

**Suramin inhibits osteoarthritic cartilage degradation by increasing extracellular levels
of chondroprotective tissue inhibitor of metalloproteinases 3 (TIMP-3).**

Anastasios Chanalaris, Christine Doherty, Brian D. Marsden, Gabriel Bambridge, Stephen P.
Wren, Hideaki Nagase, Linda Troeberg

*Arthritis Research UK Centre for Osteoarthritis Pathogenesis, Kennedy Institute of
Rheumatology, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7FY, UK
(A.C., C.D., G.B., H.N., L.T.); Alzheimer's Research UK Oxford Drug Discovery Institute,
University of Oxford, Oxford, OX3 7FZ, UK (S.P.W.); Structural Genomics Consortium,
University of Oxford, Old Road Campus Research Building, Old Road Campus, Roosevelt
Drive, Headington, Oxford, OX3 7DQ (BDM).*

Running title: Repurposing suramin to inhibit osteoarthritic cartilage loss.

Corresponding author: Linda Troeberg

Address: Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive,
Headington, Oxford OX3 7FY, UK

Phone number: +44 (0)1865 612600

E-mail: linda.troeberg@kennedy.ox.ac.uk

Number of text pages: 25

Number of tables: 0

Number of figures: 4

Number of references: 39

Number of words in *Abstract*: 162

Number of words in *Introduction*: 569

Number of words in *Discussion*: 1588

Abbreviations

ADAMTS, adamalysin-like metalloproteinase with thrombospondin motifs; DMEM, Dulbecco's modified Eagle's medium; DMMB, dimethylmethylene blue; ECM, extracellular matrix; FCS, fetal calf serum; MMP, matrix metalloproteinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; LRP1, low-density lipoprotein receptor-related protein 1; OA, osteoarthritis; PPS, pentosan polysulfate; RAP, receptor-associated protein; TCA, trichloroacetic acid; TIMP-3, tissue inhibitor of metalloproteinases 3.

ABSTRACT

Osteoarthritis is a common degenerative joint disease for which no disease-modifying drugs are currently available. Attempts to treat the disease with small molecule inhibitors of the metalloproteinases that degrade the cartilage matrix have been hampered by a lack of specificity. We aimed to inhibit cartilage degradation by augmenting levels of the endogenous metalloproteinase inhibitor, tissue inhibitor of metalloproteinases 3 (TIMP-3), through blocking its interaction with the endocytic scavenger receptor, low-density lipoprotein receptor-related protein 1 (LRP1). We discovered that suramin ($C_{51}H_{40}N_6O_{23}S_6$) bound to TIMP-3 with a K_D value of 1.9 ± 0.2 nM and inhibited its endocytosis via LRP1, thus increasing extracellular levels of TIMP-3 and inhibiting cartilage degradation by the TIMP-3 target enzyme, adamalysin with thrombospondin motifs 5 (ADAMTS-5). NF279, a structural analogue of suramin, has increased affinity for TIMP-3 and increased ability to inhibit TIMP-3 endocytosis and protect cartilage. Suramin is thus a promising scaffold for the development of novel therapeutics to increase TIMP-3 levels and inhibit cartilage degradation in osteoarthritis.

Introduction

Osteoarthritis (OA) is a common degenerative joint disease, in which cartilage degradation and subchondral bone remodeling cause pain and impaired movement of affected joints. The most common risk factors for OA are age, joint injury and obesity, which all alter the mechanical environment of the joint and initiate catabolic joint remodeling. The disease is estimated to affect 10 % of men and 18 % of women over the age of 60 (Wolf and Pfleger, 2003), and its incidence is predicted to rise with increasing population age and obesity. No disease-modifying drugs are currently available, and treatment is currently limited to management of symptoms by analgesia or joint replacement surgery. There is thus significant clinical need for the development of novel therapeutic strategies.

Articular cartilage covers and protects the ends of bones in articulating joints, enabling smooth, frictionless joint articulation. Degradation of this cartilage layer is a key feature in the pathogenesis of OA. Type II collagen and aggrecan are the major components of the cartilage extracellular matrix, and their degradation underlies the structural failure of the tissue. Studies on transgenic mice have confirmed the central role of two groups of related metalloproteinases in cartilage matrix degradation: matrix metalloproteinases (MMPs) such as MMP-13 degrade type II collagen (Little et al., 2009), while adamalysin with thrombospondin motifs 5 (ADAMTS-5) degrades aggrecan (Glasson et al., 2005; Stanton et al., 2005). These enzymes are thus considered to be potential therapeutic targets for OA, but their conserved catalytic domains have hampered development of sufficiently selective inhibitors to date.

We have adopted an alternative strategy of attempting to block cartilage degradation by increasing levels of the endogenous metalloproteinase inhibitor, tissue inhibitor of metalloproteinases 3 (TIMP-3), in the joint. The chondroprotective role of TIMP-3 is illustrated by studies showing that mice lacking the gene for *Timp3* develop accelerated OA as they age (Sahebjam et al., 2007), and conversely that recombinant TIMP-3 inhibits development of OA

in a rat model of disease (Black et al., 2006). TIMP-3 levels are reduced in OA cartilage, although the mRNA levels are not altered (Morris et al., 2010).

We found that TIMP-3 levels are primarily controlled post-translationally, and that TIMP-3 is readily endocytosed from the extracellular environment by the endocytic scavenger receptor low-density lipoprotein receptor-related protein 1 (LRP1) (Troeberg et al., 2008; Scilabra et al., 2013). We engineered mutants of TIMP-3 that do not bind to LRP1, and showed that they have a longer half-life in cartilage and protect cartilage better than wild-type TIMP-3 (Doherty et al., 2016). Sulfated glycosaminoglycans such as heparin, heparan sulfate and pentosan polysulfate (PPS) are also able to inhibit cartilage degradation by inhibiting TIMP-3 binding to LRP1 and thus increasing extracellular levels of TIMP-3 (Troeberg et al., 2009; Scilabra et al., 2013; Troeberg et al., 2014). However, such sulfated glycosaminoglycans have poor pharmacokinetics and limited clinical scope. We thus sought to identify a small-molecule inhibitor of TIMP-3 endocytosis that could serve as a lead compound for the development of novel OA therapeutics.

Yu et al. (2000) showed that TIMP-3 could be solubilized from extracellular matrices by suramin, a historic anti-parasitic and anti-helminthic drug. Here we show that suramin binds to TIMP-3 and inhibits its endocytosis by LRP1, and that suramin blocks degradation of both normal porcine cartilage and human OA cartilage in explant culture. We thus propose that suramin is a promising scaffold from which to develop a new type of therapeutic inhibitor to treat OA.

Materials and Methods

Materials. C-terminally FLAG-tagged human TIMP-3 was expressed in HEK-293 cells and purified as previously described (Troeberg et al., 2009). Receptor-associated protein (RAP) was expressed in *E. coli* and purified as described previously (Yamamoto et al., 2013). C-

terminally FLAG-tagged ADAMTS-5 lacking the C-terminal thrombospondin domain was expressed in HEK293 cells and purified as previously described (Gendron et al., 2007). The catalytic domain of MMP-1 and -3 were expressed in *E. coli* and purified as previously described (Suzuki et al., 1998; Chung et al., 2000).

Suramin hexasodium salt and suramin analogues (NF023, NF110, NF157, NF279, NF340, NF449, NF546) were from Tocris Bioscience (Bristol, UK). Pentosan polysulfate was from Bene-PharmaChemie (Geretsried, Germany). Amphotericin B and M2 anti-FLAG antibody were from Sigma-Aldrich (Dorset, UK). TIMP-3 antibody (clone 183551, catalogue number MAB973) and mouse IgG1 isotype control (clone 11711, catalogue number MAB002) were from R&D (Abingdon, UK), and anti-LRP1 (8G1, catalogue number ab20384) was from AbCam (Cambridge, UK). Quenched fluorescent substrates for MMPs and ADAMTS-5 were from Bachem (Bubendorf, Switzerland)

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, amphotericin, HEPES, trypsin-EDTA were from PAA Laboratories (Somerset, UK). Foetal calf serum (FCS) was from Gibco (Paisley, UK). Eppendorf Protein LoBind tubes were from VWR (East Grinstead, UK).

TIMP-3 binding to suramin and analogues. Glycosaminoglycan-binding ELISA plates (BD Life Sciences, Swindon UK) were coated with suramin or its analogues (10 µg/ml in Tris-buffered saline, 18 h, 25 °C) (Mahoney et al., 2004) and wells blocked with 0.2 % gelatin in PBS (1 h, 37 °C). Wells were washed in PBS containing 0.1 % Tween 20 after this and every subsequent step. Purified FLAG-tagged human TIMP-3 (0.4 - 50 nM) in blocking solution was applied to wells (3 h, 37 °C), and binding detected with anti-FLAG M2 primary antibody and anti-mouse HRP-conjugated secondary antibody. 3, 3', 5, 5'-tetramethylbenzidine (Becton Dickinson, Swindon, UK) substrate was added, the reaction stopped when appropriate by addition of 2 N H₂SO₄, and absorbance at 450 nm measured using a FLUOstar OMEGA

microplate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK). Data (mean \pm SE, $n = 3$ technical repeats) were analysed using Prism (GraphPad Software, La Jolla, CA) and EC₅₀ values determined using a one site specific binding model. Flat planar dimensions of suramin analogues were estimated using ICM-Pro.

TIMP-3 binding to LRP1. LRP1 (5 nM, BioMac, Leipzig, Germany) was coated (overnight, 4 °C) onto medium-binding ELISA plates (Greiner Bio-One, Stonehouse, UK) in 20 mM HEPES, 150 mM NaCl, 5 mM CaCl₂, 0.05 % Tween-20, pH 7.4. Wells were blocked with 10 % BSA in TNC buffer (50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.05 % Brij 35). Wells were washed in TNC buffer containing 0.1 % Tween 20 after this and every subsequent step. FLAG-tagged human TIMP-3 (0.4 - 50 nM), either alone or pre-incubated with suramin (150 µg/ml, 1 h, 37 °C) was applied to wells in TNC buffer containing 5 % BSA (3 h, 25 °C). Binding was detected with anti-FLAG M2 primary antibody and anti-mouse HRP-conjugated secondary antibody in the same buffer. 3, 3', 5, 5'-tetramethylbenzidine (Becton Dickinson, Swindon, UK) substrate was added, the reaction stopped when appropriate by addition of 2 N H₂SO₄, and absorbance at 450 nm measured using a FLUOstar OMEGA microplate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK). Data (mean \pm SD, $n = 3$) were analysed using Prism software (GraphPad Software, La Jolla, CA).

Cell and cartilage explant culture. HTB94 chondrosarcoma cells (American Culture Type Collection, Manassas, VA) were maintained in DMEM with 10 % FCS, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C in 5 % CO₂.

Porcine and human cartilage explants and chondrocytes were maintained in DMEM with 10 % FCS, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mg/ml amphotericin B and 10 mM HEPES at 37 °C in 5 % CO₂. Porcine articular cartilage was dissected from metacarpophalangeal joints of 3 - 9 month old pigs within 24 h of slaughter. Explants were prepared using a biopsy punch to ensure uniformity of size and rested for 48 h before use.

Chondrocytes were isolated by incubating dissected cartilage with type 2 collagenase (1 mg/ml, Worthington, Lakewood, NJ USA) in DMEM with 10 % FCS (18 h, 37 °C). Cells were passed through a cell strainer and washed twice before plating.

Osteoarthritic human articular cartilage was obtained from patients undergoing knee replacement surgery. Tissue samples were obtained from the Oxford Musculoskeletal Biobank and were collected with informed donor consent in full compliance with national and institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki (HTA License 12217 and Oxford REC C 09/H0606/11). Human cartilage explants and chondrocytes were prepared as described for porcine cartilage above.

TIMP-3 endocytosis assays. Cells (HTB94 chondrosarcoma, primary porcine chondrocytes or human osteoarthritic chondrocytes) were plated overnight (6×10^5 cells per well of a 12-well plate) in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS and washed 3 times in serum-free DMEM.

To evaluate endocytosis of exogenously added TIMP-3, cells were incubated with recombinant TIMP-3 (1 nM in 1.5 ml DMEM with 0.1 % FCS) for 0 - 24 h. Conditioned media were concentrated by precipitation with trichloroacetic acid [TCA, 5 % (v/v), 4 °C, 18 h]. After centrifugation (13 000 rpm, 4 °C, 10 h), protein-containing pellets were resuspended in SDS sample buffer (30 μ l), electrophoresed (7 μ l) on a 10 % polyacrylamide gel, and immunoblotted onto polyvinylidene fluoride. After blocking in 5 % (m/v) BSA in Tris-buffered saline, TIMP-3 levels were analysed using an M2 anti-FLAG antibody (Sigma-Aldrich, Dorset, UK), an alkaline phosphatase-conjugated anti-mouse secondary antibody (Promega, Southampton, UK) and Western Blue stabilized substrate for alkaline phosphatase (Promega, Southampton, UK). Immunoblots were analysed by densitometry using Phoretix 1D densitometry software (TotalLab, Newcastle-upon-Tyne, UK), and TIMP-3 remaining in the medium (mean \pm SD, n = 3) calculated relative to pixel volume at t = 0 h (defined as 100 %). TIMP-3 in the medium

(mean \pm SD, $n = 3$) was calculated relative to the pixel volume of untreated cells (defined as 1).

To evaluate accumulation of endogenous TIMP-3, cells were incubated with suramin (50 - 200 μ g/ml) in serum-free DMEM for 30 h. Media were harvested, TCA precipitated and analyzed by immunoblotting using a rabbit anti-TIMP-3 polyclonal antibody (AB6000, Millipore, Hertfordshire, UK) and an alkaline phosphatase-conjugated anti-rabbit secondary antibody (Promega, Southampton, UK).

mRNA analysis. HTB94 chondrosarcoma or primary human chondrocytes (1×10^6 cells) were treated in triplicate with 0-250 μ g/ml suramin in serum-free DMEM for 18-48 h. Total RNA was isolated from cells using a QIAGEN RNeasy mini kit (Qiagen, Crawley UK) and cDNA synthesized (Reverse Transcriptase kit, Applied Biosystems, Foster City, CA). Levels of TIMP1, TIMP2, TIMP3 and RPLP0 internal reference mRNA were quantified by real-time PCR on a Corbett Rotor-Gene 6000 (Corbett Life Science) using a TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and TaqMan probes (Applied Biosystems, Foster City, CA) Hs01092512_g1 for TIMP1, Hs00234278_m1 for TIMP2, Hs00165949_m1 for TIMP3, Hs00233856_m1 for LRP1, Hs99999902_m1 for RPLP0. $\Delta\Delta C_t$ values (mean \pm SD, $n = 3$ technical replicates) are shown relative to control untreated HTB94 cells (defined as 1).

Cartilage explant cultures. The effects of suramin on cartilage degradation were assessed using porcine or human cartilage explants. Explants were rested for 48 h after dissection, and washed with serum-free DMEM. Explants were then treated with retinoic acid (1 μ M, Sigma-Aldrich, Dorset UK), IL-1 (10 ng/ml, Peprotech, London, UK) and/or suramin (0 - 250 μ g/ml) in serum-free DMEM for 48 h.

Aggrecan degradation was analyzed by quantifying aggrecan fragments released into the conditioned media using the dimethylmethylene blue (DMMB) dye-binding assay (Farndale et

al., 1986) (mean \pm SD, n=3), as well as immunoblotting with neo-epitope antibodies that recognise ADAMTS-cleaved but not intact aggrecan. For immunoblotting, conditioned media were deglycosylated by adding an equal volume of 200 mM sodium acetate, 50 mM Tris/HCl, pH 6.8 containing chondroitinase ABC and β -endoglycosidase (0.05 units each, Sigma-Aldrich, Dorset UK, 18 h, 37 °C). Aggrecan fragments were precipitated by addition of 5 volumes of ice-cold acetone (18 h, -20 °C). After centrifugation (13 000 rpm, 10 min, 4 °C), pellets were resuspended in SDS sample buffer, separated by electrophoresis on a 6% (v/v) polyacrylamide gel and blotted onto polyvinylidene fluoride. Membranes were blocked with 5 % (m/v) BSA-TBS and incubated with a rabbit antibody recognizing the AGE_G neo-epitope generated by ADAMTS cleavage of the TAQE^{1771~1772}AGE_G bond of aggrecan (Troeberg et al., 2008), or a mouse antibody that recognizes the ARG_{SV} neo-epitope generated by ADAMTS cleavage of the NITEGE^{373~374}ARG_{SV} bond (Hughes et al., 1995).

Cell viability assays. HTB94 chondrosarcoma or human OA chondrocytes (10³ cells per well in 96 well plates) were plated overnight in DMEM with 10% FCS. Cells were washed into serum-free cartilage medium and treated for 48 - 72 h with suramin (0 - 250 μ g/ml) or sodium nitroprusside (SNP, 10 mM) as a control to induce cell death. Cell viability was then assessed using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] CellTiter Cell Proliferation Assay (Promega, Southampton, UK) according to the manufacturer's instructions (mean \pm SD, n = 3).

TIMP-3 inhibition of target metalloproteinases. The inhibition constant, K_i (app), for TIMP-3 inhibition of ADAMTS-5, MMP-1 and MMP-3 was determined under equilibrium kinetic conditions using the tight binding equation (Bieth, 1995). TIMP-3 (0.5 nM) was incubated with target metalloproteinase (0.05 nM ADAMTS-5 or MMP-1) and/or suramin (0.05 μ g/ml) or pentosan polysulfate (0.05 μ g/ml) for 1 h at 37 °C, and residual enzyme activity against a quenched fluorescent substrate determined. ADAMTS-5 activity was monitored

using 20 μ M ortho-aminobenzoyl-Thr-Glu-Ser-Glu~Ser-Arg-Gly-Ala-Ile-Tyr-(N-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Lys-Lys-NH₂ and MMP-1 and MMP-3 activity was monitored using 1.5 μ M 7-methoxycoumarin-4-yl) acetyl-Pro-Leu-Gly-Leu-(N-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH₂, as described previously (Troeberg et al., 2009). Steady-state velocities were determined using a Gemini microplate spectrofluorimeter (Molecular Devices, Wokingham, UK). K_i (app) (mean \pm SD, n = 3 - 5 independent experiments) was calculated using GraphPad Prism to fit data the data to the tight binding equation (Bieth, 1995):

$$\frac{v_s}{v_o} = 1 - \frac{(E_o + I_o + K_{i(app)}) - [(E_o + I_o + K_{i(app)})^2 - 4E_o K_{i(app)}]^{1/2}}{2E_o}$$

where v_o is equilibrium rate of substrate hydrolysis in the absence of inhibitor, E_o is the total enzyme concentration, I_o is the total inhibitor concentration and $K_{i(app)}$ is the apparent inhibition constant.

Results

Suramin inhibits the endocytosis of TIMP-3 by LRP1 scavenger receptor. As a first step to evaluating the effect of suramin on TIMP-3 endocytosis by the scavenger receptor LRP1, we examined TIMP-3 binding to immobilized suramin in a solid-phase binding assay. TIMP-3 bound strongly to suramin, with a K_D value of 1.9 ± 0.2 nM (Fig 1A). TIMP-3 also bound strongly to immobilized LRP1 (Fig 1B), and this binding was abolished when TIMP-3 was pre-incubated with suramin (200 μ g/ml, Fig 1B).

We have previously shown that the rate of TIMP-3 endocytosis by LRP1 can be quantified by adding purified FLAG-tagged recombinant TIMP-3 (1 nM) to HTB94 chondrosarcoma cells and monitoring its disappearance from the medium (Doherty et al., 2016). In the absence of

suramin, TIMP-3 was taken up from the medium with a half-life of 4.0 ± 1.3 h (Fig 1C). Pre-incubation of TIMP-3 with suramin (200 $\mu\text{g/ml}$, 1 h, 37 °C) markedly inhibited this uptake, and more than 85 % of TIMP-3 remained in the medium after 8 h and a half-life could not be accurately calculated.

Suramin also inhibited endocytosis of endogenously-expressed TIMP-3 in HTB94 cells. No TIMP-3 was detectable in the medium of untreated HTB94 cells, but TIMP-3 accumulated when cells were treated with suramin (50 - 200 $\mu\text{g/ml}$, Fig 1D). This increase in TIMP-3 in the medium was not associated with any significant change in TIMP-3 mRNA levels (Fig 1E). Suramin had no effect on cell viability, in contrast with sodium nitroprusside, a known cytotoxic agent (Fig 1F).

Suramin similarly inhibited endocytosis of endogenously-expressed TIMP-3 in primary chondrocytes isolated from human OA cartilage or normal porcine cartilage (Fig 1G), without any significant change in TIMP3 mRNA levels (Fig 1H) or cell viability (Fig 1I).

Suramin had no effect on TIMP1 or TIMP2 mRNA levels in HTB94 cells (Fig 2A, B). Expression of LRP1 was unaffected by suramin at concentrations up to 200 $\mu\text{g/ml}$, although a 3-fold increase in expression was detected at 250 $\mu\text{g/ml}$ ($P < 0.001$, Fig 2C). Shedding of LRP1 ectodomain into the medium was also not affected by suramin (50 – 250 $\mu\text{g/ml}$, Fig 2D).

Suramin does not alter TIMP-3 activity. We evaluated whether TIMP-3 binding to suramin had any effect on its inhibition of target metalloproteinases *in vitro*. ADAMTS-5 is considered to be the primary aggrecan-degrading enzyme in murine (Glasson et al., 2005; Stanton et al., 2005) and human (Ismail et al., 2015) cartilage. TIMP-3 had an apparent affinity constant, $K_i(\text{app})$, of 2.03 ± 0.65 nM for ADAMTS-5 in the absence of suramin (Fig 2E), in line with previous reports (Doherty et al., 2016). Pre-incubation of TIMP-3 with suramin had no significant effect on this affinity, with $K_i(\text{app}) = 1.60 \pm 0.62$ nM (Fig 2C). In contrast, PPS

reduced $K_i(\text{app})$ to a value too low to be calculated (Fig 2E), as previously reported (Troeborg et al., 2012).

Suramin also had no effect on TIMP-3 affinity for MMP-1, with $K_i(\text{app}) = 0.66 \pm 0.16$ nM in the absence of suramin (Fig 2F) and $K_i(\text{app}) = 0.65 \pm 0.09$ nM in the presence of suramin (Fig 2F). Affinity for MMP-3 was similarly unaffected, with $K_i(\text{app}) = 1.14 \pm 0.93$ nM in the absence of suramin, and $K_i(\text{app}) = 0.76 \pm 0.09$ nM in the presence of suramin.

Suramin inhibits cartilage degradation. The effect of suramin on cartilage degradation was evaluated by measuring its effect on aggrecan release from cartilage explants in vitro. Explants of human knee cartilage were obtained at the time of joint replacement surgery for osteoarthritis. Treatment of the explants with IL-1 or retinoic acid stimulated matrix catabolism, with the dimethylene methylene blue (DMMB) assay indicating a 2- to 3-fold increase in the amount of aggrecan released into the conditioned medium (Fig 3A, C). This aggrecan degradation was dose-dependently inhibited by suramin, with an IC_{50} of 62 ± 16 $\mu\text{g/ml}$ (Fig 3C). Similar efficacy was observed in cartilage from 5 other donors.

Suramin also effectively inhibited aggrecan release from IL-1 or retinoic acid-stimulated porcine cartilage explants, with an IC_{50} of 98 ± 9 $\mu\text{g/ml}$ (Fig 3D). Immunoblotting with the anti-ARGSV and anti-AGEG neo-epitope antibodies showed that suramin inhibited aggrecan degradation at the ADAMTS-susceptible NITEGE^{373~374}ARGSV and TAQE^{1771~1772}AGEG sites (Fig 3E), indicating that aggrecanase activities were blocked.

To investigate whether protection by suramin was dependent on TIMP-3, we stimulated cartilage with retinoic acid and added suramin in combination with a TIMP-3 antibody or an isotype control antibody. In the presence of the isotype antibody, suramin significantly inhibited aggrecan release (Fig 3F). However, in the presence of the anti-TIMP-3 antibody, suramin was unable to inhibit aggrecan degradation.

Suramin analogue NF279 shows improved efficacy. To further understand the mode of suramin action and to identify additional bioactive compounds, we tested the ability of 7 commercially available suramin analogues to inhibit cartilage degradation. Porcine cartilage explants were stimulated with retinoic acid in the presence of 200 $\mu\text{g/ml}$ of suramin or a suramin analogue for 48 h, and cartilage degradation quantified using the DMMB assay. NF279 showed markedly improved activity, inhibiting both retinoic acid-stimulated and unstimulated release of aggrecan fragments (Fig 4A), with $\text{IC}_{50} = 15.6 \pm 10 \mu\text{g/ml}$.

The affinity of the analogues for TIMP-3 was evaluated using a solid-phase binding assay. NF279 had the highest affinity for TIMP-3, with a K_D value of $0.85 \pm 0.09 \text{ nM}$ (Fig 1A). NF157 had similar affinity for TIMP-3 ($K_D = 1.5 \pm 0.11 \text{ nM}$) as was seen for suramin ($K_D = 1.9 \pm 0.2 \text{ nM}$, as in Fig 1A), with the remaining analogues showing lower affinity than suramin.

The ability of the analogues to promote TIMP-3 accumulation was assessed by incubating HTB94 chondrosarcoma cells with suramin or the suramin analogues (200 $\mu\text{g/ml}$) for 36 h and quantifying TIMP-3 levels in the medium by immunoblotting. Highest levels of TIMP-3 accumulated in cells treated with NF279 (Fig 4C, $233 \pm 73 \%$ relative to suramin, defined as 100 %). NF340 and NF546 showed minimal efficacy.

A strong exponential correlation was observed between the level of TIMP-3 accumulation (relative to suramin, defined as 100%) and the \log_{10} of analogue affinity for TIMP-3, with $r = -0.8654$ ($p = 0.0119$, Fig 4D). The level of TIMP-3 accumulation negatively correlated with aggrecan release, although this was non-significant (Fig 4E, $r = -0.6238$, $p = 0.0984$).

Discussion

OA has long been thought of a disease arising from passive wear-and-tear of the joints, and as an inevitable consequence of aging. However, studies on knockout mice over the last decade have conclusively shown that OA is an active disease, in which various risk factors stimulate

the cells in joint tissues to increase their catabolic activity. The roles of metalloproteinases in cartilage degradation are well established, with MMP-13 known to drive cleavage of type II collagen (Little et al., 2009) and ADAMTS-5 to drive cleavage of aggrecan (Glasson et al., 2005; Stanton et al., 2005). The endogenous inhibitor of these enzymes, TIMP-3, is an important chondroprotective molecule, with *Timp3*-null animals developing accelerated OA (Sahebjam et al., 2007). The observation that TIMP-3 levels are reduced in human OA cartilage (Morris et al., 2010) led us to examine the molecular mechanisms regulating TIMP-3 levels, and to the discovery that TIMP-3 is regulated post-translationally by endocytosis via the scavenger receptor LRP1 (Troeberg et al., 2008; Scilabra et al., 2013).

We recently engineered LRP1-resistant mutants of TIMP-3, and found that these have a longer half-life in cartilage and inhibit cartilage degradation at lower concentrations and for longer than wild-type TIMP-3 (Doherty et al., 2016). This illustrates that targeting the TIMP-3 endocytosis pathway is a potential strategy for inhibiting cartilage loss in OA. Administration of recombinant protein is unlikely to be a feasible therapeutic option for OA treatment, so we sought to develop small molecule inhibitors of TIMP-3 endocytosis that could increase levels of the endogenous inhibitor in cartilage. We show here that suramin, a polysulfonated naphthalene derivative of urea, binds to TIMP-3 and inhibits its endocytic uptake by cells through the LRP1 scavenger receptor, inhibiting degradation of aggrecan by ADAMTSs in cartilage.

TIMP-3 has a region of basic amino acids on its surface, and lysine and arginine residues in this area have been shown to mediate binding to sulfated proteoglycans (Lee et al., 2007; Troeberg et al., 2012) and LRP1 (Doherty et al., 2016). Suramin is likely to interact with this region of TIMP-3 via its negatively charged polysulfonated naphthylamine groups, and hence to block interaction with LRP1. This basic region is on the opposite side of TIMP-3 to the one that interacts with target metalloproteinases, suggesting that suramin should not impair TIMP-

3 inhibition of metalloproteinases. Indeed, we found that suramin had no effect on TIMP-3 affinity for ADAMTS-5, MMP-1 and MMP-3 *in vitro*. We previously found that sulfated molecules like heparin, heparan sulfate and pentosan polysulfate could increase TIMP-3 affinity for ADAMTS-4 and ADAMTS-5 (Troeberg et al., 2008; Troeberg et al., 2012; Troeberg et al., 2014) , but this was not observed for suramin. This is likely to be because suramin is too short to simultaneously bind to basic regions on TIMP-3 and the enzymes, and so is unable to support formation of high affinity trimolecular complexes.

Previous crystallography studies indicated that LRP1 ligands bind to the receptor through a pair of lysine residues 21 Å apart, that interact with 2 acidic pockets on sequential complementary repeats of LRP1 (Fisher et al., 2006). Mutation of several lysine residues on RAP, a prototypic LRP1 ligand, reduce binding to LRP1, indicating that the extended charge landscape is important for orienting the 2 lysine residues that interact with the complementary repeat pockets (van den Biggelaar et al., 2011; Dolmer et al., 2013; Prasad et al., 2016). Our mutagenesis study of TIMP-3 supports this model, with LRP1 binding being reduced by mutation of several pairs of lysine residues separated by 21 ± 5 Å (Doherty et al., 2016). In an extended flat planar conformation, suramin has an estimated maximal length of 38 Å. While the molecule is likely to adopt numerous conformations in solution, this indicates it is of sufficient length for the two clusters of sulfonate groups on either end of the suramin molecule to bind to the LRP1-interacting di-lysine motif on TIMP-3 and thus to block TIMP-3 interaction with the endocytic receptor.

We evaluated 7 commercially available suramin analogues, and found that NF279 exhibited an improved chondroprotective activity, with a 6-fold improved IC_{50} value of 15 µg/ml (11.6 µM). This correlated with an increased affinity for TIMP-3 and accumulation of higher TIMP-3 levels in NF279-treated cells. The structure of NF279 is very similar to that of suramin, with 2 clusters of sulfonate groups at each end of the molecule (Fig 4F) and an

estimated maximal length of 39 Å in an extended planar conformation, suggesting it has a similar binding mode to suramin. Shorter analogues (such as NF340 with an estimated maximal length of 23 Å) and or analogues with fewer sulfonate groups (such as NF546) were less effective at protecting cartilage and supporting TIMP-3 accumulation. NF279 may adopt a more extended conformation in solution than suramin, since the phenyl links in the middle of the compound are in para, rather than meta, orientations. This may enable NF279 to more effectively bridge LRP1-interacting residues on TIMP-3. Further analogues will need to be evaluated to improve understanding of the structure-activity relationship of the suramin scaffold.

Suramin has been shown to ameliorate cartilage damage in a rat inflammatory arthritis model of rheumatoid arthritis (Sahu et al., 2012), with reduced levels of pro-inflammatory cytokines in the plasma and joints of treated animals. While osteoarthritis is associated with metalloproteinase degradation of cartilage extracellular matrix components, rheumatoid arthritis is an inflammatory disease driven by pro-inflammatory cytokines, such as TNF and interleukin 1. TIMP-3 inhibits release of active TNF by inhibiting the activity of the metalloproteinase ADAM17 (or TNF α -converting enzyme, TACE)(Mohammed et al., 2004), so we hypothesise that suramin also protected cartilage in this inflammatory arthritis model by blocking endocytosis of TIMP-3 by LRP1. While the pathological role of inflammation in OA is speculative rather than proven, suramin's ability to inhibit inflammation as well as metalloprotease-driven cartilage breakdown is likely to further augment its chondroprotective effect.

Suramin has been shown to have several biological effects, so it is possible that suramin protects cartilage through molecular mechanisms other than inhibiting TIMP-3 endocytosis. Since its development in 1916, suramin has been used to treat human infection with protozoan *Trypanosoma* and helminthic *Onchocerca* parasites (Hawking, 1978; Voogd et al., 1993). Its

mechanism of anti-parasitic action is unclear. Suramin inhibits several trypanosomal glycolytic enzymes *in vitro* (Misset and Opperdoes, 1987) and reduces ATP generation *in vivo* (Fairlamb and Bowman, 1980). Suramin has been shown to inhibit MMP-9 activity (Taniguti et al., 2012), and we found it can also inhibit MMP-2 activity *in vitro* (data not shown). This is unlikely to contribute to the chondroprotection observed in the current study, where cartilage degradation in the first 1 - 3 days after explant stimulation is mediated by ADAMTSs, with MMP-dependent degradation only evident after 14 - 21 days (Pratta et al., 2003; Lim et al., 2010). Suramin is also known to antagonise ATP purinergic signaling through the P₂X receptor (Dunn and Blakeley, 1988). NF279 is a less effective than suramin at inhibiting purinergic signaling in chondrocytes (Varani et al., 2008), so its increased chondroprotection argues against P₂X antagonism being the primary mechanism of suramin chondroprotective action. It is likely that suramin will have additional mechanisms of action *in vivo*, but our observation that the protective effects of suramin were inhibited by a TIMP-3 blocking antibody suggest that TIMP-3 is required for suramin's chondroprotective effect and that analogues with improved ability to block TIMP-3 uptake will have improved ability to protect cartilage.

Suramin is not orally bioavailable (Voogd et al., 1993) and violates several of Lipinski's rule of 5. It has a molecular mass of 1297 g/mol, more than 5 hydrogen bond donors and more than 10 hydrogen bond acceptors. Osteoarthritis patients are commonly treated with intra-articular injection of corticosteroids, indicating that intra-articular administration of suramin is feasible. Further development of orally bioavailable suramin analogues could be pursued by 'lead-hopping' to a smaller chemical series once structure-activity relationships are more fully understood.

Suramin's clinical use has been limited by its adrenal toxicity. While suramin did not affect chondrocyte viability at the concentrations and durations tested here, its toxicity profile would prevent its systemic use in OA patients. It would also be undesirable to increase TIMP-3 levels

systemically, since metalloproteinase activity is required for numerous physiological processes, including wound healing and angiogenesis. It would thus be necessary to target suramin derivatives to cartilage, for example, through use of a cartilage-targeting peptide (Rothenfluh et al., 2008). Our limited scan of suramin analogues has identified a more effective analogue and suggested some basic structure-activity requirements for activity, indicating the potential for engineering more effective variants with improved safety profiles. We thus propose suramin as a promising scaffold for the development of novel therapeutics to target osteoarthritic cartilage loss.

Authorship Contributions

Participated in research design: Chanalaris, Marsden, Wren, Nagase, and Troeberg

Conducted experiments: Chanalaris, Doherty, Bambridge, Troeberg

Performed data analysis: Chanalaris, Doherty, Bambridge, Marsden, and Troeberg

Wrote or contributed to the writing of the manuscript: Chanalaris, Nagase, and Troeberg

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FOOTNOTES:

The work was supported by Arthritis Research UK [Grants 19466, 20205 and 20887], the Kennedy Trust for Rheumatology Research, and NIAMS, National Institutes of Health [Grant AR40994]. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

FIGURE LEGENDS:

Fig 1. Suramin binds to TIMP-3 and inhibits its cellular endocytosis by LRP1. (A) Glycosaminoglycan-binding 96-well plates were coated with suramin (10 µg/ml in PBS, ●) or PBS (○) and blocked in 0.2 % gelatin in PBS. Wells were then incubated with TIMP-3 (0.4 - 50 nM) and binding detected using an M2 anti-FLAG antibody (mean ± SD, n = 3). (B) Medium-binding 96-well ELISA plates were coated with LRP1 (5 nM) and blocked with 10 % BSA in TNC buffer. Wells were then incubated with TIMP-3 (0.4 - 50 nM), either alone (○) or pre-incubated with suramin (●, 200 µg/ml 1 h, 37 °C) and binding detected using an M2 anti-FLAG antibody (mean ± SD, n = 3). (C) HTB94 cells were incubated with recombinant TIMP-3 (1 nM) or TIMP-3 pre-incubated with suramin (200 µg/ml, 1 h, 37 °C) for 0 – 8 h and TIMP-3 remaining in the medium analysed by immunoblotting and densitometry (mean ± SD, n = 4). TIMP-3 (○) was taken up from the medium with a half-life of 4.0 ± 1.3 h, while TIMP-3 pre-incubated with suramin (●) was minimally endocytosed. (D) HTB94 chondrosarcoma cells were incubated with suramin (50 - 200 µg/ml) in serum-free DMEM for 30 h. Conditioned media were concentrated by TCA precipitation and TIMP-3 levels analysed by immunoblotting and densitometry. Values are expressed relative to the amount of TIMP-3 in the medium of untreated cells, defined as 1 (mean ± SD, n = 5, *** indicates $P \leq 0.001$ by one-way ANOVA with Bonferroni's correction). (E) HTB94 chondrosarcoma cells were treated with suramin (0 - 250 µg/ml, 18 h) and expression of TIMP3 mRNA analysed by quantitative PCR relative to RPLP0. TIMP-3 expression in the absence of suramin is defined as 1 (mean ± SD, n = 3, $P > 0.05$ by one-way ANOVA with Bonferroni's correction). (F) HTB94 were treated for with suramin (0 - 250 µg/ml) or sodium nitroprusside (SNP, 10 mM) for 72 h and cell viability assessed using MTS (mean ± SD, n = 3, *** indicates $P \leq 0.001$ by one-way ANOVA with Bonferroni's

correction). (G) Primary chondrocytes were isolated from human OA or porcine cartilage and incubated with suramin (0 - 250 μ g/ml) in serum-free DMEM for 48 h. Conditioned media were concentrated by TCA precipitation and TIMP-3 levels analysed by immunoblotting. (H) Human OA chondrocytes were treated with suramin (0 - 250 μ g/ml, 48 h) and expression of TIMP-3 mRNA analysed by quantitative PCR relative to RPLP0, with expression in the absence of suramin defined as 1 (n = 5 donors, mean \pm SD, $P > 0.05$ by one-way ANOVA with Bonferroni's correction). (I) Human OA chondrocytes were treated with suramin (0 - 250 μ g/ml) and/or retinoic acid (RA, 1 μ M) for 48 h and cell viability assessed using MTS (mean \pm SD, n = 3 donors, $P > 0.05$ by two-way ANOVA with Bonferroni's correction).

Fig 2. Suramin does not impair inhibitory activity of TIMP-3. (A) HTB94 were incubated with suramin (0 – 250 μ g/ml, 18 h) and expression of TIMP1 mRNA analysed by quantitative PCR relative to RPLP0. TIMP1 expression in the absence of suramin was defined as 1 (mean \pm SD, n = 3, $P > 0.05$ by one-way ANOVA with Bonferroni's correction). (B) HTB94 were incubated with suramin (0 – 250 μ g/ml, 18 h) and expression of TIMP2 mRNA analysed by quantitative PCR relative to RPLP0. TIMP2 expression in the absence of suramin was defined as 1 (mean \pm SD, n = 3, $P > 0.05$ by one-way ANOVA with Bonferroni's correction). (C) HTB94 were incubated with suramin (0 – 250 μ g/ml, 18 h) and expression of LRP1 mRNA analysed by quantitative PCR relative to RPLP0. LRP1 expression in the absence of suramin is defined as 1 (mean \pm SD, n = 3, *** indicates $P \leq 0.001$ by one-way ANOVA with Bonferroni's correction). (D) HTB94 were treated with suramin (0 - 250 μ g/ml, 30 h), conditioned media concentrated by TCA precipitation and analysed by immunoblotting using an 8G1 anti-LRP1 antibody. (E) ADAMTS-5 (0.5 nM) was incubated (1 h, 37 °C) with TIMP-3 (0.5 – 5 nM) and combinations of suramin (0.05 μ g/ml) or pentosan polysulfate (PPS, 0.05 μ g/ml). Residual

activity against a fluorescent peptide substrate was determined, and $K_i(\text{app})$ values (nM) calculated from equilibrium rates of substrate hydrolysis using the tight-binding equation (mean \pm SD, $n = 4-5$, * indicates $P \leq 0.05$ by one-way ANOVA with Bonferroni's correction). (F) TIMP-3 (0.3 – 50 nM) was incubated (1 h, 37 °C) with MMP-1 (0.5 nM) or MMP-3 (1 nM) in the presence or absence of suramin (0.05 $\mu\text{g/ml}$). Residual activity against a fluorescent peptide substrate was determined, and $K_i(\text{app})$ values (nM) calculated from equilibrium rates of substrate hydrolysis using the tight-binding equation (mean \pm SD, $n = 3$, $P > 0.05$ by Student's t-test).

Fig 3. Suramin inhibits aggrecan degradation in retinoic acid-stimulated porcine and human cartilage explants. (A) Human OA cartilage explants were stimulated with IL-1 (10 ng/ml) in the presence of suramin (0 - 100 $\mu\text{g/ml}$) for 48 h and degraded aggrecan fragments released into the conditioned medium quantified using the DMMB assay (mean \pm SD, $n = 3$, * indicates $P \leq 0.05$, and *** indicates $P \leq 0.001$ by two-way ANOVA with Bonferroni's correction). (B) Porcine cartilage explants were stimulated with IL-1 (10 ng/ml) in the presence of suramin (0 - 250 $\mu\text{g/ml}$) for 48 h and degraded aggrecan fragments released into the conditioned medium quantified using the DMMB assay (mean \pm SD, $n = 3$, *** indicates $P \leq 0.001$ by two-way ANOVA with Bonferroni's correction). (C) Human OA cartilage explants were stimulated with retinoid acid (1 μM) in the presence of suramin (0 - 200 $\mu\text{g/ml}$) for 48 h and degraded aggrecan fragments released into the conditioned medium quantified using the DMMB assay (mean \pm SD, $n = 3$, *** indicates $P \leq 0.001$ by two-way ANOVA with Bonferroni's correction). (D) Porcine cartilage explants were stimulated with retinoid acid (1 μM) in the presence of suramin (0 - 250 $\mu\text{g/ml}$) for 48 h and degraded aggrecan fragments released into the conditioned medium quantified using the DMMB assay (mean \pm SD, $n = 3$, *** indicates $P \leq 0.001$ by two-

way ANOVA with Bonferroni's correction). (E) Conditioned media from (D) were analysed using neo-epitope antibodies that recognise the 1772 AGEG or 374 ARGSV termini generated by ADAMTS cleavage of aggrecan. (F) Porcine cartilage was incubated with retinoic acid (1 μ M, 30 h) in the presence of either a TIMP-3 antibody (MAB973, 50 μ g/ml) or an isotype control (mouse IgG1, 50 μ g/ml). Aggrecan degradation was quantified using the DMMB assay (mean \pm SD, n = 4, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$ by two-way ANOVA with Bonferroni's correction).

Fig 4. Suramin analogue NF-279 shows improved ability to block TIMP-3 uptake and to protect cartilage. (A) Porcine cartilage explants were treated with retinoic acid (RA, 1 μ M) and/or suramin analogues (200 μ g/ml) for 48 h. Cartilage degradation was assessed by quantifying aggrecan fragments (mean \pm SD, n = 3, *** indicates $P \leq 0.001$ by two-way ANOVA with Bonferroni's correction) released into the medium using the DMMB assay. (B) Glycosaminoglycan-binding 96-well plates were coated with 10 μ g/ml suramin (red circle, $EC_{50} = 1.90 \pm 0.21$ nM), NF279 (green triangle, $EC_{50} = 0.85 \pm 0.09$ nM), NF110 (■, $EC_{50} = 6.88 \pm 0.96$ nM), NF157 (▲, $EC_{50} = 1.55 \pm 0.11$ nM), NF449 (◆, $EC_{50} = 4.66 \pm 0.69$ nM), NF023 (○, $EC_{50} = 26.5 \pm 7.5$ nM), NF340 (□, $EC_{50} > 100$ nM) or NF546 (△, $EC_{50} > 100$ nM) in PBS and blocked in 0.2 % gelatin in PBS. Wells were then incubated with TIMP-3 (0.4 - 50 nM) and binding detected using an M2 anti-FLAG antibody (mean \pm SE, n = 3 technical repeats). (C) HTB94 chondrosarcoma cells were cultured in the presence of suramin analogues (200 μ g/ml) for 36 hours and TIMP-3 levels in the conditioned medium evaluated by western blotting and quantified by densitometry (mean \pm SD, n = 4, suramin defined as 100%, *** indicates $P \leq 0.001$ by one-way ANOVA with Bonferroni's correction). (D) For each analogue, TIMP-3 accumulation (from C) was plotted against the \log_{10} of analogue affinity for TIMP-3

(EC₅₀ from B) and Pearson correlation coefficients calculated using GraphPad Prism. (E) For each analogue, TIMP-3 accumulation (from C) was plotted against aggrecan release (from A) and Pearson correlation coefficients calculated using GraphPad Prism. (F) Structural formulae of suramin and its analogue, NF279.

FIGURES:

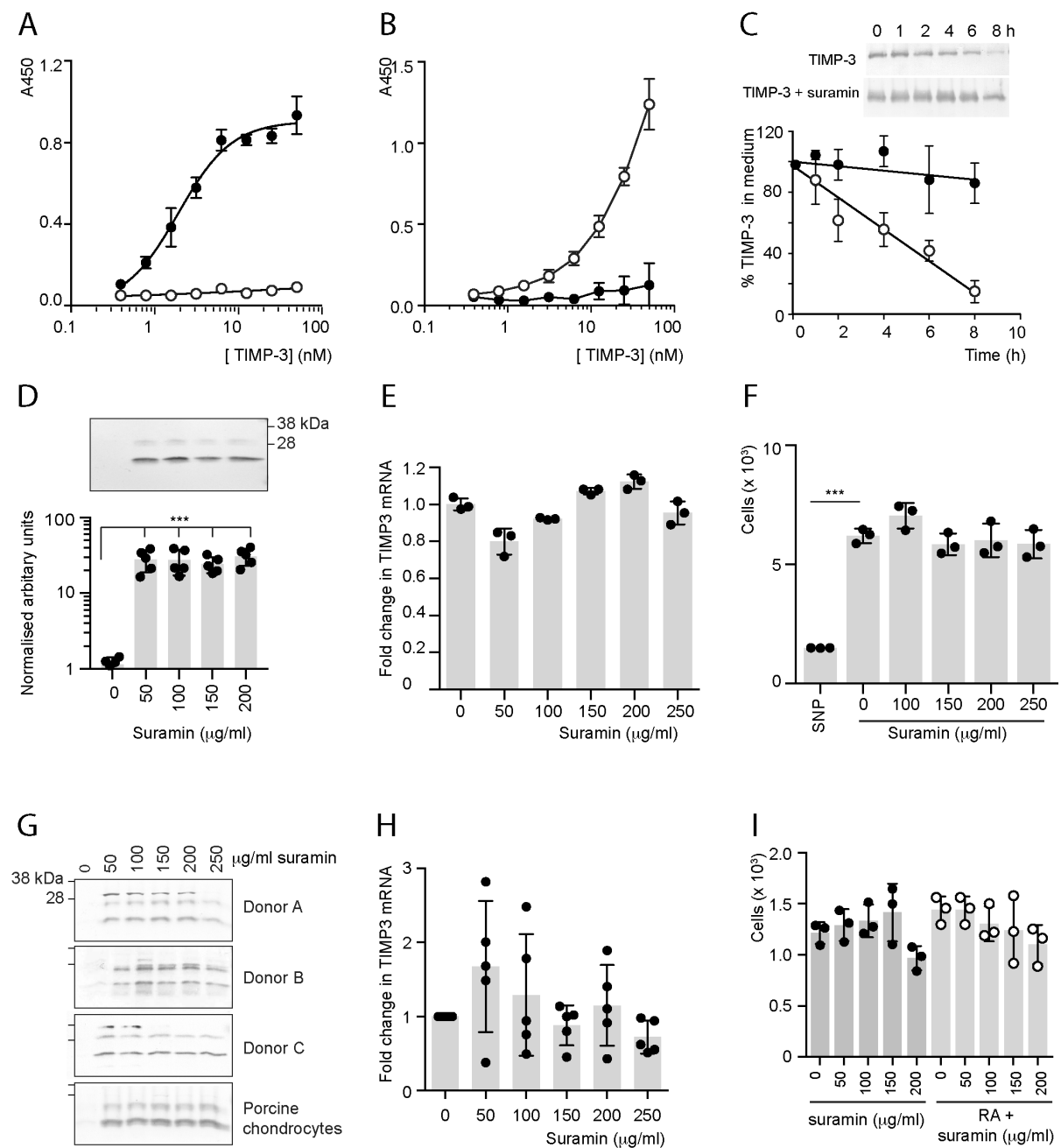


FIGURE 1

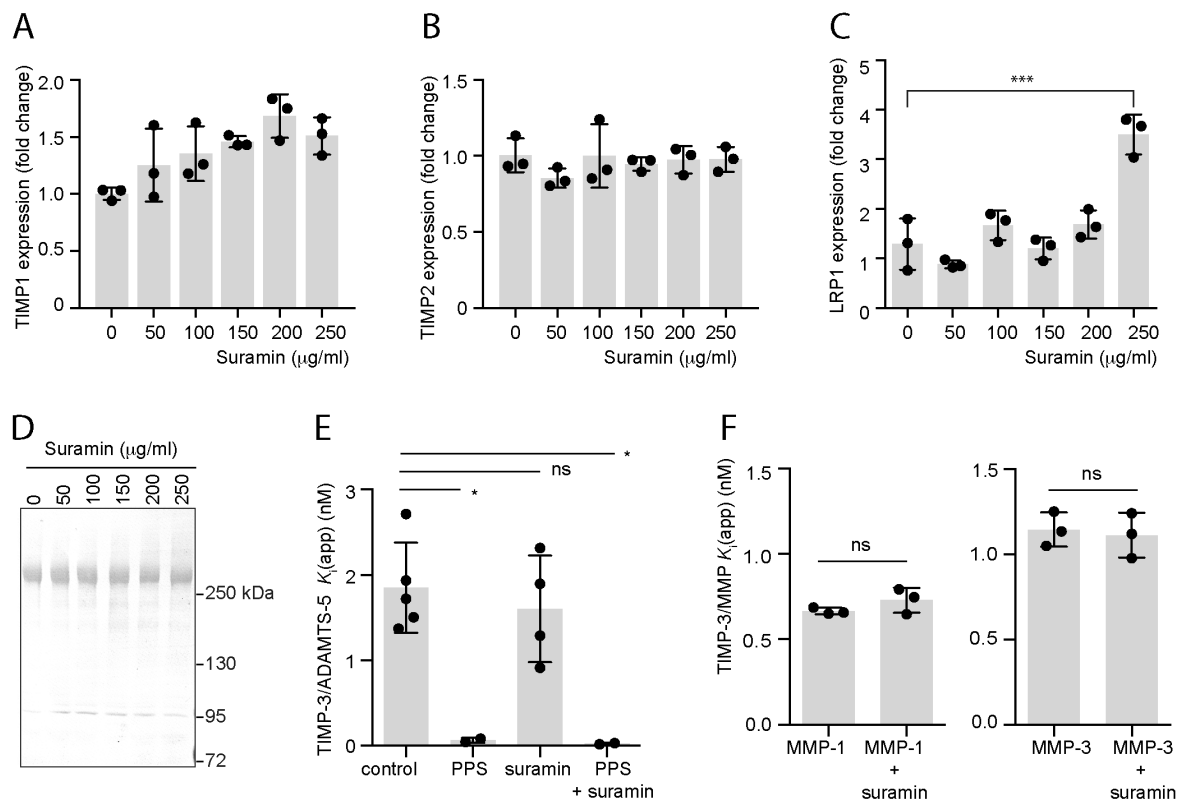


FIGURE 2

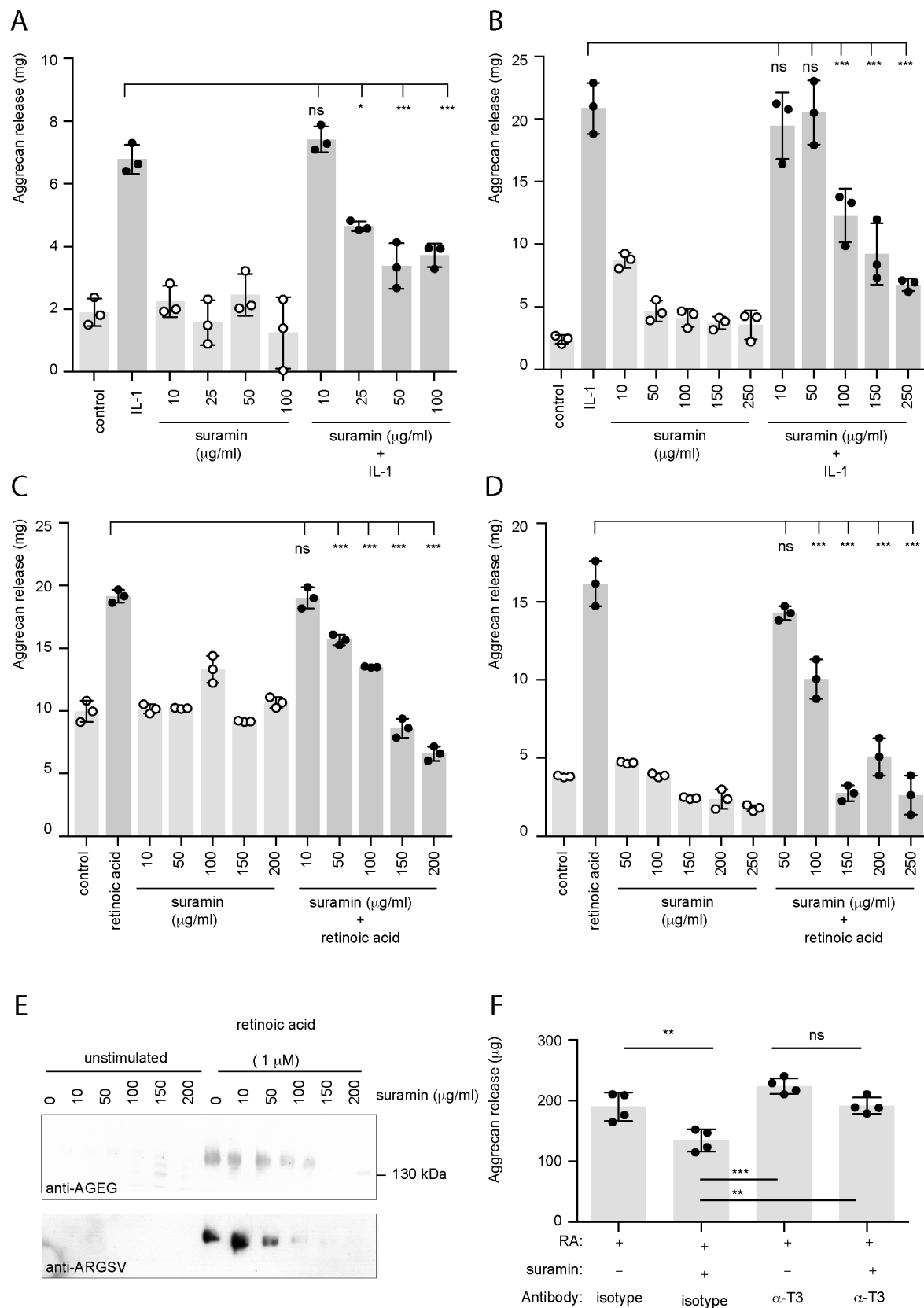


FIGURE 3

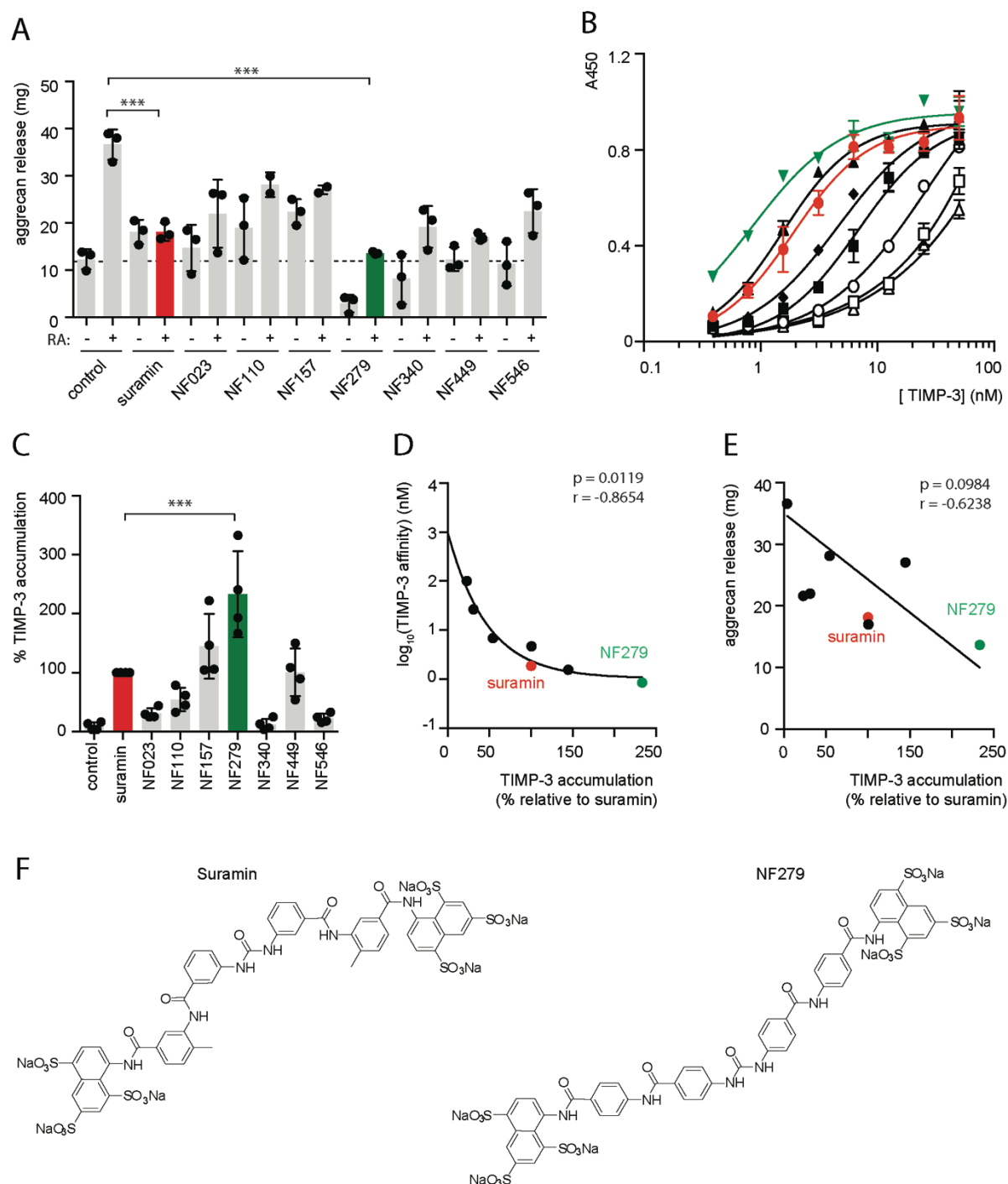


FIGURE 4