In Vitro and in Vivo Endochondral Bone Formation Models Allow Identification of Anti-Angiogenic Compounds

Gabri van der Pluijm,* Martine Deckers,*
Bianca Sijmons,* Henny de Groot,* John Bird,[†]
Ruth Wills,[†] Socrates Papapoulos,* Andy Baxter,[†]
and Clemens Löwik*

From the Department of Endocrinology,* Leiden University Medical Center, Leiden, the Netherlands and CellTech R&D Limited,[†] Cambridge, United Kingdom

A major obstacle in the study of angiogenesis and the testing of new agents with anti-angiogenic potential has been the lack of experimental models with predictive *in vivo* value. We describe here the combined use of *in vitro* and *in vivo* angiogenesis models that are based on endochondral bone development. This approach led to the identification of a new inhibitor of matrix metalloprotease (MMP) activity that inhibits neovascularization *in vitro* and *in vivo* while osteoclast invasion, which occurs simultaneously during bone development, remained unaffected. In contrast, the broad-spectrum MMP-inhibitor marimastat inhibited both *in vitro* angiogenesis and osteoclastogenesis dose-dependently but displayed severe toxic side effects *in vivo*. The combined use of these experimental models may, therefore, facilitate the discovery of mechanisms underlying angiogenesis and lead to identification of new pharmacological compounds with clinical efficacy and appropriate selectivity in the treatment of angiogenesis-dependent disorders like arthritis and cancer. (*Am J Pathol 2003, 163:157–163*)

Acquisition of a blood supply is a fundamental requirement for organ development and differentiation during embryogenesis and postnatal development. During endochondral bone formation the avascular calcified cartilaginous matrix is replaced by bone via the simultaneous invasion of osteoclasts and capillary endothelial cells that direct osteoblast progenitors to the cartilaginous scaffolds to synthesize bone.[1–8]

Invasive processes during osteoclastogenesis and angiogenesis share several similarities. For example, both cell types express matrix metalloproteases (MMPs) that are required for invasion, migration, and degradation of extracellular matrices and the release of growth factors and inhibitors from the matrix.[4,8,9–20] MMPs are a family of Zn-dependent endopeptidases that are able to cleave extracellular matrix molecules during normal tissue (re) modeling (eg, embryonic growth) and remodeling of pathological tissues (eg, rheumatoid arthritis and cancer).[1,21–26] MMPs may also directly facilitate angiogenesis by stimulating matrix degradation and, indirectly, by releasing matrix-associated endothelial growth factors and inhibitors.[27,28]

There is compelling evidence that MMPs are involved in skeletal development. Before vascular invasion of calcified cartilage various MMPs are expressed in late hypertrophic chondrocytes, osteoclasts, endothelial cells and osteoblasts.[4,11–13] MMP knockout mice (MMP$-9^{-/-}$ and MT1-MMP$^{-/-}$) show disturbances in skeletal development, which include defects in vascularization, osteoclast migration and resorption of the growth plate.[9,11,29,30] The use of MMP inhibitors (MMPIs) provided further evidence that MMPs are involved in the migration of osteoclasts and endothelial cells *in vitro* and *in vivo*.[12,26,31,32]

To date, a major impediment to the study of angiogenesis and the testing of new agents with anti-angiogenic potential has been the lack of experimental models that are predictive of *in vivo* responses. Here we describe the combined use of an *in vitro* and an *in vivo* angiogenesis model that are based on endochondral bone development. The strength of this approach is illustrated by the identification of a new inhibitor of MMP activity that selectively inhibits neovascularization *in vitro* and *in vivo* while osteoclast invasion remains unaffected.

Materials and Methods

Compound Preparation

For *in vitro* assays, MMPIs were suspended in dimethyl sulfoxide (DMSO; 2×10^{-2} M stock) and stored at $-20°C$.[33] MMPIs were diluted in culture medium and 0.05% DMSO in culture medium was used as vehicle control. For *in vivo* studies, MMPIs were prepared daily in 50% DMSO + 50% PBS at ambient temperature. MMPIs or vehicle (10 µl) were subcutaneously administered to neonatal mice for 4 consecutive days.

Table 1. IC$_{50}$ Values (nM) of MMP-Inhibitors for Various MMPs (MMP Enzyme Assays)

MMPI	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-13	MMP-14
marimastat	2	5	20	3	7	3	4
CH5902	2061	15	133	16	545	58	305
CH3921	13	24	89	153	449	338	877

Enzymatic Assays and in Vitro *Activities of Tested MMPIs*

Human recombinant MMPs were expressed in *Escherichia coli* as inclusion bodies and purified as described.[34] CH5902 and CH3921 are small synthetic MMPIs which were rationally designed to inhibit a broad range of MMP activities while sparing those of the sheddases.[33] The IC$_{50}$ values, which are depicted in Table 1, were determined *in vitro* under reducing conditions using purified human MMPs as described previously.[34]

Additionally, CH5902 only weakly inhibits TACE (IC$_{50}$>10,000 nmol/L) and does not inhibit release of tumor necrosis factor (TNF)-α, TNF-RII, L-selectin, interleukin (IL)-1-RII and IL-6-R in cell-based *in vitro* assays (thought to be mediated by tumor necrosis factor-alpha converting enzyme (TACE)). CH5902 also does not inhibit (IC$_{50}$ values >30,000 nmol/L) the *in vitro* activity of serine or cysteine proteinases, which include chymotrypsin, elastase, plasmin, cathepsin B, trypsin, thrombin, and urokinase plasminogen activator (data not shown). CH3921 does not inhibit the release of TNF-α in a cell-based assay but has not been tested in the other systems. These results, therefore, demonstrate that CH5902 and CH3921 are selective, potent nanomolar inhibitors of MMP *in vitro* activities. Importantly, they do not inhibit closely-related sheddase activities responsible for the release of cell-associated molecules.

In Vitro *Angiogenesis Assay*

17-day-old fetuses were removed from pregnant Swiss albino mice and metatarsals were dissected as described previously.[10] The isolated metatarsals were cultured for 10 days in 24-well plates in the presence or absence of MMPIs, and fixed and stained for platelet/endothelial cell adhesion molecule 1 (PECAM-1).[10] Total area of PECAM-1-positive tubular structures was determined by image analysis.[12] Data are depicted as treatment control ratios from triplicate experiments.

In Vitro *Osteoclast Invasion and Bone Resorption Assay*

Pregnant Swiss albino mice were injected with 30 μCi ^{45}Ca (1Ci/mmol) at day 16 of gestation and were sacrificed at day 17.[35] ^{45}Ca prelabeled metatarsals were dissected and cultured as described earlier in the presence or absence of MMPIs.[35] Metatarsals were cultured for 6 days and medium was replaced after 3 days. The %^{45}Ca release was used as a measure of bone resorption and osteoclast-precursors and osteoclasts were identified by TRAcP staining as described earlier.[35]

Angiogenesis and Osteoclastogenesis in Vivo

All animal procedures were approved by the Leiden University Committee on Animal Experiments. In the neonatal mouse tail primary centers of endochondral ossification are still developing in the most distal vertebrae and provide a suitable model to study bone angiogenesis and osteoclastogenesis *in vivo*.[8,36] At birth, capillaries and osteoclasts have not yet invaded the 28 neonatal tail vertebrae. Between day 1 and day 6 primary ossification centers appear with a distal progression in all caudal vertebrae,[10,33] and at day 7 these are all remodeled.[10] Neonatal Swiss albino mice were treated with the MMPIs marimastat (6, 12, or 60 μg/day), CH5902 (60 μg/day), or CH3921 (60 μg/day) or vehicle (1:1 DMSO in PBS) for 4 consecutive days, starting at the second day after birth. Every group contained 5 littermates and the experiment was repeated twice. At the start of each experiment, 2 days after birth, the tails of two non-treated mice were fixed in Zinc Macrodex formalin (ZnMF) as described previously.[8] After 4 consecutive days of treatment the animals were sacrificed, total tail-length was determined and tails were processed histochemically. *Dolichus biflorus* agglutinin (DBA) histochemistry was used to identify endothelial cells.[8] For double staining, TRAcP staining was followed by lectin staining as described.[8]

Results

In 17-day-old fetal mouse metatarsals, endothelial and osteoclast precursors are still confined to the perichondrium. At this stage of development invasion of the avascular calcified cartilage by capillary endothelial cells and osteoclasts occurs simultaneously in these long bone explants.[10,35] When cultured for more than 7 days capillaries will grow out of the bone explant and the extent of capillary outgrowth can be used to test the antiangiogenic potential of novel compounds.[10] Due to these properties the same bone explants can be used as *in vitro* models of angiogenesis and osteoclastic bone resorption.[10,35]

Effect of MMPIs on Angiogenesis in Vitro

Continuous treatment of the fetal bone explants for ten days with various doses of CH3921, CH5902, and marimastat prevented the outgrowth of PECAM-1-positive
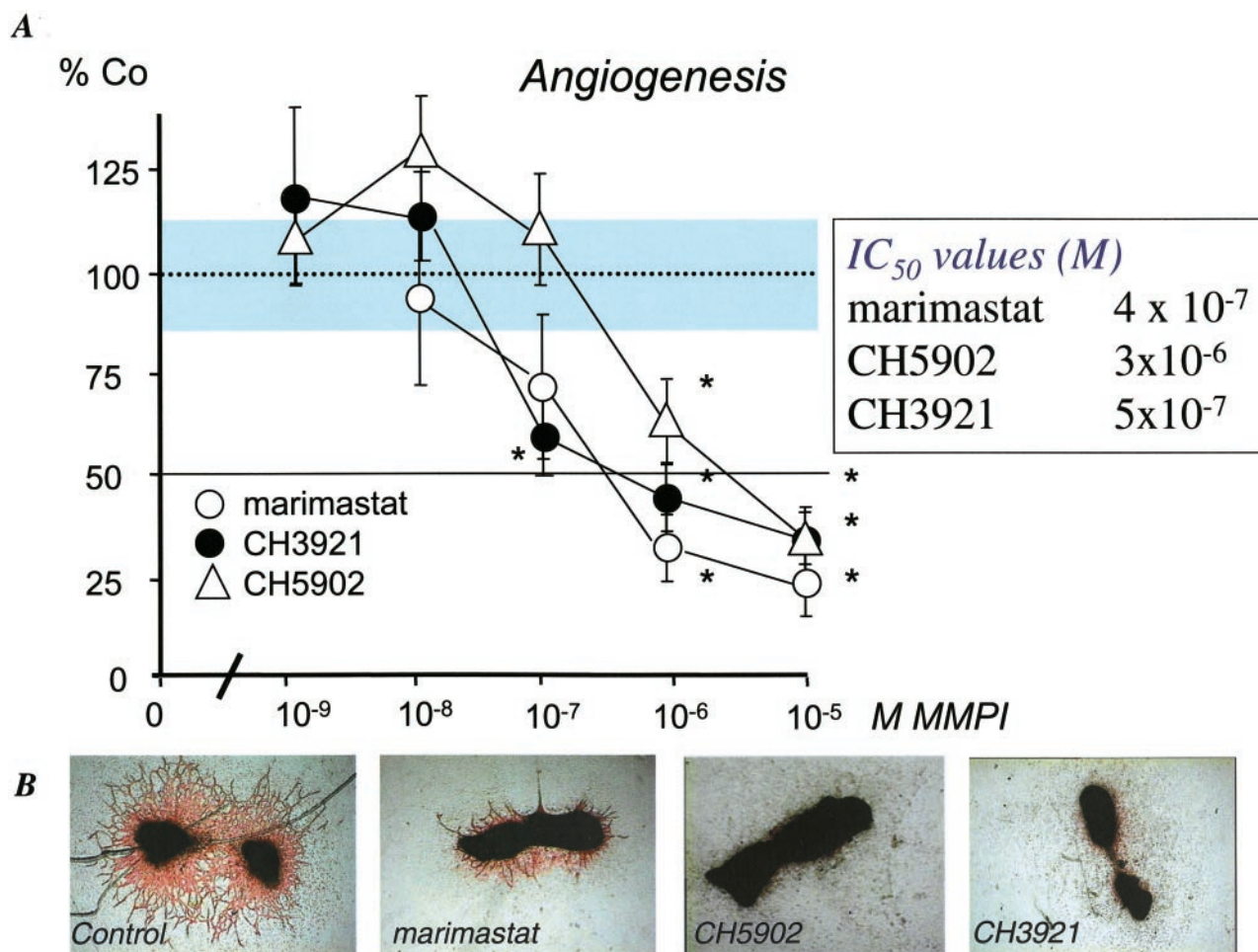
Figure 1. MMPIs inhibit angiogenesis *in vitro*. 17-day-old fetal mouse metatarsals were treated with marimastat (**open circle**), CH3921 (**filled circle**), CH5902 (**open triangle**), or vehicle for 10 days. After 10 days, cultures were stained for PECAM-1. **A:** The total area of PECAM-1-positive capillary-like structures was determined by computerized image analysis. Data are expressed as treatment control of sextuple cultures ± SEM. The experiment was repeated three times. **B:** Representative images of control cultures, marimastat-, CH5902-, and CH3921 (10^{-6}M)-treated cultures are shown after staining for PECAM-1. Original magnification, ×40.

tube-like structures in a dose-dependent manner (Figure 1A). As shown in Figure 1B, only scattered endothelial cells were present in the cultures treated with MMPIs. Strikingly, degradation of the calcified matrix (central part of the explant) was not inhibited by CH3921 (Figure 1B), whereas the other MMPIs strongly prevented the breakdown of the mineralized matrix. Therefore, we tested whether MMPIs can differentially inhibit osteoclast development and subsequent resorption of mineralized matrix in similar bone explants.

Effect of MMPIs on Osteoclast Invasion *in Vitro*

Osteoclastic bone resorption in (^{45}Ca-prelabeled) fetal bones entirely depends on differentiation of osteoclast-precursors that are present in the perichondrium at the start of the experiment, and subsequent invasion and resorption of the calcified matrix.[35] Treatment of bone explants with marimastat and CH5902 dose-dependently inhibited bone resorption in fetal long bones (Figure 2A). In contrast, CH3921 did not appear to have an effect on

osteoclastic bone resorption (Figure 2A). In control sections, mature resorbing osteoclasts were identified in the mineralized cartilaginous matrix (Figure 2B). In marimastat- and CH5902-treated long bones, however, osteoclast-precursors were still confined to the perichondrium, whereas there was no inhibitory effect with CH3921 (Figure 2B).

Effect of MMPIs on Angiogenesis and Osteoclastogenesis *in Vivo*

We further tested the effects of these MMPIs in the *in vivo* model of endochondral bone formation.[8] At the start of the experiment (day 2 after birth) the 28 caudal vertebrae of the mouse tail can be divided into different developmental stages (Figure 3A). From proximal to distal vertebrae are composed of developing trabecular bone with a primitive bone marrow cavity (caudal vertebrae 1 to 10), calcified avascular cartilage[11–21] and non-mineralized avascular cartilage (vertebrae 22 to 28).[8,36] After double-
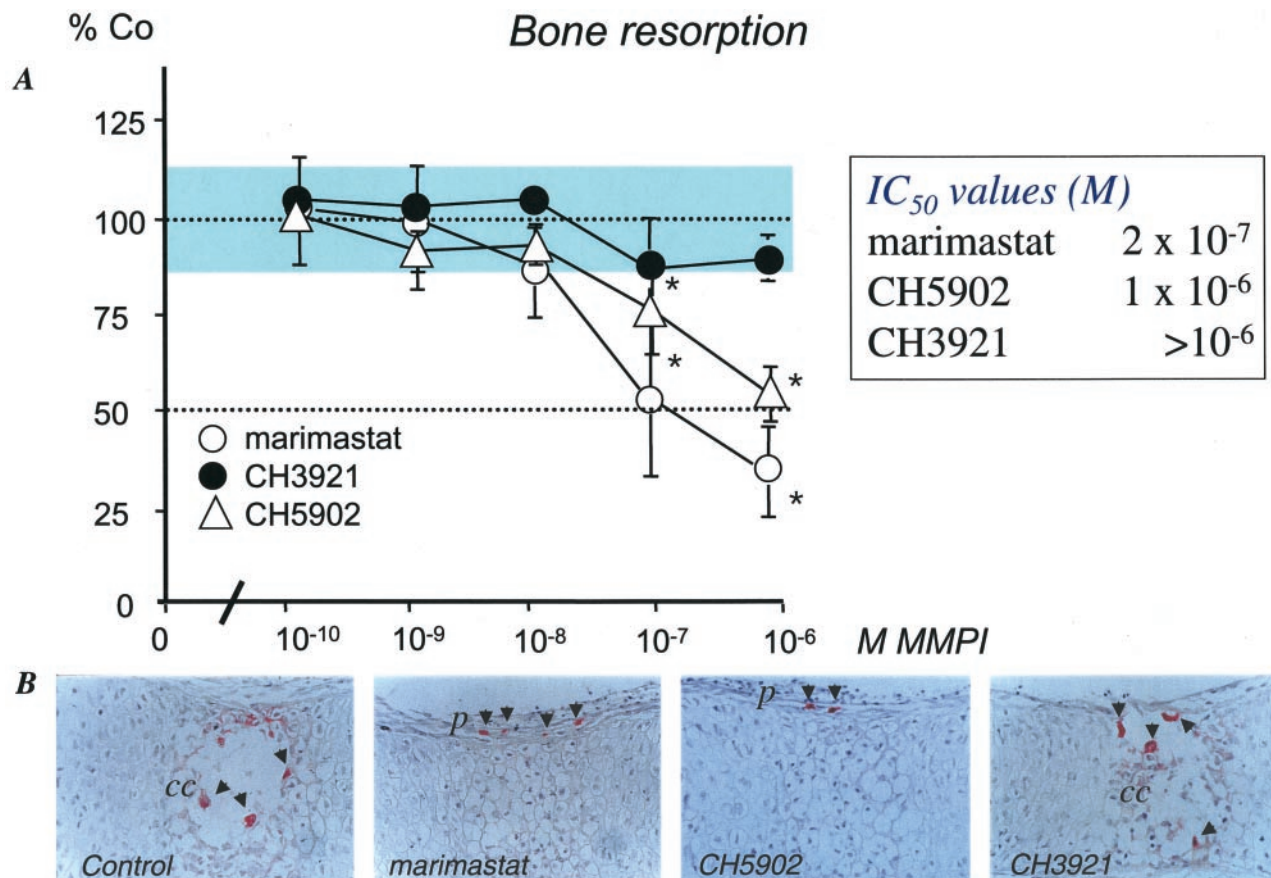
Figure 2. MMPIs and osteoclastic resorption of ^{45}Ca-prelabeled 17-day-old fetal mouse metatarsals *in vitro.* **A:** The fetal bone explants were incubated with various doses of marimastat (**open circle**), CH3921 (**filled circle**), CH5902 (**open triangle**) or vehicle (DMSO). **B:** MMPIs marimastat and CH5902 inhibit the invasion of osteoclast-precursors from the perichondrium into the calcified cartilage. Fetal bones were incubated with 10^{-6}M marimastat, CH5902, or vehicle for 4 consecutive days and processed for paraffin embedding and TRAcP staining.[39] TRAcP-positive multinuclear cells were identified in calcified cartilage (ml), while in the marimastat- and CH5902-treated metatarsal TRAcP-positive cells resided in the perichondrium (p). CH3921 did not affect the invasion of the calcified cartilage by osteoclasts. Original magnification, ×200.

staining for TRAcP and lectin (DBA), TRAcP-positive osteoclasts were closely associated to DBA-positive endothelial cells in the primitive bone marrow cavity (vertebrae 1 to 10). More proximal lectin-positive endothelial cells were identified in the perichondrium or surrounding mesenchyme (vertebrae 10 to 28) in close proximity to mononuclear TRAcP-positive osteoclast-precursors. After 4 days, the invasion front progressed to the distal end and was located at approximately vertebra 18 (Figure 3A).

Treatment of 2-day-old neonatal mice with the MMPIs CH5902 and CH3921 (60 μg/day for 4 days) did not affect the body weight of the animals (not shown). The total length of the tails after 4 days of treatment, however, was significantly inhibited from 2.00 cm ± 0.06 in control animals to 1.70 cm ± 0.04 ($P < 0.001$) in CH5902-treated animals and from 2.24 cm ± 0.06 in control animals to 2.00 cm ± 0.05 ($P < 0.05$) in CH3921-treated mice. In contrast, administration of marimastat at various doses (6, 12, and 60 μg/day) to neonatal mice led to severe cytotoxic effects, such as severe tail deformities and significant decreases in body weight, and eventually to death. In most cases the young growing animals died after 2 consecutive injections with marimastat; few ani-

mals survived after 4 days of treatment with all tested doses of marimastat (<5% after 4 days).

Longitudinal sections of neonatal mouse tails revealed that administration of the MMPIs CH3921 and CH5902 (60 μg/day for 4 consecutive days) resulted in a significant delay in angiogenesis (Figure 3B and Table 2). The invasion front was determined as the first sign of vascular invasion and/or osteoclast invasion of a developing caudal vertebra (Figure 3A). In line with our *in vitro* observations, CH3921 did not affect invasion of osteoclasts *in vivo*, while vascular invasion was significantly delayed ($P < 0.001$, Figure 3B and Table 2). As depicted in Figure 3B and Table 2 the vascular invasion front in CH3921-treated animals did not progress and was retained at the level of day 2. In contrast, CH5902 significantly delayed both the formation of a primitive marrow cavity by osteoclasts and vascular invasion (Figure 3B and Table 2). These data again show that CH3921 selectively inhibits angiogenesis without affecting osteoclastogenesis. In contrast, histological evaluation of the tail vertebrae of the marimastat-treated animals revealed severely affected vertebrae with poorly developed blood vessels and osteoclasts (Figure 3C).
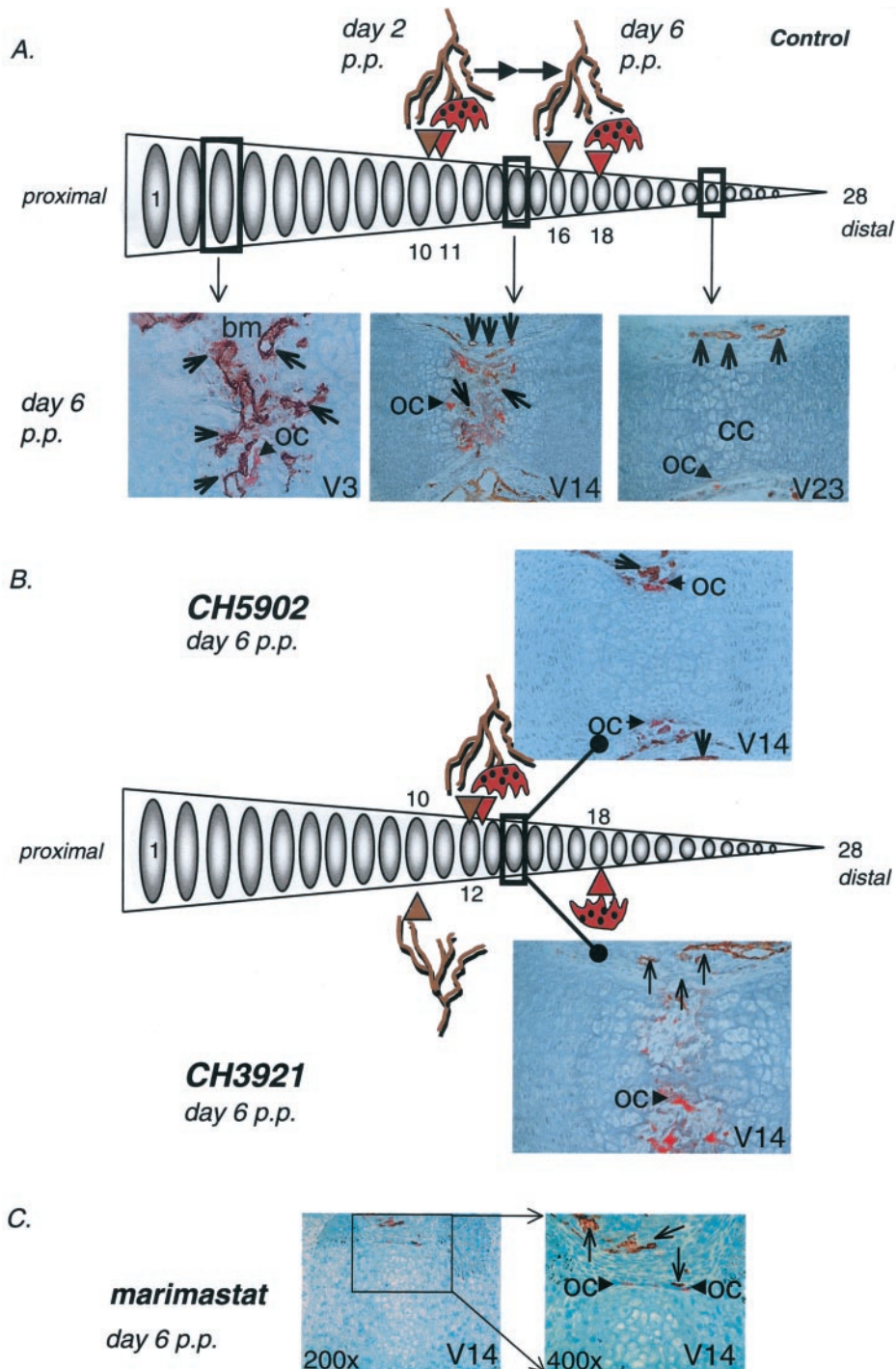
Figure 3. The effect of MMPIs on angiogenesis and osteoclast invasion of caudal vertebrae of neonatal mice *in vivo*. **A:** Schematical representation of angiogenesis and osteoclastogenesis in the caudal vertebrae in the neonatal mouse tails under control conditions (vehicle). Tails were processed and double-stained for TRAcP (red) and lectin (brown). At day 2 post partem (p.p.) osteoclast-precursors and blood capillaries have started to invade the bone collar at caudal vertebrae 11 and 10 respectively (V11, V10). Four days later, at day 6 p.p., osteoclast-precursors and capillaries have progressed in a distal manner and their invasion fronts can now be identified at caudal vertebra 16 (capillaries, V16) and 18 (osteoclasts, V18). Representative images of vertebra 3, 14, and 23 are shown. Original magnifications, ×200 (V14, 23) and ×400 (V3). **Open arrowheads** denote lectin-positive capillaries. oc, osteoclast; bm, bone marrow; ml, calcified cartilage. **B:** Distal progression of the invasion front of blood capillaries and osteoclast-precursors in neonatal mouse tails after 4 days of treatment (day 6 p.p.) with CH5902 or CH3921. Angiogenesis was significantly inhibited by both MMPIs (V12 and V10 for CH5902 and CH3921, respectively). In contrast to CH5902, CH3921 did not affect osteoclastogenesis *in vivo*. Representative images of vertebrae 14 (V14) are shown. Original magnification, ×200. **Open arrowheads** denote lectin-positive capillaries. oc, osteoclast. **C:** Administration of various doses of marimastat was found to be cytotoxic and lead to severe side effects such as growth retardation and, in most cases, death (see text). Morphological changes of osteoclasts and sinusoids after four days of treatment with 6 mg/kg/day marimastat in caudal vertebra 14 (V14; **C**). Neither osteoclasts nor blood capillaries have invaded the calcified cartilage and show signs of severe side effects. Original magnifications, ×200 and ×400. **Open arrowheads** denote lectin-positive capillaries. oc, osteoclast.

Table 2. Caudal Vertebra Number Displaying First Signs of Blood Vessel or Osteoclast Invasion

MMPI	Angiogenesis	Osteoclastogenesis
Control (vehicle)	$16 \pm 0.3^{\dagger}$	18 ± 0.1
CH5902	$12 \pm 1.2^{*}$	$12 \pm 1.0^{*}$
CH3921	$10 \pm 0.9^{*\dagger}$	18 ± 0.7

Data show angiogenesis and osteoclastogenesis, respectively, after 4 days of treatment with MMPIs (60 μg/mouse/day).
*, $P \leq 0.001$ (*vs.* control animals).
$\dagger$, $P \leq 0.005$ (angiogenesis *vs.* osteoclastogenesis in same animal), $n = 6$.

Discussion

Invasion of endothelial cells and osteoclasts into the avascular calcified cartilage has been recognized for a long time but only recently the factors that modulate these processes have been identified.[3–10] Knowledge about the regulation of these events is limited, which is mainly due to the lack of suitable *in vivo* models in which both invasive processes can be studied simultaneously. In the present study we describe the combined use of *in vitro* and *in vivo* models of angiogenesis and osteoclast invasion that allow rapid screening for putative pharmacological agents (drug discovery), and provide information about underlying mechanisms and assessment of *in vivo* toxicities.

Altered expression of MMP activity constitutes part of the pathogenic mechanism associated with a wide range of diseases. These include the destruction of cartilage and bone in rheumatoid and osteoarthritis,[25] tissue breakdown, and remodeling during invasive tumor growth and tumor angiogenesis.[19,26] Given the crucial role of various MMPs in pathological tissue remodeling, targeting MMP activity through synthetic MMPIs has become an attractive strategy.[34,37–41] The redundancy and overlap in function of MMPs requires the development of compounds with improved oral bioavailability that selectively inhibit the MMP-driven process of angiogenesis without affecting normal remodeling of extracellular matrices.[27] We have, therefore, designed selective MMPIs that potently inhibit MMP activities while minimally affecting those of other metalloproteinases (eg, sheddases) involved in the release of cell-associated molecules.

All tested MMPIs inhibited the outgrowth of capillary structures from fetal mouse bone explants in a dose-dependent manner, indicating that all tested MMPIs inhibited the activities of MMP that are required for neovascularization. A different picture emerged when these MMPIs were tested in similar bone explants for their ability to interfere with osteoclast invasion and subsequent osteoclastic resorption. Both marimastat and CH5902 inhibited osteoclastic resorption in a dose-dependent manner. Strikingly, however, continuous treatment of fetal metatarsals with the CH3921 did not inhibit osteoclast invasion and osteoclastic bone resorption. The *in vitro* assays described here may therefore provide the necessary tools to prescreen for active compounds that selectively inhibit angiogenesis or osteoclastogenesis *in vivo*. In addition, an *in vivo* assay (developing mouse tail vertebrae) is essential to monitor toxic side effects. In line with the *in vitro* observations, CH5902 inhibited angiogenesis and osteoclastogenesis during endochondral bone formation *in vivo*. Again, CH3921 selectively inhibited angiogenesis and did not affect osteoclastogenesis in the same vertebrae *in vivo*, which is in agreement with our *in vitro* observations.

Some of the general shortcomings of broad-spectrum MMPIs, despite their favorable effects on tumor progression, are the musculo-skeletal side effects that often occur, which are most likely due to inhibition of MMP activity at those physiological remodeling sites.[37,39–41] Because fast-growing neonatal mice exhibit extensive tissue remodeling, these animals can be of invaluable help to rapidly identify compounds that affect tissue remodeling and to establish potential side effects. This is best illustrated by marimastat, which displayed severe toxic effects characterized by stunted growth and decreased body weight, eventually leading to death. The more selective compounds did not show this general toxicity but still affected tail-length. These effects are compatible with the observation that MMPIs inhibit angiogenesis *in vitro* and *in vivo*.

Taken together, the combined use of these *in vitro* and *in vivo* experimental models may, therefore, greatly facilitate the discovery of mechanisms underlying angiogenesis and lead to identification of pharmacological compounds with potential clinical efficacy and appropriate selectivity in the treatment of angiogenesis-dependent disorders. The identification of the selective MMPI CH3921 may be illustrative for this approach.

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