radiochemistry**

The Fmoc-cHK–HYNIC conjugate (Fig. 1A) was prepared by direct conjugation of Fmoc-cHK peptide with
HYNIC-NHS. After the removal of Fmoc group, the final product HYNIC–cHK was confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry. The HPLC purity of HYNIC–cHK was >95% before being used for 99mTc radiolabeling. The 99mTc-labeling procedure was done within 30 min with a yield ranging from 85% to 90%. The radiochemical purity was >95% after purification, and the specific activity was >30 MBq/nmol.

**Cell-binding assay**

Similar to the HK peptide, cHK peptide also inhibited the binding of 125I–RGDLATLRQEDGVGVRYK (HYK) to integrin αvβ6-expressing BxPC-3 cells in a concentration-dependent manner, but the integrin αvβ6 binding affinity of cHK was lower compared to that of HK peptide. The IC\textsubscript{50} values for cHK and HK were 20.25 ± 1.17 and 3.55 ± 0.09 nmol/L, respectively (Fig. 1B).

The binding specificity of 99mTc–HYNIC–cHK to BxPC-3 cells was significantly inhibited by the addition of excess doses of the cHK and HK peptides (from 0.79 ± 0.01 %AD to 0.03 ± 0.005 and 0.06 ± 0.007 %AD, respectively, P < 0.001).

**Solution and metabolic stability**

The *in vitro* solution stability of 99mTc–HYNIC–cHK in fetal bovine serum (FBS) or L-cysteine was monitored by radio-HPLC. Figure 2A shows that 99mTc–HYNIC–cHK remains stable for more than 4 h both in FBS and in the presence of L-cysteine.

We performed the metabolism studies of 99mTc–HYNIC–cHK using normal BALB/c mice. We analyzed the samples from both blood and urine to determine whether the radiotracer retains its chemical integrity at 0.5 h and 1 h postinjection. Figures 2B–F illustrate the radio-HPLC chromatograms of 99mTc–HYNIC–cHK before injection (Fig. 2B), in the blood (Fig. 2C, E) and in the urine (Fig. 2D, F). 99mTc–HYNIC–cHK retained its integrity in urine, while showing the degrees of metabolism to be 9.94% and
30.09% in blood at 0.5 h and 1 h postinjection, respectively. Compared to the linear peptide-based radiotracer $^{99m}$Tc–HYNIC–HK (Liu et al. 2014b), $^{99m}$Tc–HYNIC–cHK is much more stable in vivo.

**Biodistribution**

As shown in Fig. 3A, the uptake values of $^{99m}$Tc–HYNIC–cHK in BxPC-3 tumors were 0.63 ± 0.18, 0.43 ± 0.09, and 0.33 ± 0.16 %ID/g at 0.5, 1, and 2 h after injection, respectively. The tumor uptake of $^{99m}$Tc–HYNIC–cHK was higher than that in most of the normal organs, such as heart, liver, pancreas, bone, and muscle, at almost all time points examined ($P < 0.05$). The tumor uptake of $^{99m}$Tc–HYNIC–cHK was significantly reduced with a coinjection of an excess dose of the cold HK peptide at 1 h after injection (from 0.43 ± 0.09 to 0.24 ± 0.04 %ID/g, $n = 4$, $P < 0.01$).

The uptake of $^{99m}$Tc–HYNIC–cHK was similar to $^{99m}$Tc–HYNIC–HK in BxPC-3 tumors at 0.5 h after injection (0.63 ± 0.18 vs. 0.58 ± 0.09, $n = 4$, $P > 0.05$). However, the uptake of $^{99m}$Tc–HYNIC–cHK in muscle or bone was much lower, and the tumor-to-muscle (T/M) ratio was significantly higher than that of $^{99m}$Tc–HHK (2.99 ± 0.87 vs. 1.82 ± 0.27, $n = 4$, $P < 0.05$; Fig. 3B).

**SPECT imaging of BxPC-3 cancer xenografts**

Representative small-animal SPECT/CT images of BxPC-3 tumor-bearing mice at 0.5 h and 1 h after intravenous injection of $^{99m}$Tc–HYNIC–cHK are shown in Fig. 4A. The radiotracer showed clear tumor imaging with high contrast to the contralateral background. The in vivo receptor-binding property of $^{99m}$Tc–HYNIC–cHK was determined by the blocking study. The tumor uptake of $^{99m}$Tc–HYNIC–cHK was almost completely inhibited in the HK blocking group ($P < 0.001$; Fig. 4B, C).

**SPECT/CT imaging of bleomycin-induced pulmonary fibrosis**

As shown in Fig. 5A, markedly gray regions were observed by CT imaging in the lung areas of mice in the bleomycin (BLM)-treated mice, suggesting the evident fibrosis formation induced by BLM. Notably, an evident accumulation of $^{99m}$Tc–HYNIC–cHK in the lungs of the BLM-treated mice was observed. In contrast, no
significant uptake of $^{99m}$Tc–HYNIC–cHK was observed in the phosphate-buffered saline (PBS)-treated mice (Fig. 5A, B). After the SPECT/CT imaging, the mice were sacrificed, and the presence of fibrosis in the edge of pulmonary lobes were verified by anatomic visualization after dissection in the BLM group. The hematoxylin–eosin (H&E) and Sirius red (specific for collagen) staining further confirmed the SPECT/CT findings (Fig. 5C).

**DISCUSSION**

Overexpression of integrin $\alpha\beta_6$ has been found in approximately 100% of pancreatic cancers (Liu et al. 2014a). Integrin $\alpha\beta_6$-targeted imaging for pancreatic cancer detection and staging would contribute to improve the prospect of curing or controlling pancreatic cancer. We previously synthesized a linear peptide-based SPECT radiotracer ($^{99m}$Tc–HYNIC–HK) and demonstrated its potential for the specific detection of subcutaneous pancreatic tumor xenografts and liver metastases in mouse models (Liu et al. 2014b). However, $^{99m}$Tc–HYNIC–HK had a very poor in vivo stability, which may significantly hamper its potential clinical translation. There are several approaches to improve the in vivo stability of peptides, including changing some amino acids of the peptide into D-amino acids, cyclizing the peptide to be a cyclic peptide, or engineering the peptide into scaffold-based peptides, such as cysteine knot (Zhu et al. 2014). In this study, we synthesized a cyclic peptide based on the HK peptide, and
radiolabeled it with $^{99m}$Tc, the resulting radiotracer $^{99m}$Tc–HYNIC–cHK was evaluated both in vitro and in vivo. Through the in vitro solution stability study, $^{99m}$Tc–HYNIC–cHK was demonstrated to be rather stable in FBS or l-cysteine over 4 h. The in vivo metabolic study indicated that the stability in blood was considerably improved after cyclization (Fig. 2). Afterward, the integrin $\alpha_v\beta_6$-targeting ability of $^{99m}$Tc–HYNIC–cHK was evaluated through cell-binding assays in integrin $\alpha_v\beta_6$-positive BxPC-3 cells. Similar to the HK peptide, cHK could also inhibit the binding of $^{125}$I–HYK on BxPC-3 cells in a dose-dependent manner. However, the binding affinity of cHK to integrin $\alpha_v\beta_6$ was slightly lower than that of the HK peptide. The decreased affinity may result from the shortened peptide sequence and constrained conformation of the cyclic peptide compared to the linear peptide. $^{99m}$Tc–HYNIC–cHK retains the integrin $\alpha_v\beta_6$-targeting capability as evidenced by the significantly inhibited binding by adding an excess of cold cHK or HK peptide (Fig. 1C).

The in vivo integrin $\alpha_v\beta_6$-targeting specificity of $^{99m}$Tc–HYNIC–cHK was confirmed by the biodistribution and SPECT/CT imaging studies in the BxPC-3 xenograft tumors. $^{99m}$Tc–HYNIC–cHK exhibited rapid tumor accumulation and showed the maximum tumor-uptake values at 0.5 h after injection (Fig. 3A). Predominant kidney uptake of $^{99m}$Tc–HYNIC–cHK was also observed, most likely due to the renal clearance of this radiotracer. The absolute tumor uptake of $^{99m}$Tc–HYNIC–cHK was comparable to that of $^{99m}$Tc–HHK at 0.5 h (Fig. 3). However, the tumor-to-muscle ratio was significantly higher for $^{99m}$Tc–HYNIC–cHK compared to that of $^{99m}$Tc–HHK, resulting in a favorable tumor imaging contrast.

In addition to cancer, de novo and increased expression of integrin $\alpha_v\beta_6$ also occur during fibrogenesis. The increased expression of integrin $\alpha_v\beta_6$ has been found in fibrotic lung tissue in patients with IPF and was demonstrated to play an important role in the progression of lung fibrotic disease in several different studies (Horan et al. 2008; Puthawala et al. 2008; Xu et al. 2009). To date, the only approach to detect the expression of integrin $\alpha_v\beta_6$ in the fibrotic lung is immunohistochemical analysis of biopsy samples (Raghu et al. 2011). This procedure is clinically impractical for many patients and suffers from sampling bias, resulting in incomplete information. Considering the high short-term mortality following lung biopsy (Utz et al. 2001), repeated sampling is unrealistic. Hence, noninvasive molecular imaging of integrin $\alpha_v\beta_6$ expression would offer a remarkable improvement for immunophenotyping patients with IPF. $^{18}$F–FDG and $^{68}$Ga-labeled somatostatin analogs targeting somatostatin receptor as PET tracers have been used for IPF.
stratification, but neither of these radiotracers targets well-validated pathways implicated in IPF (Ambrosini et al. 2010; Win et al. 2012). Cai et al. recently developed an optical activatable probe for noninvasive diagnosis of IPF by targeting matrix metalloproteinase type 2 (MMP-2), which was also correlated with IPF development. However, MMP-2 could be secreted into the bloodstream, the nonspecific fluorescence signal recovery in tissues (e.g. liver) other than lung was noticed over time (Cai et al. 2013). John et al. developed an 111In-labeled αvβ6-specific peptide (111In–DTPA–A20FMDV2) as a SPECT radiotracer and for the first time used it for noninvasive measurement of integrin αvβ6 expression in lungs of mice with BLM-induced fibrosis (John et al. 2013). Their results showed that the lung uptake of 111In–DTPA–A20FMDV2 is quantifiable and correlates with the levels of αvβ6 protein, itgb6 messenger RNA, and hydroxyproline in the lungs.

The low uptake of 99mTc–HYNIC–cHK in normal lungs makes it a potential tool for quantitative and global analyses of integrin αvβ6 expression with high sensitivity in imaging the lung disorders. In the murine model of pulmonary fibrosis induced by BLM, a significant accumulating of 99mTc–HYNIC–cHK in lungs of BLM-treated mice was observed, compared with the PBS group (Fig. 5A, B). The H&E and Sirius red staining confirmed the lung fibrotic lesions in the lungs of BLM-treated mice (Fig. 5C). Compared with 111In, 99mTc is more suitable for labeling peptide-based probes because that the radioactive half-life of 99mTc matches the metabolic half-life of peptides. Moreover, 99mTc-labeled radiotracer is more widely available and cost effective. The high labeling yield of 99mTc chelator systems also allows the formulation of kits for the rapid preparation of radiotracers for widespread applications.

Although the metabolic stability of 99mTc–HYNIC–cHK after cyclization was significant improved compared to the linear radiotracer 99mTc–HYNIC–HK, the receptor-binding affinity of 99mTc–HYNIC–cHK was slightly decreased. In order to increase the receptor-binding affinity and further improve the in vivo pharmacokinetics of 99mTc–HYNIC–cHK, efforts such as polyethylene glycol (PEG)ylation and multimerization may be required to further optimize this radiotracer.

Fig. 5 A Representative SPECT/CT images of 99mTc–HYNIC–cHK in BLM-treated and PBS-treated C57/BL6 mice at 0.5 h after injection. B Quantitation of lung uptakes of 99mTc–HYNIC–cHK in BLM-treated and PBS-treated C57/BL6 mice from the SPECT scanning, **P < 0.001. C Gross observation of the lungs, and H&E and Sirius red staining of the lung tissues from BLM-treated and PBS-treated C57/BL6 mice.
CONCLUSION

A cyclic peptide-based radiotracer ⁹⁹mTc–HYNIC–cHK with improved in vivo metabolic stability was prepared and evaluated both in vitro and in vivo. ⁹⁹mTc–HYNIC–cHK exhibited specific integrin αvβ6-targeting ability and was demonstrated to specific detection of integrin αvβ6 expression in subcutaneous pancreatic cancer xenografts and pulmonary fibrosis in animal models. Further optimization of ⁹⁹mTc–HYNIC–cHK may eventually yield a clinical applicable radiotracer for SPECT imaging of integrin αvβ6 expression, disease staging, and monitoring of therapy efficacy.

EXPERIMENTAL SECTION

Materials and reagents

All commercially available chemical reagents were used without further purification unless otherwise stated. The peptides Fmoc-cHK, HYK and HK were synthesized by ChinaPeptide Co., Ltd (Shanghai, China). Na⁹⁹mTcO₄ was obtained from a commercial ⁹⁹Mo/⁹⁹mTc generator (Beijing Atom High Tech Co., Beijing, China). The reversed-phase high-performance liquid chromatography (HPLC) system was Agilent Technologies 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA) coupled with the Raytest Gabi radioactivity detector (Raytest, Straubenhardt, Germany). The female BALB/c nude mice (4–5 weeks of age), BALB/c normal mice (4–5 weeks of age), and C57BL6 mice (7–8 weeks of age) were purchased from Department of Laboratory Animal Science, Peking University Health Science Center (Beijing, China). BLM was purchased from Aladdin (Shanghai, China).

Synthesis of HYNIC-conjugated cHK peptide

The Fmoc-cHK peptide was conjugated with HYNIC-NHS using a standard procedure. Briefly, a solution of Fmoc-cHK was mixed with HYNIC-NHS at a mole ratio of 1:1.2. The pH was adjusted to 8.5–9.0 using N,N-Diisopropylethylamine. After stirring for 4 h at room temperature, the Fmoc was removed by adding piperidine with a final volume fraction of 20%. The HYNIC-cHK was isolated by semi-preparative HPLC and lyophilized to afford the final product as a white powder (yield: 56%). Analytical HPLC (Retention time = 14.99 min) and mass spectrometry (MALDI-TOF–MS: m/z, 1380.66 for [MH]+ (C₅₀H₈₅N₁₇O₁₈S₃, calculated molecular weight 1380.57) confirmed the identity of the product.

Preparation of ⁹⁹mTc–HYNIC–cHK

For ⁹⁹mTc labeling, a mixture of 20 mg tricine (100 mg/mL in 25 mmol/L succinate buffer, pH 5.0), 740 MBq (20 mCi) Na⁹⁹mTcO₄, and 30 μl SnCl₂ (1 μg/μL in 0.1 mol/L HCl) was successively added to 20 μg of HYNIC–cHK with constant stirring at 99 °C for 10 min. Then 100 μl of ethylenediamine-N,N'-diacetic acid (100 mg/mL) was added to the mixture with constant stirring at 99 °C for 20 min. After allowing it to cool down to room temperature, the mixture was purified with Sep-Pak C18 cartridges (Waters) as previously described (Jia et al. 2006).

Cell culture and animal models

The BxPC-3 human pancreatic cancer cell line was obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 °C in humidified atmosphere containing 5% CO₂.

All animal experiments were performed in accordance with the guidelines of Peking University Animal Care and Use Committee. To establish the BxPC-3 subcutaneous tumor model, BxPC-3 cells (1 × 10⁷ in 100 μl of PBS) were inoculated subcutaneously into the right front flanks of female BALB/c nude mice. The animals were used for in vivo studies when the tumor size reached 200–300 mm³ (3–4 weeks after inoculation). For the pulmonary fibrosis mouse model, BLM (1.5 units/kg, 50 μl in PBS) or PBS (50 μl; as a vehicle control) was administered once into the C57/BL6 mice by intratracheal injection. On day 21 (based on pilot studies), the mice with well-established pulmonary fibrosis were used for SPECT/CT imaging.

Integrin αvβ6 binding specificity

In vitro integrin αvβ6 binding affinities of cHK and HK were compared via displacement cell-binding assays (Dong et al. 2015) using ¹²⁵I–HYK as the radioligand. ¹²⁵I–HYK was prepared by labeling HYK with Na¹²⁵I using the iodogen method as previously reported (Liu et al. 2010). Experiments were performed on high integrin αvβ6-expressing BxPC-3 cells. The best-fit 50% inhibitory concentration (IC₅₀) values were calculated by fitting the data with nonlinear regression using Graph-Pad Prism (GraphPad Software, Inc.).

In vitro integrin αvβ6 binding specificity of ⁹⁹mTc–HYNIC–cHK was tested using the integrin αvβ6-positive BxPC-3 cells. Briefly, cells were seeded into 12-well plates and incubated overnight at 37 °C to allow adherence. After brief washing with PBS, tumor cells
were incubated with 3.7 kBq $^{99m}$Tc–HYNIC–cHK with or without an excess dose of cold cHK or HK peptide at 4 °C for 4 h. Tumor cells were then washed with chilled PBS and harvested by trypsinization with 0.05% trypsin. The cell suspensions were collected and measured in a $\gamma$-counter (Wallac 1470-002, Perkin-Elmer, Finland). The cell uptake was expressed as the percent added dose (%AD). Experiments were performed twice with triplicate samples.

**Solution and metabolic stability**

$^{99m}$Tc–HYNIC–cHK was incubated in FBS or l-cysteine (1.0 mg/mL) for 0, 0.5, 1, 2, and 4 h at 37 °C to test the *in vitro* solution stability. After passing through a 0.22-μm Millipore filter, the samples were analyzed by radio-HPLC.

The metabolic stability of $^{99m}$Tc–HYNIC–cHK was evaluated in female BALB/c normal mice. Each mouse was administered with the radiotracer at a dose of 1 mCi in 100 μL saline via intravenous injection. At 0.5 h and 1 h postinjection, the blood and urine samples were collected. The samples were centrifuged at 8000 r/min for 15 min. The supernatant was collected, filtered through a 0.22-μm Millipore filter, and then analyzed by radio-HPLC.

**Biodistribution**

Biodistribution studies were performed using female BALB/c nude mice bearing BxPC-3 xenografts. Mice received an injection via the tail vein of 370 kBq (10 μCi) of $^{99m}$Tc–HYNIC–cHK to evaluate the distribution of the radiotracer. The blocking experiments were also performed by coinjection of $^{99m}$Tc–HYNIC–cHK with a saturating dose of the HK peptide (500 μg per mouse). At 0.5, 1, and 2 h after injection, the animals were sacrificed, and the tumors and the organs/tissues of interest were dissected and wet-weighed, and the radioactivity in the tissue was measured using a $\gamma$-counter. The results are presented as percentages of injected dose per gram of tissue (%ID/g). Values are expressed as mean ± SD ($n = 4$ per group).

**Small-animal SPECT/CT imaging**

Small-animal SPECT/CT scans of subcutaneous BxPC-3 tumor and pulmonary fibrosis mouse models were performed using a SPECT/CT system (NanoScan; Mediso, Budapest, Hungary). Each BxPC-3-bearing mouse was injected via tail vein with 37 MBq (1 mCi) of $^{99m}$Tc–HYNIC–cHK. At 0.5 h and 1 h after injection, the mice were anesthetized by inhalation of 2% isoflurane and imaged using the Nano-SPECT/CT camera. The SPECT and CT fusion images were obtained using the automatic fusion software (InterView Fusion; Mediso Medical Imaging Systems, Budapest, Hungary).

Each BLM- or PBS-treated mouse was administered with 37 MBq of $^{99m}$Tc–HYNIC–cHK via tail vein. After SPECT/CT imaging, the BLM- and PBS-treated mice were euthanized. Lungs were excised and fixed in 5% buffered formalin, embedded in paraffin, and cut into sections for staining with H&E or Sirius red as previously described (Yu *et al.* 2016).

**Statistical analysis**

Quantitative data were expressed as mean ± SD. Results were compared using the Student *t* test. *P* values of less than 0.05 were considered statistically significant.

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**Compliance with ethics standards**

**Conflict of interest** Hao Liu, Liquan Gao, Xinhe Yu, Lijun Zhong, Jiyun Shi, Bing Jia, Nan Li, Zhaofei Liu, and Fan Wang declare that they have no conflicts of interest.

**Human and animal rights and informed consent** All institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any studies with human subjects performed by any of the authors.

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