# A matrix metalloproteinase inhibitor which prevents fibroblast-mediated collagen lattice contraction

Kate A. Scott[a], Edward J. Wood[a,*], Eric H. Karran[b]

[a]*School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK*
[b]*Molecular Neurobiological Research, SmithKline Beecham Pharmaceuticals, Harlow CM19 5AW, UK*

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**Abstract Matrix metalloproteinases (MMPs) and the specific tissue inhibitors of metalloproteinases (TIMPs) are involved in tissue turnover in normal and pathological processes including wound healing. Marimastat, a potent inhibitor of MMPs, was used to investigate the role of MMPs in an in vitro wound contraction model, the dermal equivalent, in which fibroblasts are grown in a collagen matrix. Marimastat inhibited fibroblast-mediated lattice contraction and this inhibition was reversible upon removal of the inhibitor, indicating that MMPs play an important role in fibroblast-mediated collagen lattice contraction, modelling what may happen when granulation tissue contracts in a healing wound.**



*Key words:* Lattice contraction; Marimastat; Interstitial collagenase activity

## 1. Introduction

The dermal equivalent (DE) model consists of dermal fibroblasts grown in a hydrated native collagen lattice and offers an environment in which to observe fibroblast behaviour in physiologically relevant conditions similar to those in dermis. Fibroblasts cultured within such a collagen matrix are observed to reorganise the collagen fibrils and contract the matrix [1]. This model has several advantages over monolayer culture (in which fibroblasts are known to behave differently in many respects) and is now widely used as a simplified, in vitro model of dermis. This system also has therapeutic potential for grafting [2]. Many researchers have studied fibroblast-mediated lattice contraction and more generally have used the DE model to investigate cell-matrix interactions, for example as a model for wound healing [3–6]. In the course of wound healing, fibroblasts invade the wound space and not only secrete collagen and glycosaminoglycans, but also produce matrix metalloproteinases (MMPs) that control the remodelling of the newly formed granulation tissue [7]. At least one metalloproteinase, MMP-1, is also produced by keratinocytes during wound healing and may be involved in the process of re-epithelialisation.

MMPs form a group of extracellular proteinases that share several common characteristics [8]. Each MMP degrades at least one component of the extracellular matrix (ECM) and in fact with the exception of MMP-1, 8 and 13 (the collagenases), most have a broad substrate specificity. To be catalytically active, MMPs must contain a zinc ion within the active site and functional stability is conferred by a secondary zinc ion and calcium ions [9]. MMP-1 degrades collagen by a single specific cleavage per chain that allows partial unwinding of the triple helical structure [10]. This results in the formation of a denatured gelatin structure which is susceptible to further attack by other members of the MMP family [11].

MMPs are secreted as latent enzymes requiring proteolytic cleavage or some other modification (e.g. treatment with sulphydryl reagents) for activation [11–13] and their activity is specifically controlled by extracellular proteins, tissue inhibitors of metalloproteinases (TIMPs). The function of these is now beginning to be understood and to date four human TIMPs have been characterised [14]. TIMPs are 28 kDa glycoproteins with a 21 kDa protein core that are secreted by fibroblasts as well as other cells, that also produce MMPs, and which bind non-covalently to active MMPs in a 1:1 molar ratio [11–13]. TIMP production is regulated by agents such as growth factors [8].

To investigate the role that MMP activity may play in collagen lattice contraction in vitro as a model of wound contraction in vivo, a specific MMP inhibitor, Marimastat, was used. This is one of a range of synthetic compounds designed to bind tightly to the zinc atoms in the active site of MMPs, thus disabling the enzymes and blocking their function. Marimastat has a peptido-mimetic backbone to facilitate binding to the active site of MMP, and a hydroxamate structure to chelate the zinc ion [15].

## 2. Materials and methods

### 2.1. Materials

Marimastat was synthesised by SmithKline Beecham, Harlow, UK. [$^3$H]Acetic anhydride (specific activity 50 mCi/mmol) was purchased from New England Nuclear Research Products, Stevenage, UK. Acid-soluble type I collagen from calf skin for the MMP-1 activity assay, 1,4-dioxane, trypsin, APMA and EDTA were purchased from Sigma Chemical Co., Poole, UK. DMEM, NBCS, penicillin (5000 U/ml) and streptomycin (5000 μg/ml) were purchased from Gibco, Paisley, UK.

### 2.2. Cell culture

Monolayer cultures of primary human adult dermal fibroblasts were established from collagenase-treated dermis [16]. Fibroblasts were grown in monolayer culture in 1×DMEM supplemented with 5% (v/v) NBCS and used between passages 5 and 10. Prior to formation of dermal equivalents the cell monolayers were washed twice with PBS and detached by treatment with 0.05% (w/v) trypsin-0.02% (w/v) EDTA in PBS. Cells were washed with DMEM containing 5% (v/v) NBCS and were pelleted by centrifugation at 100×*g* in an MSE bench top centrifuge for 5 min at room temperature. A viable cell count was determined using 0.4% (w/v) trypan blue. Fibroblasts were resuspended in DMEM at a density of $2.5\times10^5$ cells per ml.

*Corresponding author. Fax: (44) (113) 233 3167.
E-mail: e.j.wood@leeds.ac.uk

*Abbreviations:* MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; ECM, extracellular matrix; DE, dermal equivalent; MMP-1, interstitial collagenase; APMA, *p*-aminophenylmercuric acetate; DMEM, Dulbecco's modified Eagle's medium; NBCS, newborn calf serum


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*2.3. Dermal equivalents*

DEs were fabricated essentially as described by Bell and co-workers [1] using type I collagen extracted from rat tail [16]. DEs were prepared by the addition of 4 ml 2×DMEM, 3 ml of 5 mg/ml collagen in 13 mM HCl and 1 ml of 0.1 M NaOH to 1 ml of cell suspension (final concentration 1.5 mg/ml collagen). The DE suspension was swirled to mix and 800 μl was pipetted into each well of a 12-well tissue culture plate to give $2\times10^4$ cells per well. The plate was incubated at 37°C to allow gel formation and then DEs were always incubated in 1×D-MEM containing 5% (v/v) NBCS.

Unless otherwise stated, Marimastat in DMSO was added to DEs at a final concentration of 1 or 10 μM immediately after polymerisation in 1 ml medium. Toxicity studies indicated that Marimastat at concentrations below 15 μM did not affect fibroblast viability as measured by the tetrazolium (MTT) assay [17] (data not shown). DEs were subsequently detached from the walls of the culture plate 3 days (unless otherwise stated) after polymerisation by 'ringing' with a pasteur pipette. Fresh medium with and without Marimastat was added and subsequently changed every 48 h following DE detachment. Lattice contraction was measured by determining the diameter of the DE using a ruler.

*2.4. Assay for MMP-1 activity*

Commercially available acid-soluble type I collagen from calf skin was radiolabelled using [$^3$H]acetic anhydride as described previously [18]. 5×MMP-1 assay buffer (250 mM Tris-HCl pH 7.4, 1 M NaCl, 25 mM $CaCl_2$) was added to 3 μg radiolabelled collagen in 13 mM HCl (specific activity $1.45\times10^6$ cpm/mg) to raise the solution to neutral pH yielding final concentrations of 50 mM Tris-HCl pH 7.4, 200 mM NaCl and 5 mM $CaCl_2$. Fibroblast-conditioned medium in 1×MMP-1 assay buffer was incubated with the neutralised radiolabelled collagen solution for 20 h at room temperature. To stop the reaction 20 mM EDTA was added. To activate latent MMP-1, samples were incubated with the $^3$H-labelled collagen in the presence of 1 mM APMA.

For each experiment a negative control containing 20 mM EDTA to inhibit MMP activity was included in which typically less than 4% of the total radioactivity present was released. The samples were mixed with 1 volume of 1,4-dioxane to precipitate native collagenous material and placed on ice for 10 min. The tubes were then centrifuged for 10 min at $12\,000\times g$ in a microfuge at 4°C to pellet undigested collagen. The amount of digested collagen was determined by removing the supernatant and determining the radioactivity by scintillation counting. For each time point five replicates were used to determine the radioactivity released into solution and the linear response region was used. The radioactivity measured in the negative control was subtracted from each sample to allow a measurement of proteolysis of collagen by MMP-1.

## 3. Results

*3.1. MMP-1 activity produced by fibroblasts is inhibited by Marimastat*

Fig. 1 shows the effect of Marimastat on the measured MMP-1 activity in the first 8 h after DE fabrication. In control DEs the MMP-1 activity began to rise, detectable 4 h after polymerisation, and increased linearly from this point. The measured MMP-1 activity in Marimastat-treated DEs in contrast was almost zero throughout this time course at both Marimastat concentrations used. Fig. 2 shows the measured MMP-1 activity 1 and 3 days after DE detachment. It can be seen that 1 day after detachment MMP-1 activity was completely inhibited at both concentrations of Marimastat. After 3 days the level of MMP-1 activity had increased two-fold in control DEs. A concentration of 1 μM Marimastat produced a 60% inhibition of MMP-1 activity, whereas MMP-1 activity was 90% inhibited in DEs incubated with 10 μM Marimastat, after 3 days. Partial inhibition of MMP-1 activity with 1 μM Marimastat correlated with the partial inhibition of fibroblast-mediated lattice contraction observed (see Fig. 3).

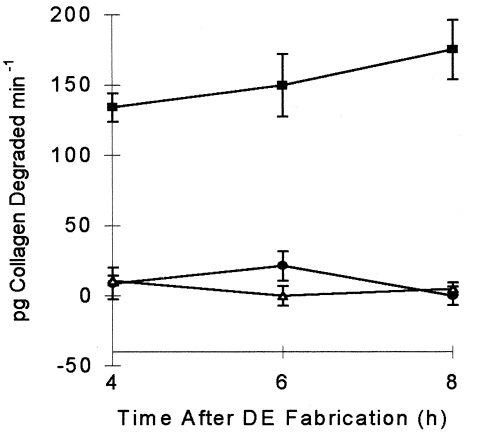


Fig. 1. Effect of Marimastat on MMP-1 activity after DE fabrication. DEs were incubated in the presence of 0, 1 or 10 μM Marimastat following fabrication. Samples of DE conditioned medium were removed at intervals after fabrication and assayed for MMP-1 activity as described in Section 2. Results are expressed as pg collagen degraded/min ± S.E.M. of five replicates. ■, control; ●, 1 μM; △, 10 μM.

*3.2. Marimastat inhibits fibroblast-mediated lattice contraction*

Fig. 3 shows the effect of Marimastat on DE contraction. Under the conditions used, control DEs contracted, typically with approx. 40% of the contraction occurring during the first 4 days. In the presence of 10 μM Marimastat, lattice contraction was completely inhibited. Incubation with 1 μM Marimastat resulted in a similar contraction pattern to that observed in control DEs but occurred at a slower rate resulting in only 25% contraction after 4 days in culture.

To establish whether the effect of Marimastat could be reversed after 5 days in culture, the medium was aspirated and the DEs were washed twice with 1×DMEM for 1 h and then incubated in medium without Marimastat. The medium was replaced every 24 h to ensure that any residual

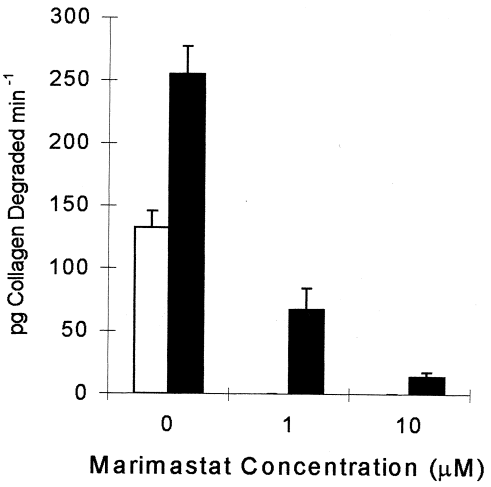


Fig. 2. Effect of Marimastat on MMP-1 activity after DE detachment. DEs were incubated in the presence of 0, 1 or 10 μM Marimastat following fabrication. DEs were detached after 3 days in culture. Samples of DE conditioned medium were removed at 1 and 3 days after detachment and assayed for MMP-1 activity as described in Section 2. Results are expressed as pg collagen degraded/min ± S.E.M. of five replicates. Open bars, 1 day and solid bars, 3 days after DE contraction (absence of bar is equivalent to no measurable activity).

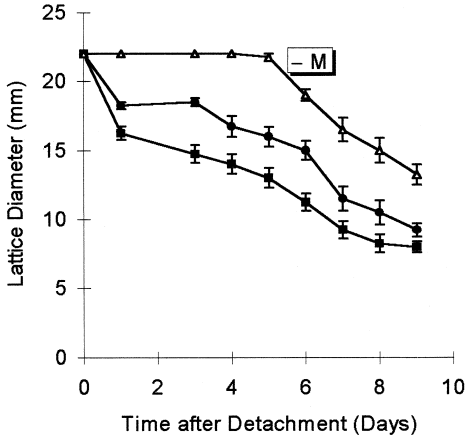


Fig. 3. Effect of Marimastat on fibroblast-mediated lattice contraction. DEs were incubated in the presence of 0, 1 or 10 μM Marimastat following fabrication. DEs were detached after 3 days in culture. Lattice diameter was measured every 24 h. After 5 days following detachment Marimastat was removed, DEs were washed and incubated in medium without Marimastat. Results are expressed as mean lattice diameter ± S.E.M. of four replicates. ■, control; ●, 1 μM; △, 10 μM.

Marimastat trapped within the collagen lattice was eventually removed.

Removal of Marimastat after 5 days in culture resulted in the reversal of the inhibition of contraction. DEs which had been cultured with 10 μM Marimastat immediately began to contract at a rapid rate. The rate of contraction was even greater in DEs that had been incubated with 1 μM Marimastat to result in almost the same size lattices as control ones after 9 days in culture.

Fibroblasts released from the plastic of culture plates by trypsinisation need to attach to a new substrate and spread before they can proliferate and begin other activities. In order to investigate whether Marimastat was impairing initial fibroblast spreading in the collagen lattice which then resulted in an inhibition of contraction, DEs were prepared and the experiment was repeated exactly as described in Section 2 except that for the first 24 h after lattice fabrication the DEs were incubated in medium without Marimastat. After 24 h incubation the medium was replaced with medium with 0, 1 or 10 μM Marimastat. The DEs were incubated for a further 48 h before the lattices were detached. A similar pattern of Marimastat inhibition of contraction was observed to that previously seen in Fig. 3 (data not shown).

To investigate whether Marimastat inhibited cell attachment fibroblasts were seeded onto glass coverslips in fibroblast medium containing 1 or 10 μM Marimastat. Cell attachment and cell spreading were examined microscopically after 4 h and 48 h. Marimastat inhibited cell attachment after 4 h incubation but after 48 h the fibroblasts had spread out indicating that Marimastat can initially retard cell attachment (data not shown).

## 4. Discussion

Many attempts have been made to identify and isolate the factors required for fibroblast-mediated lattice contraction such as occurs in wound contraction and which is modelled by the DE system [19–21]. Fibroblast-mediated gel contraction in DEs is biphasic [22]: there is an initial phase of rapid contraction followed by a subsequent period of slower contraction and this was observed in the present work. Investigation of the role of MMP-1 in DE contraction using radiolabelled collagen [22] showed that during the first 24 h after fabrication radioactivity was being released into the culture medium implying that the 'remodelling' of collagen fibres was occurring. The enzymic activity of MMPs in a tissue or in a model system such as the DE, is dependent on the concentration of TIMPs present. In the model system activation of MMP-1 by APMA results in the production of active enzyme to which TIMP-1, if present, will bind instantly to form an enzyme:inhibitor complex [18]. Degradation of [$^3$H]collagen is therefore due only to MMP-1 molecules not bound to TIMP-1. Release of MMP-1 into the medium is an indication of the production of MMP-1 as it is well established that activation of MMPs occurs close to the cell allowing 'focalised' proteolysis, even in the presence of high concentrations of inhibitors [23]. Although other MMPs may degrade type I collagen to a limited extent, in the dermal equivalent fabricated using native collagen as reported in the present work, MMP-1 is the most likely candidate.

We have shown that MMP-1 production increases during normal collagen lattice contraction and that typically the amount of *active* enzyme detectable under the conditions described here is approx. 15% of the total potential MMP-1 activity (data not shown). The presence of 10 μM Marimastat inhibited both MMP-1 activity and collagen lattice contraction. Partial lattice contraction was observed in DEs treated with 1 μM Marimastat and this correlated with 60% inhibition of MMP-1 activity. Upon removal of Marimastat from the medium by washing out, the DEs then contracted after a short lag phase. This confirmed that the fibroblasts seeded into these lattices had remained viable throughout the treatment.

Previous studies have shown that the time course of collagen gel reorganisation correlates with extent of initial cell spreading, and that cells which spread poorly because of cytoskeletal or other defects, have a decreased ability to cause lattice contraction [4,20]. Marimastat initially reduced cell attachment but subsequent fibroblast spreading was not compromised by Marimastat. Fibroblasts allowed to spread in the collagen lattice for 24 h prior to addition of Marimastat and lattice contraction showed a similar pattern of lattice contraction inhibition. This indicated that impaired fibroblast attachment and spreading was not a significant factor in the inhibition of contraction by Marimastat. It is also unlikely that Marimastat exerts its action by inhibiting cell proliferation since cell numbers in DEs do not begin to increase until after 6 days in culture under the conditions used here (data not shown) although it is well established that inhibition of cell attachment inhibits cell proliferation [24].

It should be noted that the concentrations of Marimastat used in this study are in the μM range and yet the $K_i$ is 5–8 nM [25]. It is possible that the accessibility of the compound to the fibroblasts may be impaired by the surrounding collagen matrix. For example, Marimastat may have become trapped within, or bound to, components in the lattice, or may not get close enough to the active site on collagenase unless much higher concentrations are used. Another possibility is that a different metalloprotease which may play a more important role in fibroblast-mediated lattice contraction was

inhibited and that higher concentrations of Marimastat were required to ensure complete inhibition.

We have shown that Marimastat, a potent inhibitor of MMPs, can retard or stop fibroblast-mediated collagen lattice contraction. The viability of fibroblasts within the collagen lattice was not affected and inhibition of cell spreading did not play a role in contraction inhibition. This study has shown that the presence of exogenously added MMP inhibitors may have a potential use for treating chronic wounds where the correct balance of enzyme to inhibitor has been lost, or to prevent rapid contraction of burn wounds to reduce scarring.

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