

In Vivo Target Analysis by MRI in a Murine Model of Pulmonary Fibrosis

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Introduction:

Pulmonary fibrosis is a progressive and lethal lung disease that results as a consequence of an overexuberant repair process, characterized by an accumulation of inflammatory cells, excessive fibroblast proliferation, an increase in collagen content, and deposition of extracellular matrix in the lungs [1]. Currently no effective treatment exists for lung fibrosis. Animal models are important to investigate pathological mechanisms and for preclinical evaluation of novel targets and therapies. Local instillation of bleomycin in small rodents is the most used model of lung fibrosis [2]. In the present work MRI has been applied to non-invasively investigate two knockout mouse lines in the bleomycin model with the aim of providing potential therapeutic targets to intervene in the disease development. The rationale for the analysis of cancer Osaka thyroid (COT) kinase deficient mice is the fact that COT regulates the production of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) [3], which have been shown to be implicated in fibrosis [4] and wound healing [5]. Cadherin-11 knockout mice were investigated in the bleomycin model because it has been shown that cadherin-11 is overexpressed in human renal tubular cells during epithelial to mesenchymal transition (EMT) [6], a process that can adversely cause organ fibrosis [7], and because EMT has been reported to occur in bleomycin-induced pulmonary fibrosis [8].

Materials and Methods:

Animal handling, care, and experimental use were in line with the Swiss Federal Law for animal protection (license BS No. 1989).

Animals: Seven- to 9-week-old C57BL/6 (male and female) mice (Charles River, L'Arbresle, France; ~20 g body weight) were used. COT-kinase deficient mice were bred at Charles River Europe (Kisslegg, Germany). A partial deletion of exons 2 and 3 of COT kinase was introduced in a 129SV mouse background. This mutation deletes the ATP binding pocket. Animals were backcrossed on a C57BL/6 background over 11 generations [9]. Cadherin-11 knockout mice were bred in-house following a description by Horikawa et al [10]. COT kinase and cadherin-11 mice were 9-10 weeks old at the time of the study.

Bleomycin administration: Mice were lightly anesthetized with 1.5% isoflurane (Abbott, Cham, Switzerland) in a chamber and bleomycin hydrochloride (Euro Nippon Kayaku, Frankfurt am Main, Germany; 0.25 mg/kg) in 25 μ l of saline or vehicle (25 μ l of saline) was administered intranasally (i.n.) via a micropipette (12.5 μ l per nostril). This procedure was performed on days -7, -6, -5, -2, -1 and 0.

MRI: Performed at 4.7 T with a Biospec 47/40 system (Bruker Medical Systems, Ettlingen, Germany). During image acquisition, anaesthesia was maintained with 1.3% isoflurane, in a mixture of O₂/N₂O (1:2), administered via a nose cone. Measurements were performed on spontaneously breathing animals; neither cardiac nor respiratory triggering was applied. A gradient-echo sequence with the following parameters was used: TR 5.6 ms, TE 3.5 ms, flip angle ~10°, matrix size 256x128, slice thickness 0.75 mm, FOV 3x3 cm². A single slice image (acquisition time of 74 s) was obtained by computing the 2DFT of the averaged signal from 60 individual acquisitions and interpolating the data set to 256x256 pixels. The volume of the MRI signals in the lung was quantified by applying a semi-automatic segmentation procedure as described previously [11].

Histology: Slices were stained with (i) hematoxylin and eosin to assess the general morphology, (ii) Masson trichrome for the demonstration of fibrosis, and (iii) Picro-sirius red for the identification of collagen fibers and newly synthesized collagen. Collagen was quantified using the image analysis software "Histolab" (Microvision Instruments, Evry, France). The color corresponding to picrosirius was extracted by threshold setting and the area corresponding to picrosirius staining automatically calculated.

Results and Discussion:

A lasting response was obtained following multiple bleomycin administration, translated by patchy, diffuse MRI signals being detectable in male C57BL/6 mice up to day 70, when measurements were interrupted. Histology showed that from day 14 onwards fibrosis was the predominant component of the response elicited in the lungs of male C57BL/6 mice by repeated bleomycin administration. The response in female C57BL/6 mice as detected by MRI was less pronounced and resolved more quickly than in male animals. This observation is consistent with relaxin and estrogen playing protective roles against airway fibrosis in female mice [12].

Figure 1 summarizes the MRI signals obtained in heterozygous (HE) and homozygous (HO) COT kinase knockout mice and in wildtype animals following bleomycin administration. Overall, the responses induced by the antibiotic were substantially more pronounced in male compared to female mice. Moreover, the signals detected in male mice were attenuated in COT HO compared to male wildtypes. Statistical analyses on unparsed data showed a significant increase of signal volumes with respect to baseline values until day 21 after bleomycin for male HO COT kinase knockout animals, whilst signals were significantly increased throughout the experiment (until day 42) in male wildtype and HE COT kinase deficient mice. In female animals, the response was smaller and of shorter duration in HE COT kinase compared to wildtype mice. These data suggest protection against bleomycin treatment for male HO and female HE knockout mice, as further evidenced by comparisons of the areas under the curves (AUCs). Moreover, at day 42, the collagen areas in histological samples from male HO and female HE COT kinase knockout mice challenged with bleomycin were significantly smaller than in wildtype mice treated with bleomycin, and comparable to those detected in naive mice treated with saline.

Repeated bleomycin dosing elicited a comparable response in wildtype and in HO cadherin-11 deficient mice as detected by MRI, suggesting that the cadherin-11 knockout mice were not protected against bleomycin-induced lung injury.

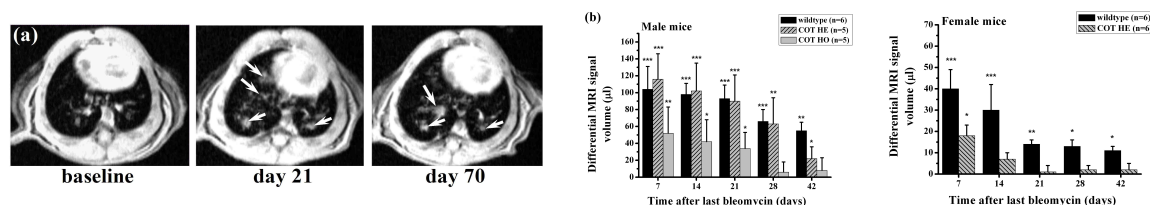


Fig. 1 – Repeated bleomycin dosing. (a) Transverse MR images of a C57BL/6 male mouse acquired prior to (baseline) and at days 21 and 70 after last bleomycin. Arrows show diffuse signals detected by MRI after bleomycin. **(b)** Volumes of signals (baseline-subtracted data; means \pm sem) evaluated on the MR images following repeated dosing of bleomycin to wildtype or COT kinase knockout mice. Levels of significance *** p <0.001, ** p <0.01, * p <0.05 correspond to comparisons of raw signal volumes with respect to baseline values.

The present study shows that MRI applied without gating can quantify, in spontaneously breathing mice, bleomycin-induced lung injury. The data obtained here demonstrate that repeated bleomycin administration at a low dose led to consistent and sustained fibrosis formation with moderate initial inflammation. With the ability for repetitive measurements in the same animal, the technique will be useful for in vivo target analysis and compound profiling in this murine model of lung fibrosis. MRI will facilitate compound testing as the responses at several time points during the course of therapy can be easily compared. The availability of a murine model of pulmonary fibrosis provides the opportunity to study, in wildtype animals or using transgenic technology, novel pharmacological approaches to prevent or treat this disease.

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