Eur Respir J 2002; 19: 1124–1127
DOI: 10.1183/09031936.02.00281602
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European Respiratory Journal
ISSN 0903-1936

The rapamycin analogue SDZ RAD attenuates bleomycin-induced pulmonary fibrosis in rats

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ABSTRACT: Pulmonary fibrosis is characterized by excessive deposition of extracellular matrix proteins within the pulmonary interstitium. The new macrolide immunosuppressant SDZ RAD, a rapamycin analogue, inhibits growth-factor dependent proliferation of mesenchymal cells and might therefore be of therapeutic interest for the treatment of fibrotic lung disease.

In this study the effect of SDZ RAD on lung-collagen accumulation in the bleomycin model of pulmonary fibrosis in rats was investigated. SDZ RAD (2.5 $mg \cdot kg^{-1} \cdot day^{-1}$) or drug vehicle were administered orally by daily gavage. Successful dosing was confirmed by measuring splenic weight. Total lung-collagen content was measured by high-performance liquid chromatographic quantitation of hydroxyproline.

In animals given bleomycin and drug vehicle, total lung collagen was increased by 182±11% (mean±SEM) compared with saline controls at 14 days ($p<0.001$). The increase in lung-collagen accumulation was reduced by 75±12% ($p<0.01$) in animals given SDZ RAD and was accompanied by a concomitant 56±6% ($p<0.001$) reduction in lung weight.

SDZ RAD is currently in clinical trials for the prevention of solid organ graft rejection, another condition characterized by excessive extracellular matrix production. The authors propose that SDZ RAD warrants evaluation as a novel therapeutic agent for fibrotic lung disease.

Eur Respir J 2002; 19: 1124–1127.

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Keywords: Bleomycin, collagen, idiopathic pulmonary fibrosis, RAD, rapamycin

Received: September 19 2001
Accepted after revision January 11 2002

The present study was supported by the Medical Research Council (UK), the Wellcome Trust (programme grant no. 051154) and the Middlesex Hospital Special Trustees.

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders characterized by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium [1]. There is good evidence that this process is driven by growth factors and cytokines produced by activated inflammatory and immune cells, which induce fibroblasts to proliferate or produce excess collagen [2]. Current therapeutic guidelines recommend immunosuppressive treatment with glucocorticoids, azathioprine and cyclophosphamide [3, 4]. Unfortunately, these agents have significant side-effects and poor success rates [5]. Therefore better tolerated and more efficacious therapies are urgently required.

Rapamycin (RPM) is a 31-membered macrolide immunosuppressant which exerts potent antiproliferative effects on lymphoid and nonlymphoid cells by inhibiting cytokine and growth-factor mediated cell signalling [6]. RPM has been successfully used in the carbon tetrachloride model of hepatic fibrosis [7], but its usefulness as a therapeutic agent is limited by its suboptimal pharmacokinetic properties. Problems associated with poor oral bioavailability were overcome with the development of the new orally-active RPM analogue, 40-O-(2-hydroxyethyl)–rapamycin SDZ RAD [8]. This agent was developed to prevent chronic graft rejection in solid organ transplantation. The short-term safety and tolerability of SDZ RAD in stable transplant recipients has been established [9, 10], and studies assessing its efficacy in these patients and other solid organ recipients are ongoing. More recently, SDZ RAD has been shown to attenuate pulmonary arterial hypertension and neointimal formation induced by administration of monocrotaline in pneumonectomized rats [11].

The aim of this study was to evaluate SDZ RAD as a novel antifibrotic agent by assessing its effect on lung-collagen accumulation in the bleomycin model of pulmonary fibrosis.

Methods

Animals

Experiments were performed using male Lewis rats aged 6 weeks. All procedures were approved by the

Home Office and were in accordance with the Animals Scientific Procedures Act 1996.

Experimental protocol

Rats were anaesthetized by intramuscular injection of 0.75–1.0 mL·kg^{-1} Hypnorm (fentanyl citrate (0.315 mL·kg^{-1}) and fluanisone (10 mL·kg^{-1}); Janssen Pharmaceutical, High Wycombe, UK). A tracheotomy was performed and bleomycin sulphate (BLM; Lundbeck, Luton, UK) was administered by a single intratracheal (*i.t.*) injection (1.5 mg·kg^{-1} body weight in 0.3 mL sterile saline) as described previously [12]. Control animals received 0.3 mL sterile saline (SA) alone.

SDZ RAD microemulsion (2.5 mg·kg^{-1}·day^{-1}) (Novartis Pharma AG, Basel, Switzerland), was diluted with distilled water (DW) and administered by daily gavage, beginning 1 day prior to BLM administration. Control animals received DW by daily gavage. Animals were divided into four experimental groups: 1) SA *i.t.*+DW orally; 2) SA *i.t.*+RAD orally; 3) BLM *i.t.*+DW orally; and 4) BLM *i.t.*+RAD orally. Groups of 10 animals, six for biochemical analysis and four for histological analysis, were sacrificed on day 14 using an overdose of intraperitoneal pentobarbitone followed by transection of the aorta. For lung-collagen assessment, the vasculature was perfused with 5 mL normal saline containing 100 U·mL^{-1} heparin. The lungs were removed *en bloc* after removing the major visible bronchi, blotted dry, weighed and snap-frozen in liquid nitrogen. For histological studies, lungs were fixed by *i.t.* instillation of formalin at a pressure of 25 cmH_2O. The trachea was ligated and the thoracic contents were removed *en bloc*.

Collagen measurement

Lung-collagen content was assessed by high-performance liquid chromatographic (HPLC) quantitation of hydroxyproline as previously described [13]. Briefly, 100-mg aliquots of powdered lung tissue were hydrolyzed in 3 mL of 6 M hydrochloric acid at 110°C for 16 h. Hydrolysates were mixed with 30 mg activated charcoal and filtered (Millipore, type DA, 0.65 μm). A 150 μL aliquot of a 1-in-10 dilution of filtered hydrolysate was dried using a centrifugal vacuum concentrator. Hydroxyproline was isolated and measured by reverse-phase HPLC after derivation with 7-chloro-4-nitrobenz-2-oxo-1,3-diazole (NBD-Cl; Sigma, Poole, UK). The total amount of collagen in each lung was calculated, assuming that lung collagen contains 12.2% w/w hydroxyproline [14] and the results were expressed as mg collagen·lung^{-1}.

Pathological examination

Lung tissue was embedded in paraffin wax, cut into sections (5 μm), and stained with haematoxylin and eosin (H&E). Sections were examined blind by the pathologist (PSH) using light microscopy at ×100 magnification. Each successive field was individually assessed for severity of interstitial fibrosis and given a score between 0–8 using the Ashcroft scoring system [15]. The mean score of all fields examined was taken as the fibrosis score of each animal.

Statistical analysis

Results are presented as the mean±SEM. Statistical significance between groups was assessed using an unpaired t-test for normally-distributed data and a Mann-Whitney U-test for nonparametric data. A $p<0.05$ was considered significant.

Results

Spleen weights/assessment of drug efficacy

In order to assess the immunosuppressive effect of RAD, spleen weights were measured after 14 days of daily gavage. Spleen weights were reduced by 21±5% ($p<0.01$) in animals given SA+RAD compared with animals given SA+DW. The difference in spleen weights was greater (40±3%; $p<0.01$) for animals given BLM+RAD, compared with animals given BLM+DW, but there was no significant difference in spleen weights for animals given SA+DW and BLM+DW (fig. 1a).

Lung weights

There was no significant difference in lung wet weight between animals given SA+RAD and SA+DW. As expected, lung wet weight was increased by 170±4% in animals given BLM+DW compared with the SA+DW group ($p<0.01$). In BLM-treated animals, RAD administration significantly attenuated the increase in lung weight by 56±6% ($p<0.001$) (fig. 1b).

Lung collagen

The *i.t.* instillation of BLM without drug treatment caused a characteristic 182±11% ($p<0.01$) increase in lung collagen at 14 days (fig. 1c). This increase in lung-collagen accumulation was reduced by 75±12% in animals given BLM+RAD compared with animals given BLM+SA. Administration of RAD alone had no effect on basal lung-collagen levels in saline-treated animals. The results shown are representative of two separate experiments performed, in which statistically significant differences in total lung collagen between drug-treated animals and those given BLM and drug vehicle alone were obtained.

Ashcroft score

Histological assessment of lung tissue at 14 days revealed that there was no evidence of fibrosis in animals given SA+DW or SA+RAD. In contrast *i.t.* instillation of BLM was associated with a median

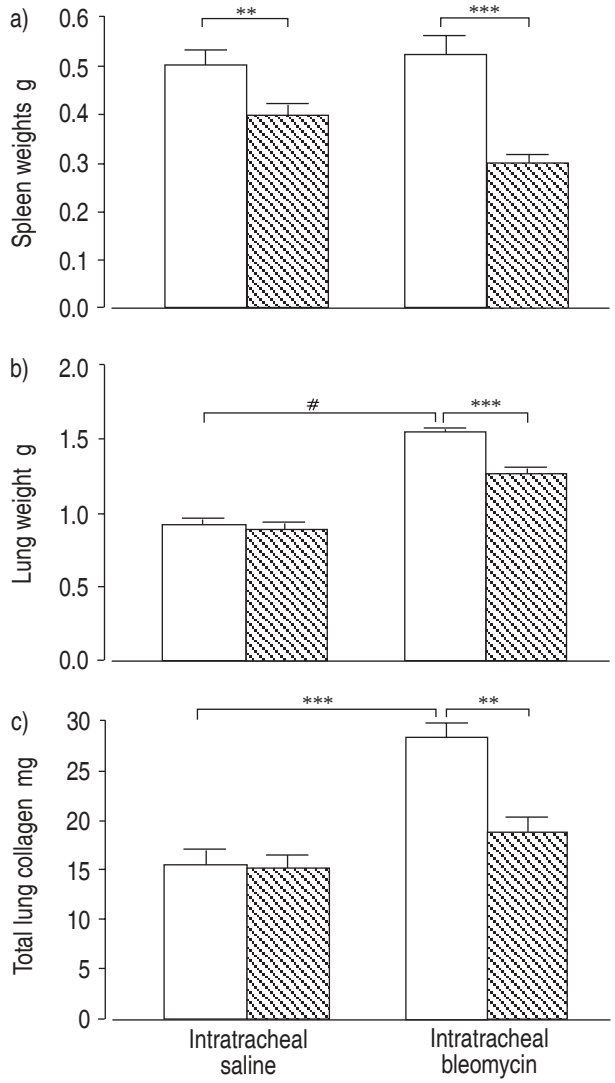

Fig. 1.–Effect of SDZ RAD in bleomycin-induced pulmonary fibrosis. a) Spleen wet weight, b) lung wet weight and c) total lung collagen. □: distilled water; ▨: RAD. **: $p \leqslant 0.01$; ***: $p \leqslant 0.001$; #: $p \leqslant 0.0001$.

Ashcroft-fibrosis score of 2.5 for animals given BLM+DW and 3.5 for the BLM+RAD group. The difference in Ashcroft-fibrosis score between these two treatment groups was not significant. The fibrosis in both BLM groups was loose, focal and myxoid, in keeping with its recent nature. Foci of normal lung were also present. As the lung injury was recent there was high levels of associated acute on chronic inflammation admixed with the fibrosis.

Discussion

The aim of this study was to examine the effect of the macrolide immunosuppressive, SDZ RAD in the BLM model of pulmonary fibrosis. It has been shown, for the first time, that this agent causes a dramatic (75%) reduction in lung-collagen accumulation in this model without any adverse effect on basal (SA control) collagen levels. This reduction is comparable to the reported effect obtained in this model with the antifibrotic agent pirfenidone, which is currently undergoing clinical trials [16]. In addition, the reduction in lung-collagen accumulation by SDZ RAD was much greater than that obtained in previous bleomycin studies with a related macrolide, erythromycin [17], as well as in studies using single anticytokine/growth factor approaches, such as administration of tumour growth factor (TGF)-β soluble receptor or TGFβ-neutralizing antibodies [18, 19]. This makes SDZ RAD a very promising candidate for further clinical evaluation.

Despite the promising effects of SDZ RAD on lung-collagen accumulation in this model, histological evaluation failed to show a significant difference in Ashcroft-fibrosis scores between animals receiving BLM alone and those receiving BLM and RAD. This was disappointing but can be explained. Assessment of a drug effect in this model by histological evaluation is notoriously difficult because *i.t.* instillation of bleomycin causes very patchy fibrosis [20] and is therefore sensitive to sampling errors. In addition, although the Ashcroft-scoring method is clearly able to distinguish between SA *i.t.* and BLM *i.t.* groups, it can be difficult to distinguish areas of acute inflammation and pneumonitis from areas of established fibrosis.The insensitivity of the Ashcroft score to discriminate between established fibrosis and pneumonitis may explain why there was no difference between the two BLM groups using the Ashcroft score. Furthermore, the authors observed the absence of a close temporal relationship between hydroxyproline change and morphological change estimated by histology, in other experiments. This suggests that histological change postdates changes in hydroxyproline and as a consequence quantifiable histological change was not seen in this experiment. Therefore, it is believed that quantification of fibrosis by biochemical assessment of hydroxyproline is a more reliable and definitive method for examining the effects of antifibrotic agents in this model and the dramatic reduction obtained with SDZ RAD in this study is encouraging.

The mechanism by which SDZ RAD attenuates lung-collagen accumulation in this model is likely to include both its immunosuppressive and antiproliferative effects. SDZ RAD, as well as RPM, are potent macrocyclic immunosuppressive agents which are only bioactive when bound to immunophilins. However, unlike other members of this class of immunosuppressants (*e.g.* tacrolimus (FK506) and cyclosporin A), SDZ RAD and RPM do not inhibit cytokine gene activation but exert their effects by blocking cytokine-dependent cell proliferation. The mammalian target of the immunophilin-RPM complex (mTOR) is a protein kinase of the phosphoinositide 3-kinase family. RPM binding to mTOR blocks the activation of ribosomal p70 S6 kinase and the synthesis of proteins required for cell-cycle progression in both lymphoid and nonlymphoid cells [21]. In RAD-treated animals, spleen weights were significantly reduced compared with animals given drug vehicle alone. Since spleen weights reflect T-cell mass,

this suggests that this agent was given at an immunosuppressive dose. The role of T-lymphocytes in the BLM model is at present controversial and has only been examined by experiments performed in mice rather than rats. Initial studies provided evidence that T-cell-dependent cytokine production may play an important role in lung-collagen accumulation in the bleomycin model of pulmonary fibrosis since "nude" mice, depleted of T-lymphocytes, show a reduced fibrotic response [22]. However, more recent studies in SCID mice have challenged this view [23]. In addition to its effects on T-lymphocytes, there is also good evidence that RPM can inhibit growth-factor dependent human-lung fibroblast proliferation *in vitro* [24], so that attenuation of the fibroproliferative response by SDZ RAD is also likely to play a role in this model. Further experiments to elucidate the exact mechanism of action of SDZ RAD in this model should prove very informative and will be the focus of future studies.

To conclude, this study showed, for the first time, that SDZ RAD has dramatic inhibitory effects on lung-collagen accumulation in the bleomycin model of pulmonary fibrosis. This agent is orally available, has favourable pharmacokinetic properties and is already being evaluated in clinical trials of allotransplantation. Since current therapies for fibrotic lung disease are at present inadequate, the authors propose that SDZ RAD requires evaluation as a novel antifibrotic agent.

Acknowledgements. The authors would like to thank Novartis Pharma AG for the kind gift of the SDZ RAD microemulsion, and in particular W. Schuler (Transplant Research, Novartis Pharma AG) for helpful advice.

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