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Ro 32-3555, an orally active collagenase inhibitor, prevents cartilage breakdown *in vitro* and *in vivo*

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1 Ro 32-3555 (3(**R**)-(cyclopentylmethyl)-2(**R**)-[(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)methyl]-4-oxo-4-piperidinobutyrohydroxamic acid) is a potent, competitive inhibitor of human collagenases 1, 2 and 3 (K_i values of 3.0, 4.4 and 3.4 nM, respectively). The compound is a selective inhibitor of collagenases over the related human matrix metalloproteinases stromelysin 1, and gelatinases A and B (K_i values of 527, 154 and 59 nM, respectively).

2 Ro 32-3555 inhibited interleukin-1α (IL-1α)-induced cartilage collagen degradation *in vitro* in bovine nasal cartilage explants ($IC_{50} = 60$ nM).

3 Ro 32-3555 was well absorbed in rats when administered orally. Systemic exposure was dose related, with an oral bioavailability of 26% at a dose of 25 mg kg^{-1}.

4 Ro 32-3555 prevented granuloma-induced degradation of bovine nasal cartilage cylinders implanted subcutaneously into rats ($ED_{50} = 10$ mg kg^{-1}, twice daily, p.o.).

5 Ro 32-3555 dosed once daily for 14 days at 50 mg kg^{-1}, p.o., inhibited degradation of articular cartilage in a rat monoarthritis model induced by an intra-articular injection of *Propionibacterium* acnes.

6 Ro 32-3555 is a potential therapy for the treatment of the chronic destruction of articulating cartilage in both rheumatoid and osteoarthritis.

Keywords: Cartilage protection agent; collagenase inhibitor; arthritis; cartilage explants; matrix metalloproteinases; pharmacokinetics

Introduction

Rheumatoid arthritis is an immuno-inflammatory disease in which inflammation-induced synovitis precedes articular cartilage destruction. In osteoarthritis, focal splitting and fragmentation of articular cartilage occur early in the disease. Synovitis, when it occurs in osteoarthritis, is slight relative to that seen in rheumatoid arthritis and is secondary to the changes which occur in cartilage and bone. Destruction of articular cartilage is therefore a common feature of both diseases (Krane, 1974) and is thought to contribute directly to their disabling nature. There are currently no therapies which address this aspect of these diseases directly (Scott & Bacon, 1985; Conaghan & Brooks, 1995).

The integrity of articular cartilage is critical for the protection of the underlying bone and for the smooth articulation of the joint. This highly specialized tissue has unique biomechanical properties which allow it to resist compressive loads during joint articulation. The ability of articular cartilage to resist compressive forces derives from the organisation of its two major structural components, type II collagen and proteoglycan. The triple helical collagen provides a dense, fibrillar network within which a high concentration of proteoglycan molecules are immobilized. The hydrophilic characteristics of the proteoglycan molecules induce a swelling pressure within the cartilage which allows it to resist compressive forces, while the type II collagen network gives the tissue its tensile strength. Thus articular cartilage is a fibre-reinforced composite matrix, the biomechanical properties of which are critically dependent on the integrity of the collagen network (Hardingham, 1988). During the physiological remodelling of articular cartilage, turnover of the proteoglycan matrix is very rapid when compared with the turnover of the collagen matrix. When pathological destruction of articular cartilage occurs, there is an initial net loss of the proteoglycan matrix. However, this step is reversible providing the supporting collagen network remains intact (Pettipher *et al*., 1986), it is the subsequent destruction of the collagen network which marks the irreversible step in this process (Fell *et al*., 1976).

The enzymes most frequently implicated in the destruction of articular cartilage in arthritic joints are the matrix metalloproteinases (MMPs). MMPs are a family of zinc endopeptidases which include collagenases 1, 2 and 3 (MMPs 1, 8 and 13), stromelysins 1 and 2 (MMPs 3 and 10) and gelatinases A and B (MMPs 2 and 9) (Birkedal-Hansen *et al*., 1993). They are produced by cells in response to inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (Krane *et al*., 1988) and collectively can degrade all the components of the extracellular matrix (Woessner, 1991). Collagenases are the only mammalian enzymes known which will cleave the triple helix of collagen at physiological pHs and the concentrations of these enzymes are raised in the arthritic joints of animals and man (Brinckerhoff, 1991). Inhibition of the MMPs and specifically the collagenases therefore represents an attractive potential drug target.

Collagenases cleave collagen at a single, well characterized locus approximately three quarters along the helical region of the collagen trimer. Peptides based on the conserved residues adjacent to this cleavage site and containing Zn^{2+} ligands in place of the cleaved amide bond have been identified as potent inhibitors of collagenase (Johnson *et al*., 1987). Subsequently, these inhibitors were shown also to inhibit other members of the MMP family (Nixon *et al*., 1991). The non-selective MMP inhibitors Ro 31-9790 and BB94, were sufficiently metabolically stable to demonstrate efficacy *in vivo* (Lewis *et al*., 1993; Beckett *et al*., 1996). However, Ro 31-9790 has limited oral absorption, while BB94 was not absorbed when dosed orally. Knowledge derived from continued improvements in the understanding of the structure activity relationships of matrix metalloproteinase inhibitors and an awareness of the three

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dimensional structures of these enzymes (Borkakoti *et al*., 1994; Bode *et al*., 1994; Borkakoti, 1997), has led to the identification of inhibitors with improved selectivity. Novel structures also allowed the identification of more orally bioavailable compounds.

The desirable features of good oral bioavailability and selective inhibition of collagenases, are combined in Ro 32-3555 (3(**R**) - cyclopentylmethyl) - 2(**R**) - [(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)methyl] - 4 - oxo-4-piperidinobutyrohydroxamic acid; (Figure 1): a rationally designed, novel, orally bioavailable collagenase inhibitor, which prevents cartilage destruction *in vitro* and *in vivo*. Some of these results have previously been communicated to the British Pharmacological Society (Lewis *et al*., 1996; Sutton *et al*., 1996).

Methods

In vitro *experiments*

Determination of matrix metalloproteinase inhibitor potencies Collagenase 1 was inhibitor affinity purified (Moore & Spilburg, 1986) from medium used to culture human dermal fibroblasts (CCD45) in the presence of recombinant human IL-1α (rH IL-1α) 25 ng ml^{-1} (Gubler *et al*., 1986). Progelatinase A was purified by gelatin-agarose affinity chromatography from the flow through from the inhibitor affinity matrix used for the purification of collagenase 1 (Ward *et al*., 1991). Human prostromelysin 1 was antibody-affinity purified from conditioned human fibroblast culture medium (Ito & Nagase, 1988). Progelatinase B was purified by gelatin-agarose-affinity chromatography from human neutrophils (Ward *et al*., 1991). Human collagenase 2 was purified and assayed according to the method of Dioszegi *et al*. (1995). The catalytic domain of human collagenase-3 was expressed in and purified from *E. coli*. The nucleotide sequence coding for residues Leu 20-Asn 274 coded for by cDNA (Freije *et al*., 1994) of the catalytic domain of procollagenase-3 was amplified from the cDNA. The amplified product was isolated and ligated into KpnI/EcoRI digested pUbPMt (Welch *et al*., 1995). An N-terminal ubiquitin-procollagenase-3 fusion protein was produced in *E. coli* by expression from the pHC3x.2 plasmid in BL21 (DE3) cells. The expression and purification conditions were identical to those described previously for a ubiquitin-promatrilysin protein (Barnett *et al*., 1994) with the following modification: the catalytic domain of collagenase-3 (after activation by 4-amino-phenylmercuric acetate (APMA) such that it no longer contains the ubiquitin fusion or prodomain) was eluted from the sepharose-Pro-Leu-Gly-NHOH affinity column with 1 M NaCl, 0.1 M CAPSO, 0.1 M $CaCl_2$, pH 10.0. Both prostromelysin and progelatinase B were activated by treatment with trypsin, progelatinase A was activated following incubation with APMA, conditions described by Bottomley *et al*. (1997). Ro 32-3555 inhibitor constants were determined by use of fluorogenic peptide substrates (Knight *et al*., 1992; Bottomley *et al*., 1997) at substrate concentrations ($[S] < < K_m$) where IC_{50} approximates to K_i.

Rat interstitial collagenase purified from rat uterine smooth muscle cell culture medium (Quinn *et al*., 1990) was obtained from Prof John J. Jeffery (Albany Medical College, New York). The enzyme was activated by treatment with trypsin (1 μg 200 μg^{-1} rat pro-collagenase, 10 min at room temperature) followed by the addition of trypsin inhibitor (5 μg 200 μg^{-1} rat pro-collagenase). Collagenase activity was assessed by monitoring the cleavage of ^{14}C-rat type I collagen after 16 h at 37°C (Johnson-Wint, 1980).

Bovine nasal cartilage explant assay: measurement of collagen metabolism Bovine nasal cartilage explants (25–30 mg) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 u ml^{-1}, streptomycin (50 μg ml^{-1}) and fungizone (250 μg ml^{-1}) (Bottomley *et al*., 1997). Degradation of collagen was induced by the addition of 114 ng ml^{-1} of rHu-IL-1α to the culture medium, the cultures were incubated for 15 days. During this period culture media containing rHu-IL-1α and inhibitors were renewed every 7 days. When a considerable portion of each cartilage piece had degraded, the assay was stopped by removing the remaining cartilage explant and then analysed for hydroxyproline (Kwok-Chu & Chi-Pui, 1989), as a marker of cartilage collagen content. Glucose utilization was determined by measuring the glucose concentration in culture medium around the explants at the end of the experiment. The samples were analysed by a COBAS Bio (Roche Diagnostics) with a GlucHK-unimate 5 test kit.

Animal experiments

All procedures were performed in accordance with Home Office guidelines, and as specifically licensed under the Animals (Scientific Procedures) Act 1986. Unless stated, Ro 32-3555 was formulated in 5% succinylated gelatin and the volume administered to rats was 10 ml kg^{-1}, p.o.

Pharmacokinetic evaluation of Ro 32-3555 in rats Three groups of three, sexually mature, male, conscious Sprague-Dawley CD rats (266–392 g) were given single doses of Ro 32-3555. One group was given a single intravenous bolus administration at 10 mg kg^{-1} via a jugular vein catheter previously implanted in the animals whilst under isoflurane anaesthesia. The second group was given a single oral dose at 25 mg kg^{-1}, by gavage. The third group received Ro 32-3555 at 10 mg kg^{-1} as a constant rate intravenous infusion over 2 h, the animals in this group had been previously implanted with a jugular vein catheter. Seven timed blood samples were taken from a caudal (tail) vein in each animal by use of a butterfly needle. The anticoagulant used was EDTA, plasma was separated by centrifugation and then stored at −20°C for analysis.

Blood samples were taken 3, 10 and 20 min and 1, 3, 5 and 7 h after the intravenous bolus dose and at 0.25, 0.5, 1, 2, 4, 8 and 24 h after the oral dose. Samples for the intravenous infusion were taken at 0.25, 1, 2, 3, 5, 7 and 24 h after the start of the infusion.

The samples were analysed for Ro 32-3555 by a specific high-performance liquid chromatographic (h.p.l.c.)-mass spectrometric method after extraction of drug related material on C8 Bond-elut cartridges. Minimal chromatography was achieved on a Phenomenex ODS (30) 30 × 4.6 mm, 5 μm guard

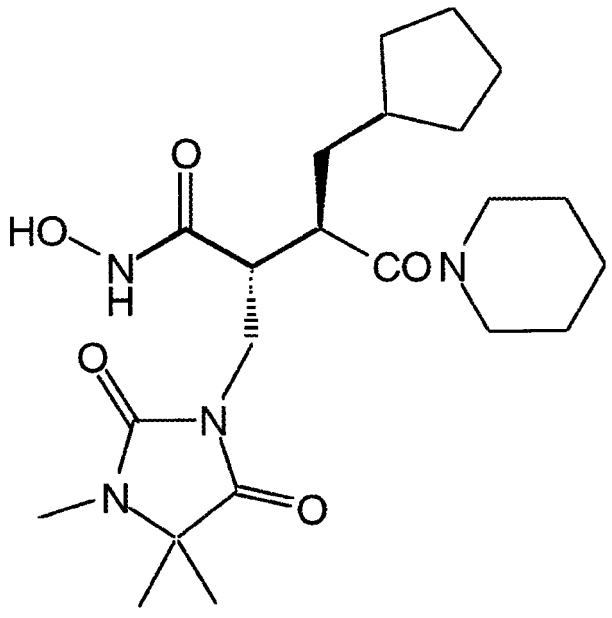

Figure 1 Chemical structure of Ro 32-3555.

column with a mobile phase of 80% acetonitrile in 2.5 mM ammonium formate (pH 3). Selective detection was accomplished on a Sciex (API 111 plus) mass spectrometer in the multiple reaction monitoring mode by atmospheric pressure chemical ionization.

Pharmacokinetic parameters were calculated from the individual data by use of model-independent methods and are quoted as the average data from the three animals in each group. Area under the plasma concentration time curve from 0 to 24 h ($AUC_{0\text{-}24h}$) was calculated by the linear trapezoidal method. Drug plasma concentrations at maximum (C_{max}) and at minimum (C_{min}), and time when plasma drug concentration reached a maximum (T_{max}) were observed values. Bioavailability was calculated from the dose adjusted $AUC_{0\text{-}24h}$ values of the oral dose and the intravenous infusion.

Efficacy of Ro 32-3555 in animal models of cartilage degradation and arthritis

Sponge/cartilage model The techniques used to form and implant sponge/cartilages into rats has been previously described in detail (Bishop *et al*., 1993) but the basic methodology is described briefly.

Sponge/cartilage preparation Nasal septum from young cattle (12 to 18 months old) was obtained from the abattoir. Bone and excess tissue overlying the cartilage was removed under sterile conditions, in an airflow hood. The septum was washed in 95% ethanol before the cartilage was cut into cylinders (2 mm in diameter) by using a leather punch. The cylinders were then trimmed to a final weight of 20 ± 2 mg then placed in a sterile vial containing 0.5 ml of RPMI 1640 medium (Gibco). Discs (6 mm in diameter) of sponge were cut from cellulose sponge (Spontex Ltd., Swansea) and a 2 mm hole cut into the centre with a leather punch. The sponges were soaked in 0.1 ml of a 10 mg ml^{-1} homogenized suspension of heat-killed, *Mycobacterium tuberculosis* in saline (Human strains C, DT and PN, obtained from the Central Veterinary Laboratory, Weybridge, Surrey). The sponges were autoclaved at 120°C, for 15 min, before a weighed cylinder of cartilage was placed into the centre of the sponge. Ten cartilages cylinders were stored at −20°C in medium; these were to measure the levels of collagen in non-implanted cartilages.

Sponge/cartilage implantation and removal Male, Allen and Hanbury hooded rats (AHH/R) were used for all the sponge/cartilage experiments and initial weights were 150–250 g. Animals used in these experiments were obtained from Roche Breeding Unit, housed in groups of five and supplied with food and water *ad libitum*. Groups of eight or ten animals were used for each dose schedule.

Rats were anaesthetized by use of a closed system (Fluovac, Cheshire, U.K.) with isoflurane (Abbott Laboratories Ltd, Kent, U.K.) and oxygen. A small incision was made through the skin overlying the backbone and by using a pair of scissors two subcutaneous channels were formed running from the incision towards the ventral sides. A sponge/cartilage was then placed in each channel and the incision closed by surgical clips (Michel 7.5 mm, Scientific Laboratories Supplies).

At the end of the experiment the animals were killed and the sponge/cartilage implant was excised, the bovine nasal cartilage removed, blotted dry, weighed, placed in medium (RPMI 1640) and then stored at −20°C until required for biochemical analysis.

Cartilage biochemical analysis The individual cartilages were first incubated at 65°C in 0.5 ml of a phosphate buffered (pH 6.5) solution of papain (600 μg ml^{-1}) containing 2 mM N-acetyl-L-cysteine and 2 mM EDTA. The resulting digested cartilages were hydrolysed in 6 M hydrochloric acid for 18 h at 110°C (Bishop *et al*., 1993). The hydrolysate was diluted 1 : 10 in acetate/citrate buffer before being assayed for hydroxyproline by using the Chloramine T/p-dimethylamino-benzaldehyde reaction (Kwok-Chu & Chi-Pui, 1989).

Propionibacterium acnes *induced arthritis* This method was adapted from Trimble *et al*. (1987).

Preparation of P. acnes *Propionibacterium acnes* was obtained from the National Collection of Type Cultures (London, U.K.) as freeze-dried cells and cells propagated in Heart/Brain infusion (Gibco, Oxoid laboratories, U.K.) for three days at 37°C. The infusion was centrifuged (1000 *g* for 10 min) to form a pellet of cells that were resuspended in 10% formalin solution for one hour. The cells were pelleted by centrifugation before resuspension in phosphate buffered saline to wash the cells, this procedure was repeated three times. After the final centrifugation step, the cell pellet was weighed and the cells resuspended in saline (40 mg ml^{-1}) and stored at −80°C until required. Before injection, the cell suspension was mixed 1 : 1 with Freund's incomplete adjuvant (Sigma Ltd., Poole, Dorset, U.K.) and sonicated to form an emulsion.

Induction of arthritis Female, AHH/R strain rats were used in all *P. acnes* experiments. The animals were housed in groups of five and provided with food and water *ad libitum*. Rats were anaesthetized in an isoflurane-closed system, and an intra-articular injection of 20 μl of the *P. acnes*/Freund's incomplete adjuvant emulsion made into the right hind knee. Twenty-eight days later the injection was repeated with the same volume and concentration of antigen to induce the monoarthritis. Animals were orally dosed once daily with either 5% succinylated gelatin as the control vehicle or Ro 32-3555 (50 mg kg^{-1}) starting on day 1 after challenge injection. After fourteen days, the animals were killed according to scheduled methods and the right hind leg removed at the hip joint. Skin and excess muscle were removed from the leg except for muscle overlying the knee joint. The legs were attached to an aluminium plate (10×10 cm with a 120° angle central bend) so that the knee was held at an angle of 120°, and then placed in a solution of 10% formol saline for tissue fixation. The fixed limbs were decalcified in a 10% EDTA solution over a 3 week period before coronal sections were taken. The final histological sections showed the patella, femoral condyles, left and right meniscoids and tibial plateau. Sections were stained with haematoxylin and eosin stain. Image analysis techniques (PC-Image, Foster-Findley Ltd., Newcastle-upon-Tyne, U.K.) were used to quantify the areas of articular cartilage and the overlying pannus. The microscopic field of view (final magnification = 145×) which showed the area from the synovial lining layer to the cruciate ligament of the lateral femoral condyle was used for all sections. The area of articular cartilage was determined morphologically as that lying between the cartilage/pannus and cartilage/bone junctions. The cartilage area was then measured by image analysis techniques with reference to a graticule scale (100 μm). The area of pannus overlying the cartilage was measured in a similar fashion.

Adjuvant arthritis Groups of eight female AHH/R rats were used in these experiments. The animals were dosed twice daily with either 50, 25 or 10 mg kg^{-1} Ro 32-3555, dexamethasone (0.1 mg kg^{-1}, s.c. once/day) or vehicle control (10 ml kg^{-1}, p.o., b.i.d.). The arthritis was induced by injection into the right hind paws with 0.1 ml of a 5 mg ml^{-1} homogenized suspension of *Mycobacterium tuberculosis* in liquid paraffin (Human strains C, DT and PN, obtained from the Central Veterinary Laboratory, Weybridge, Surrey). The volume of both the right and left hind paws was measured by water plethysmography by immersing the paw up to the hair line of the ankle. Paw volumes were determined every two or three days. The change in paw volume was expressed as the difference for days 0 to 5 for the primary inflammatory response and days 9 to 14 for the secondary inflammatory response. The

experiment was terminated after 14 days when the secondary phase of inflammation was evident. The animals were then visually scored for the appearance of inflammatory lesions in the ears, nose, forelimbs, left hind paw and tail as described by Birchall *et al*. (1994).

Carrageenan and zymosan-induced paw inflammation Groups of five female AHH/R rats were used in these experiments. The animals were orally dosed with either 100, 30 or 10 mg kg^{-1} Ro 32-3555, indomethacin (3 mg kg^{-1}) or 5% succinylated gelatin as a vehicle control. Thirty minutes after oral dosing, the animals were injected subcutaneously into the right hand paw with either 0.1 ml of 1% carrageenan (Viscarin) or 0.5% zymosan A (Sigma Co. Ltd., Poole, Dorset, U.K.) suspension formed by boiling the zymosan in phosphate buffered saline for one hour. The paw volume of both the right and left hind paws were determined by water plethysmography by immersing the paw up to the malleous bone. Paw volumes were measured at 0, 2.5 and 5 h after injection of the irritant. The degree of paw swelling was assessed as the difference in volume between the left and right paws.

Results

In vitro *assay results*

Isolated enzyme assays Ro 32-3555 was a potent, competitive inhibitor of human matrix metalloproteinases. The compound was selective for collagenase 1, 2 and 3 relative to related matrix metalloproteinases (Table 1). Ro 32-3555 was also a potent inhibitor of rat collagenase (IC_{50}, mean $\pm$ s.e.mean, $= 44.7 \pm 3.4$ nM ($n = 4$)).

In vitro *cartilage degradation* Ro32-3555 inhibited IL-1α induced cartilage degradation *in vitro* in a concentration-dependent manner with an $IC_{50} = 60$ nM (Figure 2). The inhibition was not mediated by a cytotoxic action on explant chondrocytes. Ro 32-3555, at all concentrations tested, failed to modify glucose utilization when compared to explants cultured in the presence of IL-Lα alone (data not shown).

Table 1 Inhibitory potency of Ro 32-3555 against selected human matrix metalloproteinases (MMP)

	MMP	K_i (nM)	
Collagenase 1	MMP 1	3.0 ± 0	($n = 2$)
Collagenase 2	MMP 8	4.4 ± 0.5	($n = 3$)
Collagenase 3	MMP 13	3.4 ± 1.3	($n = 3$)
Stromelysin 1	MMP 3	527.0 ± 2.7	($n = 3$)
Gelatinase A	MMP 2	154.0 ± 16.9	($n = 5$)
Gelatinase B	MMP 9	59.1 ± 13.6	($n = 4$)

Animal experiments

Pharmacokinetic evaluation of Ro 32-3555 Pharmacokinetic parameters for Ro 32-3555 after i.v. and oral administration to the rat are presented in Table 2. After an i.v. bolus dose of 10 mg kg^{-1} Ro 32-3555 was rapidly distributed and then cleared with a $t_{½}$ of approximately 3 h. The volume of distribution at steady state (1.9 l kg^{-1}) indicated that the compound is widely distributed. Clearance was also high (3.6 l kg^{-1}). Following oral administration at 25 mg kg^{-1} maximum plasma concentrations were rapidly achieved (T_{max} 0.25 h). Bioavailability at this dose was 26%.

Efficacy of Ro 32-3555 in animal models of cartilage degradation and arthritis

Sponge/cartilage model The protective effect of Ro 32-3555 in implanted cartilages is shown in Figure 3. The physical appearance of the cartilages before and after implantation in vehicle-dosed animals was compared to cartilages implanted in the group treated with Ro 32-3555 (5 mg kg^{-1}, b.i.d.). The amount of hydroxyproline in non-implanted cartilage was 119.3 ± 4.2 nmol mg^{-1} and this decreased in cartilages implanted in vehicle-dosed animals to 53.6 ± 7.1 nmol mg^{-1} over a fourteen day period. Animals administered Ro 32-3555 orally at doses of 2.5, 5, 10 and 25 mg kg^{-1} showed statistically increased levels ($P < 0.05$) of implanted cartilage hydroxypro-

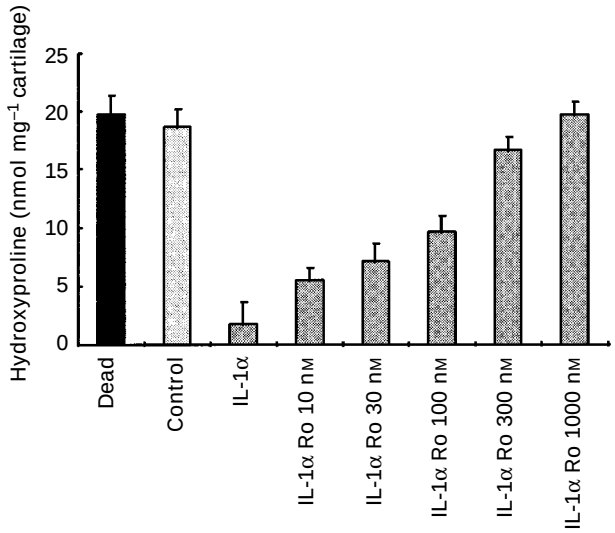

Figure 2 Inhibition of rhu-interleukin-1α (IL-1α) (114 ng ml^{-1}) induced bovine nasal cartilage degradation by Ro 32-3555 (Ro). Each column represents the mean $\pm$ s.e.mean for 10 determinations. The dead cartilages were killed by 3 successive freezing and thawing cycles resulting in chondrocyte death. The remaining explants were cultured alone, or in the presence of 114 ng ml^{-1} rH-IL-1α.

Table 2 Single dose pharmacokinetic parameters of Ro 32-3555 in the rat

Dose of Ro 32-3555	$t_{1/2}\beta$ (h)	$t_{1/2}\alpha$ (h)	T_{max} (h)	C_{max} (ng ml^{-1})	*Cl* (l h^{-1} kg^{-1})	V_{ss} (l kg^{-1})	$AUC_{(o\text{-}inf)}$ (mg h l^{-1})	*F* (%)
10 mg kg^{-1} i.v. bolus	2.95 (0.77–5.65)	0.357 (0.32–0.41)	0.05 (0.05–0.05)	7728 (4532–10140)	3.602 (2.93–4.04)	1.940 (1.295–2.54)	2.832 (2.604–3.415)	
10 mg kg^{-1} infusion	5	0.222	1.33 (1–2)	1787 (1633–2044)	3.20 (2.6–3.8)	1.50 (1.22–2.06)	3.192 (2.632–3.812)	
25 mg kg^{-1} p.o.	3.8 (3.7–3.9)	0.382 (0.31–0.48)	0.25 (0.25–0.25)	1492 (706–2566)			1.971 (1.380–3.003)	26 (17–38)

Pharmacokinetic parameters were: half-lives ($t_{1/2}\alpha$) for first phase and ($t_{1/2}\beta$) for second phase; T_{max}, time when concentration of Ro 32-3555 in plasma was observed to be at a maximum; C_{max}-maximal concentration observed in plasma. Clearance (Cl), volume of distribution of a steady state (V_{ss}); area under the plasma concentration time curve from 0 to 24 h ($AUC_{(0-24\ h)}$) and bioavailability (F). Data shown are mean (minimum–maximum) values.

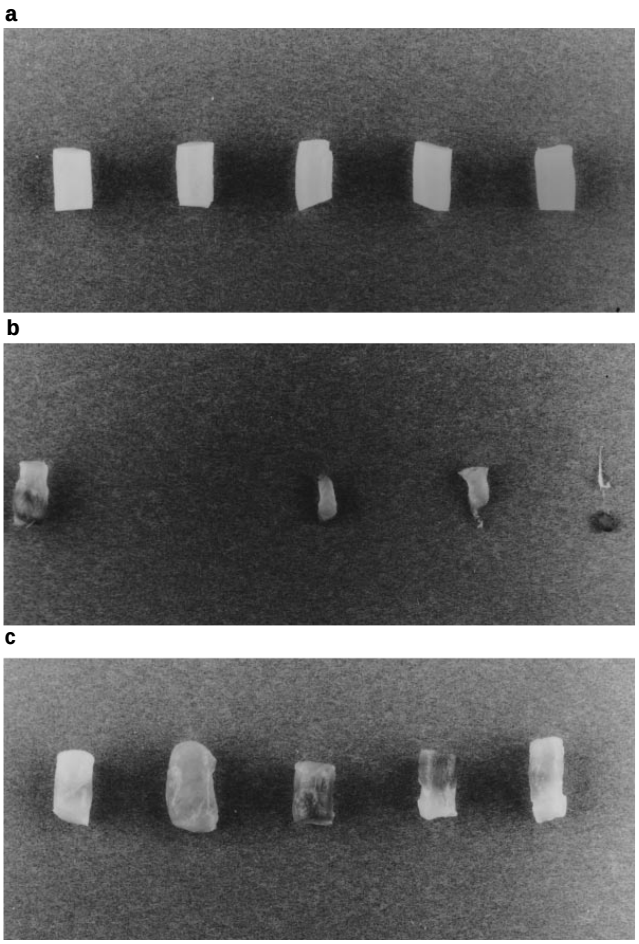

Figure 3 The appearance of bovine nasal cartilage implanted subcutaneously in rats is shown in photographs (a) to (c). The initial state of the cartilage before implantation is represented by (a) non-implanted. Fourteen days after implantation in rats dosed daily with the drug vehicle the cartilage has degraded and appears as (b) implanted cartilage (the second cartilage in the control group was totally degraded). Animals dosed with Ro 32-3555 (5 mg kg^{-1}) for fourteen days after implantation show protection of cartilages as shown in (c). The cartilages in (b) and (c) were the first of five cartilages taken from groups of ten animals implanted with sponge/cartilages where Ro 32-3555 was administered 1 to 25 mg kg^{-1}, p.o., b.i.d.

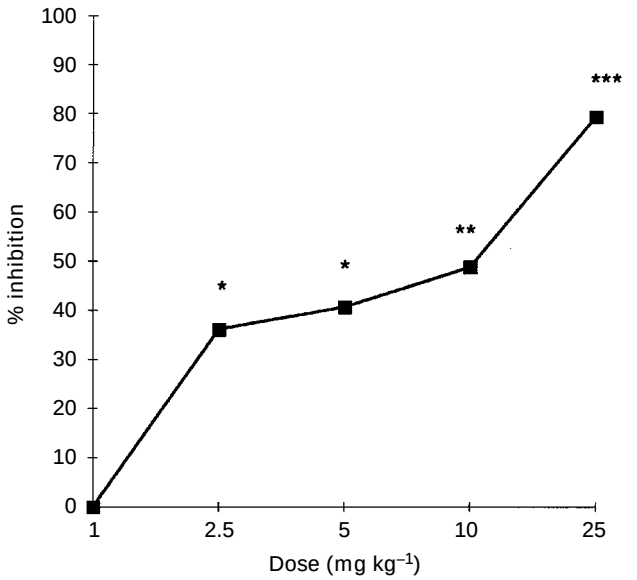

Figure 4 Efficacy of Ro 32-3555 in the rat sponge/cartilage model of cartilage degradation. Bovine nasal cartilage cylinders were placed in cellulose sponges containing 1 mg of dead *Mycobacterium tuberculosis* and implanted subcutaneously into the backs of rats. The rats were dosed orally with either drug-vehicle or Ro 32-3555. After fourteen days the animals were killed and the cartilages were removed, weighed and hydroxyproline content determined. The matrix content of cartilages implanted in vehicle dosed animals and non-implanted cartilage was determined to calculate the inhibitory effect of Ro 32-3555. The graph shows the mean value of the % inhibition of loss of hydroxyproline (■) after administration of Ro 32-3555 (1 to 25 mg kg^{-1}, p.o., b.i.d.). Ro 32-3555 significantly protected the cartilage from loss of hydroxyproline at doses of 2.5 to 25 mg kg^{-1}; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

line (Student's *t* test analysis; unpaired, two-tailed). The calculated ED_{50} for Ro 32-3555 in this experiment was 10 mg kg^{-1}, b.i.d. (Figure 4).

P. acnes *induced arthritis model* Fourteen days after the second challenge injection of *P. acnes*, histological analysis revealed synovitis and the generation of a pannus layer which had overgrown and degraded the underlying articular cartilage (Figure 5). The area of cartilage most consistently affected by pannus was the lateral femoral condyle, which was the area analysed. In non-arthritic animals the mean cartilage area was 0.17 ± 0.02 mm^2 ($n=5$). In arthritic animals there was a significant decrease ($P<0.01$) to a mean area of 0.086 ± 0.01 mm^2 ($n=10$). The group of animals dosed with Ro 32-3555 (50 mg kg^{-1}, p.o.) showed a significantly greater area of cartilage ($P<0.05$) with a mean value of 0.126 ± 0.012 mm^2 ($n=9$). The total mean area of pannus overlying the cartilage was not affected by Ro 32-3555. The pannus area in vehicle-dosed animals was 0.099 ± 0.017 mm^2 and in Ro 32-3555 dosed animals 0.102 ± 0.019 mm^2.

Adjuvant arthritis Injection of adjuvant induced two phases of swelling of the injected paw in vehicle-dosed rats. The primary swelling phase occurred between days 0 to 5 and induced an increase in paw volume of 1.9 ± 0.1 ml; the secondary phase occurred between day 9 to 14 and there was an increase in paw swelling of 0.98 ± 0.08 ml. Ro 32-3555 dosed twice daily (50, 25 and 10 mg kg^{-1}) had no statistically significant effect on the primary inflammatory response (mean paw volume was 1.9 ± 0.1 ml in animals dosed with 50 mg kg^{-1}), or on the secondary inflammatory response (mean paw volume was 0.70 ± 0.2 ml in animals dosed with 50 mg kg^{-1}) of the injected right hand paw. The total lesion score (7.8 in vehicle-dosed animals) was not reduced significantly by Ro 32-3555 at 50 (6.2), 25 (6.3), or 10 (6.2) mg kg^{-1}. The group of animals dosed with dexamethasone (0.1 mg kg^{-1}, s.c.) showed a significant reduction ($P<0.001$, Student's *t* test; unpaired, two-tailed, $n=16$) in both primary (0.2 ± 0.03 ml) and secondary inflammation (0.07 ± 0.08 ml) paw swelling as well as total inhibition of the lesion score (0).

Carrageenan- and zymosan-induced acute inflammation Ro 32-3555 administered 30 min before an injection of carrageenan or zymosan did not significantly inhibit the resultant paw swelling, whereas the positive control drug indomethacin, was effective (Table 3).

Discussion

Ro 32-3555 potently inhibited the breakdown of collagen II within bovine nasal cartilage explants cultured in the presence of IL-1α. This was not a consequence of a cytotoxic action on chondrocytes. The results show that Ro 32-3555 is able to access the tissue and attain sufficient concentrations to inhibit the enzymes involved in the destruction of the cartilage type II collagen. Protection was consistent with inhibition of collagenase(s) secreted from chondrocytes embedded within the cartilage matrix.

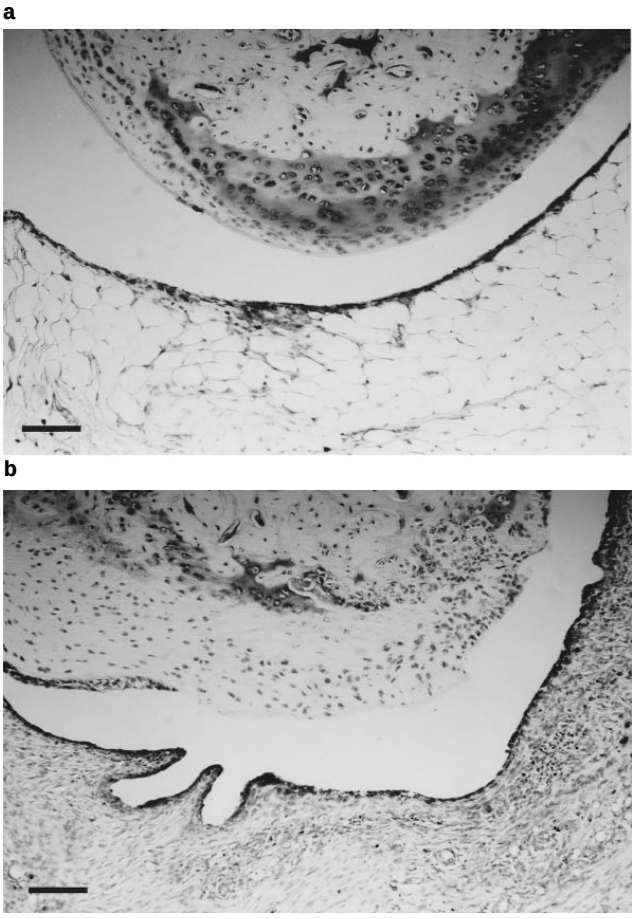

Figure 5 Photomicrograph of the lateral femoral condyle of rat knee from (a) normal, non-arthritic; (b) *P. acnes* induced arthritic rats. The coronal sections were taken through the 120° angled knee and stained with Azure A to highlight the proteoglycan in cartilage. Photomicrograph (a) from a normal rat shows the articular cartilage layer as a dark stained area below the bone matrix (top of micrograph). The space between the femoral and tibial condyle (not shown) is partly filled by a spongy fat-pad (bottom of micrograph). Photomicrograph (b) from a rat with *P. acnes*-induced arthritis shows formation of a pannus layer (dark round nucleated cells) which has overgrown the articular cartilage of the condyle. This pannus layer has caused erosion of the cartilage (loss of dark stained area) and invasion of the bone matrix (right hand side of the condyle). The fat-pad layer has been replaced by infiltrating or proliferating inflammatory cells (bottom of micrograph). The scale shown on the left hand side of the micrographs is equivalent to 100 μm.

The ability of Ro 32-3555 to prevent cartilage breakdown *in vivo* was first assessed with the sponge/cartilage model by measurement of the breakdown of subcutaneously implanted bovine nasal cartilage. Ro 32-3555, when dosed orally, inhibited loss of the collagen marker, hydroxyproline from implanted cartilage. In this model, unlike the *in vitro* cartilage degradation assay, cartilage degrading enzymes can originate not only from the implanted cartilage but also from the cells which form part of the encapsulating granuloma (Bishop *et al*., 1993). The rate and degree of implanted cartilage breakdown is principally dependent on the intensity and duration of the inflammation associated with the granuloma which surround the bovine nasal cartilage. Compounds which reduce the granuloma inflammation might therefore be expected to reduce the rate of cartilage breakdown. Ro 32-3555, at doses where cartilage protection was observed, did not inhibit the acute inflammatory changes seen in either the carrageenan/zymosan paw models or the adjuvant arthritis model. Neither does Ro 32-3555 prevent collagen breakdown because of a toxic reaction in the host animal following repeated daily administration, as in toxicology studies (data not shown), the no observed effect level in the rat after 1 month treatment was 225 mg kg^{-1} day^{-1}. At this no observed effect level, there was a tenfold greater exposure to drug than seen at the ED_{50} dose. Therefore, our results indicate that Ro 32-3555 in the sponge/implant model acts by inhibition of the enzymes involved in the breakdown of the bovine nasal cartilage.

Table 3 Effect of Ro 32-3555 on carrageenan and zymosan-induced paw oedema

Treatment (mg kg^{-1}, p.o.)	*Carrageenan* 2.5 *h*	5 *h*	*Zymosan* 2.5 *h*	5 *h*
Vehicle	0.5±0.07	0.7±0.05	0.6±0.05	0.7±0.04
Ro 32-3555 (100)	0.4±0.10	0.6±0.07	0.6±0.09	0.7±0.08
Ro 32-3555 (30)	0.4±0.04	0.6±0.07	0.7±0.02	0.8±0.08
Ro 32-3555 (10)	0.4±0.06	0.6±0.05	0.7±0.06	0.7±0.06
Indomethacin (3)	0.3±0.06	0.4±0.07*	0.4±0.03*	0.4±0.05*

Values shown are the mean±s.e.mean of 5 rats and *indicates $P<0.05$ compared to vehicle values (Student's t test, unpaired, two-tailed). Compounds were administered 30 min before subcutaneous injection of the irritants into the hind paw of rats.

A model of arthritis involving destruction of articular cartilage induced by a chronic synovitis was developed to mimic the chronic destruction of an articulating joint characteristic of rheumatoid arthritis. In rheumatoid arthritis the major cause of the destruction of articular cartilage and underlying bone is by pannus, which is composed of proliferating synovial lining layer cells plus infiltrating mononuclear cells. The *P. acnes* model shows many of the sequential changes that are associated with rheumatoid arthritis — initial infiltration of the joint by leukocytes, synovitis, formation of a pannus tissue, then degradation of both articular cartilage and underlying bone by pannus. In this model, the articular cartilage of the lateral femoral condyle was affected by pannus-induced degradation. By using strict positioning of the knee it was possible to obtain coronal sections consistently from the same area of the joint and quantify the cartilage changes. Ro 32-3555 significantly protected the articular cartilage in arthritic animals without reducing pannus formation, emphasizing that the compound does not act through an anti-inflammatory mechanism.

In early rheumatoid arthritis when either aggressive therapy or remission intervenes before structural damage occurs, then normal joint function can be restored. Unchecked destruction of articular cartilage, in either rheumatoid (RA) or osteoarthritis (OA), is believed to lead to compromised joint function, which is associated with pain and consequent reduced patient quality of life. In OA significant cartilage destruction and loss of joint function can lead to surgical joint replacement. Preserving joint integrity and function in both RA and OA are major therapeutic goals that are currently unmet by available therapies. Ro 32-3555 is a potent, orally bioavailable drug of low toxicity, which prevents destruction of articular cartilage via inhibition of the enzymes involved in this process. Treatment with Ro 32-3555 in both RA and OA should lead to prevention of structural damage and maintained joint function. Consequently, Ro 32-3555 has the potential to modify underlying disease processes which occur in both RA and OA, thereby maintaining long term joint function.

Poorly regulated MMP activity has also been implicated in a number of diseases additional to arthritis, such as cancer (Stetler-Stevenson *et al*., 1993), periodontal disease (Birkedal-Hansen *et al*., 1993) and inflammatory bowel disease (Anthony *et al*., 1994). The number and diversity of disorders in which poorly regulated MMP activity has been implicated confirm not only the importance of these enzymes in normal biological processes but also the potential for potent, non toxic inhibitors of these enzymes as the basis of future therapies for a variety of diseases.

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