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# Evaluation of resolvin E1 as a potential therapeutic for rheumatoid arthritis

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**EVALUATION OF RESOLVIN E1 AS A POTENTIAL  
THERAPEUTIC FOR RHEUMATOID ARTHRITIS**

by

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Submitted in partial fulfillment of the  
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## **DEDICATION**

This work is dedicated to Daniel Beane and Hailey Beane who are the inspiration for this  
and all my journeys.

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# **EVALUATION OF RESOLVIN E1 AS A POTENTIAL THERAPEUTIC FOR**

## **RHEUMATOID ARTHRITIS**

**JOY SACHIE MIYASHIRO**

### **ABSTRACT**

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by chronic inflammation, pain and joint remodeling. Existing RA therapies such analgesics and anti-inflammatories can treat symptoms. More recent strides in disease modifying anti-rheumatic drugs (DMARDs) can slow progression of disease. However, there is still no therapeutic that can reverse disease damage and there is no cure for RA. Resolvin E1 (RvE1) is an endogenous lipid initially identified as a key pro-resolving mediator. By tamping down expression of pro-inflammatory cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), RvE1 is able to down-modulate inflammation and return an inflamed tissue to a homeostatic state. More recently, RvE1 has been shown to act directly to inhibit inflammatory pain through central and peripheral nervous system mechanisms. RvE1 has also been shown to restore bone homeostasis by balancing osteoclast and osteoblast activity. In contrast to current therapeutics that treat symptoms and slow disease progression, a RvE1 pathway agonist has the potential to reverse RA by resolving inflammation, reversing bone remodeling and returning joints to normal homeostasis.

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## LIST OF ABBREVIATIONS

5Hp-18R-HEPE.....	5S-hydroperoxy-18R-hydroxyeicosapentaenoic acid
5-LO .....	5-lipoxygenase
12/15-LO .....	12/15-lipoxygenase
14S-HpDHA .....	14S-hydroperoxydocosahexaenoic acid
15-PGDH.....	15-hydroxyprostaglandin dehydrogenase
15S-HETE.....	15S-hydroxyeicosatetraenoic acid
15R-HETE.....	15R-hydroxyeicosatetraenoic acid
17S-HDHA .....	17S-hydroxydocosahexaenoic acid
17S-HpDHA .....	17S-hydroperoxydocosahexaenoic acid
18R-HEPE.....	18R-hydroxyeicosapentaenoic acid
AA.....	arachadonic acid
ABC.....	ATP-binding cassette
ADA.....	anti-drug antibody
AIA.....	antigen-induced arthritis
AITC.....	allyl isothiocyanate
ALX/FRL2.....	lipoxin A4 receptor/formyl peptide receptor 2
AT-LXA4.....	aspirin-triggered lipoxin A4
BLT1.....	LTB4 receptor 1
C II.....	collagen, type II
C5aR.....	complement 5a receptor

CAIA.....	collagen antibody-induced arthritis
CAP.....	capsaicin
CCL2.....	chemokine C-C motif ligand 2
CFA.....	complete Freund's adjuvant
CGRP.....	calcitonin gene related peptide
ChemR23 .....	chemerin receptor 23
CIA .....	collagen-induced arthritis
CMKLR1.....	chemokine receptor-like 1 (also known as ChemR23)
COX-1.....	cyclooxygenase-1
COX-2.....	cyclooxygenase-2
COX-3.....	cyclooxygenase-3
CRG.....	carrageenan
CRP.....	C - reactive protein
CV.....	cardiovascular
CysLT1.....	cysteinyl leukotriene receptor 1
DHA.....	docosahexaenoic acid
DKK-1.....	Dickkopf-1
DMARD .....	disease modifying anti-rheumatic drug
DRG.....	dorsal root ganglion
EPA.....	eicosapentaenoic acid
ESR.....	erythrocyte sedimentation rates

GPR32.....	G protein-coupled receptor 32
GPI.....	glucose-6-phosphate isomerase
HLA.....	human leukocyte antigen
HTS.....	high throughput screen
<i>i.a.</i> .....	intraarticular
<i>i.c.</i> .....	intracranial
<i>i.t.</i> .....	intrathecal
<i>i.p.</i> .....	intraperitoneal
IFN- $\gamma$ .....	interferon- $\gamma$
IL-1 $\beta$ .....	interleukin-1 $\beta$
IL-2.....	interleukin-2
IL-4.....	interleukin-4
IL-6.....	interleukin-6
IL-7.....	interleukin-7
IL-8.....	interleukin-8
IL-9.....	interleukin-9
IL-12.....	interleukin-12
IL-15.....	interleukin-15
IL-17.....	interleukin-17
IL-21.....	interleukin-21
IL-23.....	interleukin-23
Jak-1.....	Janus kinase-1

Jak-3.....	Janus kinase-3
LC-UV-MS-MS.....	liquid chromatography-ultraviolet-tandem mass spectrometry
LTA4.....	leukotriene A4
LTBI.....	latent tuberculosis infection
LTC4.....	leukotriene C4
LTB4.....	leukotriene B4
LXA4.....	lipoxin A4
MaR1.....	maresin 1 (7,14-dihydroxydocosa-4Z,8,10,12,16Z,19Z-hexaenoic acid)
mBSA .....	methylated bovine serum albumin
MCP-1 .....	monocyte chemoattractant protein-1
M-CSF.....	macrophage colony-stimulating factor
MHC II .....	major histocompatibility complex, class II
NF-kB.....	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells.....	natural killer cell
NSAID.....	non-steroidal anti-inflammatory drug
PD1.....	protectin D1 (10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid)
PGH2.....	prostaglandin H2
PGD2.....	prostaglandin D2
PGE2.....	prostaglandin E2
<i>p.o.</i> .....	<i>per os</i> (by mouth)
PUFA.....	polyunsaturated fatty acid

RA.....	rheumatoid arthritis
RANKL.....	receptor-antagonist of NF- $\kappa$ B ligand
RF.....	rheumatoid factor
RFC.....	reduced folate carrier
RvE1....	resolvin E1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid)
RvE2.....	resolvin E2 (5S,18R-dihydroxy-8Z,11Z,14Z,16E-eicosapentaenoic acid)
RvD1.....	resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid)
ROS.....	reactive oxygen species
sEPSC.....	spontaneous excitatory postsynaptic currents
SP.....	substance P
TB.....	tuberculosis
T <sub>H</sub> 1.....	T helper lymphocyte, subtype 1
T <sub>H</sub> 2.....	T helper lymphocyte, subtype 2
T <sub>H</sub> 17.....	T helper lymphocyte, subtype 17
TNF- $\alpha$ .....	tumor necrosis factor- $\alpha$
TNFR1.....	TNF- $\alpha$ receptor 1
TNFR2.....	TNF- $\alpha$ receptor 2
TRP.....	transient receptor potential cation channel
TRPV1.....	transient receptor potential cation channel, subfamily V, member 1
TRPA1.....	transient receptor potential cation channel, member A1
TXA2.....	thromboxane 2

Wnt.....wingless

## INTRODUCTION

### **Rheumatoid Arthritis (RA)**

#### *Epidemiology of RA*

Rheumatoid Arthritis (RA) is a chronic polyarthritic autoimmune disease characterized by swelling in at least five joints, debilitating pain, and ultimately joint remodeling (Silman AJ, *et al.* 2001). It is estimated that between 2001 and 2005, approximately 0.5-1% of adults worldwide and 0.6% of the adults in the United States suffered from RA (Sacks JJ, *et al.* 2005). As with many autoimmune diseases, RA is about three times more likely in females than in males. RA is more prevalent among adults over 60 years old (Silman AJ, *et al.* 2001).

#### *Etiology of RA*

The cause of RA is unknown, but is believed to be a combination of genetic factors, such as HLA class II genotype (Scott DL, *et al.* 2010), and environmental exposures, such as smoking, dietary factors and infections (Silman AJ, *et al.* 2001). Research into the molecular mechanisms of RA is focused on three main areas: inflammation, pain, and joint remodeling.

#### *Treatments for Rheumatoid Arthritis*

Early pharmacological intervention for RA focused on treating the symptoms of disease. Analgesics, from acetaminophen to narcotics, are used to treat RA pain as it

escalates through disease progression (TABLE 1). Inflammation can be controlled to some degree with non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen or celecoxib for chronic use or steroids, such as prednisone, for acute episode short-term use (TABLE 1). While these therapies have some efficacy at treating RA symptoms, underlying causes of the disease remained and thus joint destruction continued.

Disease-modifying anti-rheumatics (DMARDs) changed the rheumatoid arthritis treatment landscape (TABLE 1). Patients now had a therapeutic regime that could stop the RA symptoms of pain and inflammation and also slow disease progression.

Introduced to RA in the 1970's and popularized in the 1980's, one of the most notable early DMARD, the orally-administered small molecule drug, methotrexate, is now considered the “anchor” of RA treatment (Pincus T, *et al.* 2003). Methotrexate was initially used as an anti-cancer therapeutic. Although the mechanism of action as an RA therapeutic is unclear, methotrexate is known to inhibit dihydrofolate reductase, an essential enzyme in DNA synthesis and cell proliferation (Cronstein BN, *et al.* 2005). The major routes of methotrexate resistance include rapid metabolism of methotrexate to the partially inactive 7-hydroxymethotrexate, defective transport due to reduction in expression or activity of reduced folate carrier (RFC), or increased methotrexate efflux via ATP-binding cassette (ABC) subfamily (van der Heijden JW, *et al.* 2007). Gastrointestinal adverse effects are the most common complaints and are reported by as many as 66% of RA patients on methotrexate therapy (Verstappen SM, *et al.* 2010). Approximately 12% of RA patients discontinue methotrexate use due to adverse effects or ineffectiveness within the first two years of treatment (Verstappen SM, *et al.* 2010).

However, methotrexate is the standard of care in RA because it is effective, it can be taken orally, and it is inexpensive (TABLE 1).

In the 1990s, the quality of life expectation of RA patients was dramatically increased with the advent of biological therapies. Monoclonal antibodies against tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), infliximab (Remicade®) and adalimumab (Humira®), and a soluble TNF- $\alpha$  receptor F<sub>c</sub> fusion protein, etanercept (Enbrel®), work by neutralizing TNF- $\alpha$ . These treatments, either alone or in combination with methotrexate, have become the new gold standard in RA treatment. Other biologic DMARDs can also be used to treat RA if TNF- $\alpha$  blockade fails. Kineret®, a recombinant non-glycosylated IL-1 receptor agonist, (Brensihan B, *et al.* 2003; Jiang Y, *et al.* 2000) or Rituxan, a B-cell depleting anti-CD20 antibody (Roll P, *et al.* 2008) are among a growing field of immunomodulating biologics in the RA treatment arsenal. Many patients see dramatic improvement in flexibility and dramatic decrease in pain with biologics over methotrexate (TABLE 1).

Biologics are protein therapeutics, such as antibodies, receptor-Fc fusions, and as a class present many challenges. Biologics can themselves be immunogenic, and therefore a patient's immune system can mount an anti-drug antibody (ADA) response, which can render the drug inactive. About 9% of patients are primary non-responders. It is estimated that about 30% of patients discontinue anti-TNF therapy due to ineffectiveness within the first year of treatment (Calabrese LH. 2003). Possible mechanisms of non-response to TNF- $\alpha$  inhibitor therapy include existing anti-TNF- $\alpha$  antibody titers and increased anti-TNF- $\alpha$  titers over time (Calabrese LH. 2003).

Compared to methotrexate-treated patients, patients who take the anti-TNF therapies adalimumab, etanercept and infliximab, were observed to have higher rates of skin infection, pneumonia, cystitis, and tuberculosis (TB) (TABLE 1). *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* emerged as frequently observed bacterial pathogens. Varicella zoster virus and Herpes simplex virus caused most cases of viral infections. *Mucocutaneous candidiasis* accounted for most fungal infections (Pérez-Sola MJ, *et al.* 2011). Tuberculosis infection or reemergence is a risk factor for patients on anti-TNF- $\alpha$  therapies. RA patients who have been treated with biologics have a four-fold increased risk of TB over the general population (Arkema EV, *et al.* 2014). However, when patients are treated with latent TB infection (LTBI) standard of care, there is no statistically significantly increased risk of active TB in LTBI-positive TNF- $\alpha$  inhibitor patients compared with LTBI-negative TNF- $\alpha$  inhibitor patients (Kwon M, *et al.* 2014).

As a class, biologics are extremely expensive and must be injected either subcutaneously or intravenously, disqualifying them as first-line therapies. However, patients demand these therapeutics because they are clinically efficacious.

The newest addition to the RA therapeutic arsenal is Xeljanz®, a Janus kinase (Jak) antagonist small molecule drug (TABLE 1). The Jak-Stat signaling pathway transmits signals from cytokine receptors that employ the  $\gamma$ c chain; these include IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, and IL-21R. It is thought that by inhibiting Jak-3-mediated cytokine receptor signal transduction, Xeljanz® dampens autoimmune response in RA (Maeshima K, *et al.* 2012; Zerbini CAF and Lomonte ABV. 2012). Xeljanz® also

inhibits Jak-1, and carries a black box warning about the increased risk of serious infection, cancers and lymphoma (Pfizer, 2012).

Xeljanz® and biologic TNF- $\alpha$  antagonist therapeutics are more targeted to the immune system than methotrexate. However, even these more immune system-targeted drugs only inhibit on-going inflammatory damage without enhancing resolution; and therefore do not cure arthritis.

The lifestyle enhancing activity of DMARDs has set the bar high for the next generation of RA therapy. Treatment of symptoms and even slowing of disease progression will not be enough. Current treatments address pain and inflammation, but do not address bone loss or joint remodeling. The next leap in RA therapy must be a drug that can resolve inflammation and reverse joint damage.

### *Mechanisms of RA*

In order to design better therapies to treat RA, it is necessary to understand the cellular and molecular mechanisms that underlie disease progression and symptoms. Research into the molecular mechanisms of RA is focused on three main areas: inflammation, pain, and joint remodeling.

#### **Inflammation**

During normal inflammation, the immune system directs a protective program at the sight of infection or injury by recruiting neutrophil and macrophages, increasing inflammatory cytokines, and clearing debris, such as dead cells or invading pathogens. When carried out successfully, the inflammatory program is transient and gives way to a

resolution program, catabasis, which promotes healing and return to homeostasis. Catabasis is an active resolution of inflammation distinct from merely anti-inflammatory action (Serhan CN, *et al.* 2007). However in autoimmunity, the adaptive immune system chronically remains in the inflammatory state, autoreactive activated T cells continuously secrete pro-inflammatory cytokines and activated B cells produce autoreactive antibodies which damages affected tissues over time.

In the early stages of RA, dendritic cells, producing the cytokines TNF- $\alpha$  and interleukin-12 (IL-12), activate naïve T lymphocytes to differentiate into pro-inflammatory mature T-lymphocytes: IFN- $\gamma$ -producing T<sub>H</sub>1 lymphocytes or interleukin-17-producing T<sub>H</sub>17 lymphocytes (Silverman GJ and Carson DA. 2003). In response, infiltrating macrophages produced the inflammatory cytokines TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) (Figure 1A) (Choy E. 2012).

As disease progresses, activated B lymphocytes can up regulate co-stimulatory molecules to further T lymphocyte activation (Figure 1B) (Choy E. 2012). In human RA, there are three recognized autoantibodies: anti-rheumatoid factor (anti-RF), anti-citrullinated peptides (anti-CCP), and anti-type II collagen (anti-CII) (Silverman GJ and Carson DA. 2003). While all three are biomarkers for cartilage erosive arthritis, only anti-CII has been directly linked to RA pathogenesis via animal models (Rowley MJ, *et al.* 2008). Citrullination is a post-translational modification that converts the amino acid arginine to the non-standard amino acid citrulline via peptidylarginine deiminase (PAD)-catalyzed replacement of the aldimine with a ketone. Anti-citrullinated protein antibodies

form immune complexes and can drive complement cascade (Choy E. 2012). Fibrin and fibrinogen in the arthritic joint may be the source of citrullinated proteins in RA.

In addition to B cells, other immune cells also participate in RA pathogenesis. Activated T cells recruit innate immune cells such as macrophages and neutrophils into the synovial space between joints. TNF- $\alpha$ , IL-6 and IL-1 $\beta$  are usually produced by macrophages in the acute phase response, but remain up regulated in RA (Figure 1A) (Choy E. 2012). TNF- $\alpha$  is a pyrogen, can up-regulate macrophage phagocytic activity, can up regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) transcription factor expression, and is a potent chemoattractant for neutrophils (Old LJ. 1985). Signaling through the TNF- $\alpha$  and IL-1 $\beta$  pathways has been shown to up regulate cyclooxygenase-2 (COX-2), an enzyme that can convert arachidonic acid (AA) to prostaglandin H2 (PGH2), an inflammatory mediator.

Rheumatoid arthritis patients have a two-fold higher risk of developing cardiovascular (CV) disease, even after traditional CV risk factors, such sedentary lifestyle and high cholesterol, have been taken into account (Sattar N, *et al.* 2003). Indeed, while RA patients do not have increased hypertension or diabetes mellitus, traditional CV risk factors (Boyer JF, *et al.* 2011; del Ricon ID, *et al.* 2001), these patients have a higher incidence of atherosclerosis and increased mortality due to CV disease (Gonzalez-Juanatey C, *et al.* 2003; Nurmohamed MT. 2009). Atherosclerosis has some features of autoimmune disease, in particular, activated T and B cells in the vasculature. It is hypothesized that in RA, clonally expanded activated T produce high levels of TNF- $\alpha$ , endothelin and collagenases injure vasculature, thus contributing to

atherosclerosis (Hollan I, *et al.* 2013). Therefore, the chronic inflammation in RA is a mechanism, not just for joint damage but may contribute to damage to other organs as well.

## Pain

Pain is the most common symptom that drives an RA patient to initially seek out medical attention and continues to be a major patient complaint throughout disease progression. Molecular mechanisms of RA pain are associated with both inflammatory mediators and neuropeptide mediators.

The inflammatory pain associate with RA is mediated by peripheral and central mechanisms. After tissue injury, inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$  and pro-inflammatory prostaglandins) can induce peripheral sensitization, sensitization of nociceptor terminal in inflamed tissue and in nociceptor cell bodies in the dorsal root ganglion (DRG) (Cheng JK and Ji RR. 2008). TNF- $\alpha$ , IL-1 $\beta$  and prostaglandins produced in the spinal cord rapidly induce inflammatory pain via central sensitization in spinal cord dorsal horn neurons (Hess A, *et al.* 2008; Kawasaki Y, *et al.* 2008; Samad TA, *et al.* 2001).

Two neuropeptides, substance P (SP) and calcitonin gene related peptide (CGRP) are found in high abundance in RA patient synovial fluid as compared to normal controls (Black PH. 2002). SP and CGRP can act on transient receptor potential cation channel, subfamily V, member 1 (TRPV1) or capsaicin (CAP) receptors expressed locally on synoviocytes and centrally on the dorsal root ganglion neurons to promote a pain response (Geppetti P, *et al.* 2008; Lin Q, *et al.* 2007).

## Bone damage

In healthy bone, there is a homeostatic balance between bone formation and bone resorption. In RA tissue, this balance is skewed toward bone resorption on both sides of the bone homeostasis equation; osteogenesis and osteolysis. Evidence suggests that this imbalance is tightly linked to the autoimmune state, and that infiltrating activated immune cells such as macrophages, dendritic cells, B cells, T cells, and mast cells directly and indirectly contribute to bone erosion (Figure 2) (Choy E, 2012).

On the one side of the bone homeostasis equation, bone resorption is favored by inflammatory cytokines and recruitment of monocytes in the inflamed RA joint. Increased macrophage colony-stimulating factor (M-CSF) and receptor-antagonist of NF- $\kappa$ B ligand (RANKL), up regulated in RA synovial tissue, signal precursor macrophages to differentiate into bone-resorbing osteoclasts (Gravallese EM, *et al.* 2000). In addition, inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17 further drive osteoclasts to maturity (Lam J, *et al.* 2000; Lubberts E, *et al.* 2003; Sato K, *et al.* 2006; Wei S, *et al.* 2005).

On the other side of the bone homeostasis equation, bone formation is impeded in the inflamed RA joint. TNF- $\alpha$  induces Dickkopf-1 (DKK-1) proteins, which interfere with signaling through Wnt proteins. Interference with Wnt signaling prevents mesenchymal cell differentiation into osteoblast, the bone forming cells (Diarra D, *et al.* 2007). DKK-1 expression in RA patient serum was significantly higher than in healthy controls. DKK-1 expression significantly decreased in RA patients treated with anti-TNF- $\alpha$  therapies, such as anti-

TNF- $\alpha$  antibodies, or recombinant TNF- $\alpha$  receptor-F<sub>c</sub> chimeric proteins. DKK-1 levels in patient serum correlated with bone erosion as measured by Sharp radiologic change, and with other biomarkers of inflammation such as serum C-reactive protein (CRP) levels, and erythrocyte sedimentation rates (ESR) (Diarra D, *et al.* 2007).

Taken together, chronic inflammation in the RA joint results in increased osteoclast bone resorption and decreased osteoblast bone formation, which leads to joint remodeling through net bone loss.

### **Cyclooxygenase-2 (COX-2)**

The cyclooxygenase (COX) pathway is important in both inflammation and in resolution of inflammation (Figure 3) (Serhan CN and Petasis NA. 2011). There are three major isozymes, COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in most mammalian tissues. COX-2 expression is induced in activated macrophages, inflamed tissue, and some carcinomas. COX-3, also called COX-1b or COX-1v, is a splice variant of COX-1, which retains intron 1 and has a frame shift mutation rendering it non-functional in humans. COX-1 and COX-2 can convert the polyunsaturated fatty acids (PUFA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into potent pro-inflammatory or anti-inflammatory mediators (Serhan CN and Petasis NA. 2011).

COX-1 and COX-2 convert arachidonic acid, and  $\omega$ -6 PUFA, into prostaglandin H<sub>2</sub> (PGH<sub>2</sub>). PGH<sub>2</sub> is then further converted to other pro-inflammatory mediators. PGE synthase converts PGH<sub>2</sub> into PGE<sub>2</sub>, which can signal through its receptors to mediate

pain, fever and vasodilation. Homeostasis of blood clot formation is regulated by prostacyclin (prostaglandin I<sub>2</sub>, PGI<sub>2</sub>) and thromboxane 2 (TXA<sub>2</sub>), products of PGH<sub>2</sub> conversion by prostacyclin synthase and thromboxane synthase, respectively. PGI<sub>2</sub> inhibits platelet aggregation and is an effective vasodilator. In contrast, TXA<sub>2</sub> promotes platelet aggregation and is a potent vasoconstrictor. These products are lipid mediators that help direct inflammatory cell and molecular programs.

Paradoxically, COX-2 can also catalyze the production of lipid mediators that help direct pro-resolution cell and molecular programs. Pro-resolving lipid mediators include arachidonic acid-derived lipoxins, ω<sub>3</sub>-eicosapentaenoic acid-derived resolvins of the E-series, docosahexaenoic acid-derived resolvins of the D-series, protectins and maresins (Serhan CN and Chiang N. 2007).

Aspirin, one of the first NSAIDs, irreversibly acetylates serine in the active site of the cyclooxygenase enzymes, COX-1 and COX-2. Previously, it was thought aspirin's only pharmacological activity was as a COX enzyme antagonist, inhibiting the production of pro-inflammatory lipid mediators. However, now it is understood that aspirin's acetylation of COX enzymes also confers a new enzymatic activity resulting in the production of aspirin-triggered pro-resolving lipid mediators (Serhan CN and Chiang N. 2007). Currently, aspirin is the only NSAID that has shown this second, pro-resolving activity. In the context of RA, this suggests that non-aspirin NSAIDs may inhibit inflammation in the short-term, but may also block resolution of inflammation.

### *Resolvin E1 (RvE1)*

Resolvins, lipoxins and protectins have anti-inflammatory and pro-resolving activities. (Table 2) (Serhan CN, *et al.* 2008). Resolvins have also been reported to have analgesic properties that are both anti-nociceptive and anti-inflammatory (Table 3) (Ji RR, *et al.* 2011). RvE1 also has activity in bone damage healing (Gao L, *et al.* 2013; Hasturk H, *et al.* 2007). Thus, only RvE1 has the potential to treat RA in all three mechanisms of disease: inflammation, pain and bone destruction.

### RvE1 Endogenous Synthesis

RvE1 is an  $\omega$ 3-eicosapentaenoic acid (EPA)-derived resolvin of the E-series, synthesized through an orchestrated cooperation between vascular endothelial cells and activated leukocytes. During inflammation and in the presence of aspirin, vascular endothelial cells convert EPA to 18R-hydroxyeicosapentaenoic acid (18R-HEPE) and release it into the extracellular space. Proximal polymorphonuclear leukocytes (PMNs) further take up the 18R-HEPE further transform it into RvE1. Alternatively, RvE1 can also be produced through aspirin-independent cytochrome P450-mediated mechanisms (Node K, *et al.* 1999).

### RvE1 and ChemR23

Chemerin receptor 23 (ChemR23) is a G-protein coupled receptor that binds both the peptide Chemerin and the lipid mediator RvE1. It was first identified as the receptor

of RvE1 through a functional screen of LTB<sub>4</sub> receptor-related G-protein coupled receptors (GPCRs) transfected into HEK cells. RvE1 inhibits TNF- $\alpha$ -induced NF- $\kappa$ B activation in a concentration-dependent manner with an EC<sub>50</sub> of 1.0 nM in ChemR23-transfected, but not in mock-transfected HEK cells (Figure 4) (Arita M, *et al.* 2005). <sup>3</sup>H-RvE1 bound to ChemR23-transfected HEK with a high affinity (K<sub>d</sub> of 11.3 nM) (Arita M, *et al.* 2005).

Prochemerin, a 163-amino acid protein is processed to a 143-amino acid mature secreted chemerin protein via removal of the 20-amino acid signal sequence (Figure 5) (Du XY and Leung LL. 2009). Sequential proteolytic cleavage of chemerin results in chemotaxis of activated macrophages and pro-inflammatory activity (Table 4) (Du XY and Leung LL. 2009). Further processing produces to N-terminally truncated polypeptides: chemerin<sup>151-157</sup>, chemerin<sup>149-157</sup>, chemerin<sup>148-157</sup>, and chemerin<sup>146-157</sup> proteins. This results in peptides that have activity similar to the chemerin<sup>139-157</sup> protein (Figure 6) (Wittamer V, *et al.* 2004). Chemerin peptides were able to compete with <sup>3</sup>H-RvE1 for ChemR23 binding, suggesting overlapping binding sites (Arita M, *et al.* 2005).

At the amino acid level, human and mouse ChemR23 share 80% identity. Chemerin and ChemR23 orthologs can be found in mammals, birds and fish with strict conservation of six cysteines hypothesized to be important in disulfide bridging (Luangsay S, *et al.* 2009).

ChemR23 is expressed on many tissues, including three tissues that are involved in mechanisms of RA: immune cells in peripheral blood, neurons in the brain and peripheral nervous system (PNS) (Arita M, *et al.* 2005) and bone (Methner A, *et al.*

1997). Among human peripheral blood leukocytes, ChemR23 is most highly expressed on monocytes, followed by neutrophils and T lymphocytes (Arita M, *et al.* 2005). In human peripheral blood monocytes, both ChemR23 and COX-2 mRNA expression are up-regulated in response to TNF- $\alpha$  (Arita M, *et al.* 2005), which is consistent with RvE1 as a regulator of resolution of inflammation.

### RvE1 Biological Functions

RvE1 has pleiotropic biological activities that counteract the three mechanisms of RA: inflammation, pain and bone loss. RvE1 was discovered as a COX pathway lipid mediator that had pro-resolving and anti-inflammatory properties (Serhan CN, *et al.* 2000). RvE1 inhibits capsaicin-induced spontaneous excitatory postsynaptic currents (sEPCs) in patch clamp experiments on dissociated mouse DRG neurons (Park C-K, *et al.* 2011). The RvE1 receptor, ChemR23 is expressed on both osteoblasts (Gao, *et al.* 2013) and osteoclasts (Herrera BS, *et al.* 2008) RvE1 directly increases bone growth and mineralization via up-regulation of osteoprotegerin (OPG) (Gao L, *et al.* 2013), and decreases osteoclast differentiation and diminishes dentin resorption (Herrera BS, *et al.* 2008).

## **HYPOTHESIS**

RvE1 or its analogs represent a unique RA therapeutic opportunity because RvE1 is known to modulate three mechanisms involved in RA pathology and progression: (1) inflammation, (2) inflammatory and neuropathic pain, and (3) joint destruction. Unlike

the currently available therapeutics for RA, an RvE1-based therapeutic has the potential to resolve disease and return diseased joints to homeostasis.

### **PUBLISHED STUDIES**

To evaluate RvE1's potential as an RA therapeutic, there are four major areas of exploration: (1) RvE1 action in reducing inflammation and promoting resolution of inflammation, (2) RvE1 action in reducing inflammatory and neuropathic pain, (3) RvE1 action in reducing joint destruction and promoting joint repair, and finally, (4) evaluation of RvE1's drug-like properties. There are no reports of RvE1 used directly as a therapeutic in human RA or in animal models of RA. However, there is ample literature evidence of RvE1 used as a therapeutic in both autoimmune and bacterial inflammation models, in inflammatory and neuropathic pain models and in joint destruction models. To evaluate RvE1 with the current literature, similarities in molecular mechanism of action between these models and in RA can be drawn.

#### **RvE1 and Resolution of Inflammation**

RvE1 is efficacious in reversing inflammation in animal models of inflammation, including experimental autoimmune animal models such as allergic asthma (Flesher RP, *et al.* 2013) and inflammatory bowel disease (Arita M, *et al.* 2005), as well as bacterial infection such as pneumonia-induced inflammation. These models share features with RA in that they involve activation of macrophages, neutrophils, T cells and B cells. Upon infiltration of these activated immune cells into target tissue, they unleash pro-

inflammatory programs that include up-regulation of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6, antibody production, and translocation of transcription factor NF- $\kappa$ B from the cytoplasm to the nucleus.

#### *RvE1 and Ovalbumin-induced Allergic Asthma*

The ovalbumin-induced allergic airway mouse model is a well-accepted model of human allergic asthma. In this model, ovalbumin-sensitized mice are challenged with low level aerosolized antigen for 4 weeks. A single moderate level ovalbumin (OVA) challenge is administered to mimic a human allergy-induced acute asthmatic episode. As with human asthma, mice respond with rapid neutrophil, lymphocyte, eosinophil, and macrophage recruitment to the lungs. The infiltrating immune cells produce pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , and translocate the pro-inflammatory transcription factor NF- $\kappa$ B from the cytoplasm to the nucleus. Over the following 96 hours, a molecular program to resolve inflammation promotes suppression of inflammatory cytokines and down-regulation of NF- $\kappa$ B. In this model, *i.p.* administration of RvE1 2 or 8 hours post-acute challenge resulted in acceleration of the resolution program: inflammatory cells in the lung declined, inflammatory cytokine expression by activated macrophages decreased, and NF- $\kappa$ B translocation to the nucleus decreased (Figure 7) (Flesher RP, *et al.* 2013).

Although RA and asthma are autoimmune diseases that affect disparate tissues, some of the mechanisms of unchecked inflammation between these two diseases are

similar, such as high expression of pro-inflammatory cytokines TNF- $\alpha$ , and IL-1 $\beta$ , antibody production, infiltration of inflammatory immune cells into affected tissue and translocation of transcription factor NF- $\kappa$ B in activated immune cells. In the OVA model of asthma, RvE1 was able to inhibit inflammation. In RA, RvE1 may also be able to inhibit inflammation through these mechanisms common to both diseases.

#### *RvE1 and Chemically-induced Colitis*

The human autoimmune condition, IBD can be modeled in rodents through chemically-induced colitis. Administration of exogenously produced RvE1 in both 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and dextran sulfate sodium (DSS)-induced colitis results in protection against inflammation and disease progression (Arita M, *et al.* 2005; Ishida T, *et al.* 2010).

In the TNBS-induced colitis model, mice sensitized by skin painting with TNBS followed by intrarectal TNBS administration develop bloody diarrhea and weight loss which leads to death in >60% of mice treated. Physiologically, there is significant shortening, thickening and ulceration of the colon. Histologically, ulcerations are marked by transmural infiltration of inflammatory PMNs, monocytes and lymphocytes.

Mice pretreated *i.p.* with 1  $\mu$ g/ mouse RvE1 were protected from TNBS-induced colitis. Compared to vehicle controls, RvE1-treated mice had improved survival rates, sustained body weight, improved histological scores and reduced inflammatory cells. RvE1-treated mice also had significant reduction in molecular markers of disease, such as

expression of pro-inflammatory cytokines IL-12 and TNF- $\alpha$  (Figure 8), and circulating anti-TNBS antibodies (Arita M, *et al.* 2005).

The dextran sulfate sodium (DSS)-induced colitis in mice is another chemically-induced colitis animal model of human inflammatory bowel disease. In this model, after 5 days of treatment with 3.5% DSS in drinking water, mice develop bloody diarrhea and severe wasting. The colon is significantly shortened and thickened. Transmural infiltration of neutrophils and mononuclear cells cause ulcerations in the colon. In the infiltrating immune cells, NF- $\kappa$ B p65 Ser276 phosphorylation and translocation to the nucleus results in up-regulation of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Ishida T, *et al.* 2010).

1  $\mu$ g/mouse RvE1 dosed *i.p.* for 5 days concurrently with 3.5% DSS in drinking water protected mice from DSS-induced colitis. By day 8, RvE1-treated mice maintained  $90.7 \pm 2.4$  % of baseline body weight compared with PBS-treated mice the retained only  $78.1 \pm 2.6$  % baseline body weight. RvE1-treated mice also were protected from DSS-induced colon shortening and thickening, compared to PBS-treated mice. Microscopically, RvE1 treated mice also had significantly fewer neutrophil and mononuclear infiltrates in the colon. RvE1 treatment reduced NF- $\kappa$ B p65 Ser276 phosphorylation and translocation in the distal colon. In addition, RvE1 also significantly reduced transcription of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Ishida T, *et al.* 2010).

In both TNBS-induced and DSS-induced animal models of colitis, RvE1 demonstrated the ability to reduce TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 production and to reduce neutrophil and macrophage infiltration into colon tissue. Although RA and IBS are

disparate autoimmune disease affecting different tissues, they share the features of uncontrolled inflammation that features high expression of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, and infiltration of neutrophils and macrophages into affected tissue.

#### *RvE1 and Pneumonia-induced Inflammation*

In the aspiration pneumonia model, mice receive an acute left lung injury with hydrochloric acid followed by challenge with enteric *E. coli*. Subsequent infection results in neutrophil infiltration of the lung, up-regulation of IL-1 $\beta$ , IL-6, and monocyte chemoattractant protein-1 (MCP-1), and can ultimately lead to death. Mice pretreated with 5 $\mu$ g/kg RvE1 showed enhanced clearance of *E. coli*, significantly decreased neutrophil infiltration, significant reduction in pro-inflammatory cytokines and chemokines, and marked improvement in survival. RvE1 did not appear to have an anti-bacterial effect on *E. coli* in culture, suggesting that RvE1 acts to enhance immunity rather than directly inhibit *E. coli* (Figure 9) (Seki H, *et al.* 2010). While other autoimmune disease models show that RvE1 can control sterile inflammation, these experiments also suggest that RvE1 can play a role in bacteria-mediated inflammatory response and resolution. This suggests that RvE1's role in inflammation resolution is not specific to a disease, but rather a general mechanism to bring an inflammatory response back to homeostatic equilibrium.

### *RvE1 and Inflammation in RA*

The inflammation animal models outlined above have several features in common with human RA. All of these immune responses depend on inflammation that directs monocytes and B cells to target tissues. These immune cells, particularly monocytes, produce pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$ . Activation of these cells results in phosphorylation of I $\kappa$ B in the cytoplasm and nuclear translocation of the transcription factor NF- $\kappa$ B. Antibodies are produced in response to an autoantigen or bacterial component. In the animal models, therapeutic use of RvE1 results in not only a dampening but a reversal of these inflammatory responses and a return to homeostasis. If a RvE1-based therapeutic could resolve inflammation and return the immune system to homeostasis in RA patients, it would represent a paradigm shift in RA treatment.

### **RvE1 and Analgesia**

When dietary supplements containing EPA, the omega-3 fatty acid precursors of type E resolvins, were taken by RA patients for 3-4 months, patients reported significant changes in several measures of RA-related pain: decreases in minutes of morning joint stiffness, intensity of joint pain, number of painful/tender joints and NSAID consumption (Goldberg RJ and Katz J. 2007). 1 ng/mouse RvE1 was more effective than 1  $\mu$ g/mouse EPA at reducing nocifensive behavior in the complete Freund's adjuvant (CFA)-induced heat hyperalgesia model (Figure 10) (Xu ZZ, *et al.* 2010). This suggests that RvE1 could be the major component of the analgesic activity in EPA.

To understand RvE1's analgesic potency in inflammatory pain, at least three mechanisms should be considered: direct inhibition of inflammation, PNS-mediated analgesia, and the central nervous system (CNS)-mediated analgesia. RvE1 has repeatedly been shown to directly decrease inflammatory pain by inhibiting inflammation, but RvE1 also can directly provide analgesia through PNS- and CNS-mediated pathways.

#### *RvE1 and PNS-mediated Analgesia*

Local administration of RvE1, at the site of inflammation, has been shown to reduce inflammation and pain in well-established rodent models of inflammatory pain. Mice pretreated with intraplantar RvE1 showed reduced formalin-induced spontaneous pain and reduced carrageenan (CRG)-induced heat hyperalgesia (Xu ZZ *et al*, 2010). In the CRG-induced hyperalgesia model, RvE1 reduced inflammation directly as shown by inhibition in neutrophil recruitment and up regulation of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. RvE1 levels could be reduced until the analgesic effect remained intact even as neutrophil recruitment and edema increased (Figure 11) (Xu ZZ *et al*, 2010). This suggests that RvE1's analgesic properties are not solely due to inhibition of inflammation.

### *RvE1 and the CNS-mediated Analgesia*

In mice, intraplantar 0.5% formalin administration is characterized by a two-phase pain response. The first phase begins immediately after formalin administration and lasts for 10 minutes. Duration of licking and flinching is used as a measure of pain. After a transient decline in these behaviors, there is second phase of pain that lasts for 30-60 minutes. Local anesthetics such as lidocaine can inhibit first phase pain (Abbadie C, *et al.* 1997). It is hypothesized that the second phase pain is due to activity-dependent sensitization of C-fiber nociceptors in the dorsal horn of the CNS (Woolf CJ. 1983). Administration of *i.t.* NSAIDs (Malmberg AB and Yaksh TL. 1992) or morphine (Yaksh TL, *et al.* 2001) inhibits only second phase pain.

An *i.t.* dose of 1 ng/mouse of RvE1 was as effective as 100 ng/mouse of morphine, an opioid analgesic, or 10 µg/mouse NS-398, a selective COX-2 antagonist, at reducing formalin-induced 2<sup>nd</sup> phase, but neither reduced 1<sup>st</sup> phase response (Figure 12) (Xu ZZ, *et al.* 2010). This result shows that RvE1 is a potent analgesic that can act through CNS-mechanisms.

### *Molecular Mechanisms of RvE1 Analgesia*

*In vitro* cell-based experiments probe the potential molecular mechanism of RvE1's reductions of pain in animal models. Two possible mechanisms have been proposed: (1) RvE1-mediated reduction of TNF- $\alpha$ -induced inflammation, and peripherally and centrally-regulated pain; and (2) RvE1-mediated inhibition of TRPV1.

### *RvE1 and TNF- $\alpha$ in Analgesia*

Apart from inflammatory pain due simply to edema, TNF- $\alpha$  appears to have a direct role in pain. The two receptors for TNF- $\alpha$ , known as TNF- $\alpha$  receptors 1 and 2 (TNFR1 and TNFR2), appear to play different roles in transmission of inflammatory pain. Both TNFR1<sup>-/-</sup> and TNFR2<sup>-/-</sup> mice have heat sensitivity and lamina II neuron spontaneous excitatory post synaptic current (sEPSC) responses that are comparable to wild type mice. In wild type mice, TNF- $\alpha$  evokes heat hyperalgesia and significant increase in sEPSC frequency in wild type lamina II neurons. This TNF- $\alpha$ -enhanced heat hyperalgesia was decreased in both early and late phase pain response for TNFR1<sup>-/-</sup>, but reduced only in early phase pain response for TNFR2<sup>-/-</sup> mice. At the cellular level, TNF- $\alpha$ -enhanced sEPSC response was completely ablated in lamina II neurons from TNFR1<sup>-/-</sup> mice and reduced in TNFR2<sup>-/-</sup> mice (Zhang L, *et al.* 2011). These data show that TNF- $\alpha$  can enhance pain response by increasing sEPSC frequency, and there are differing contributions between TNFR1 and TNFR2. RvE1 has been shown to down-regulate TNF- $\alpha$  produced by monocytes. As a therapeutic, RvE1 or RvE1 analogs have the potential analgesic effects by inhibiting inflammation-driven edema or by inhibiting TNF- $\alpha$  actions on sEPSCs frequency.

#### RvE1 and TRP Channels in Analgesia

Resolvins have been shown to act through transient receptor potential cation (TRP) channels (Caterina MJ, *et al.* 2000; Dai Y, *et al.* 2007; Ji RR, *et al.* 2002). The TRP superfamily members are structurally similar integral membrane proteins that

function as ion channels. The 28 identified TRP superfamily members are organized into 6 subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP and TRPML (Montell C. 2005).

Two TRV family members stand out as molecular switchboards for a diverse array of stimuli. To date, they are the most studied of the TRP family members. Transient Receptor Potential Vanilloid 1 (TRPV1) is the obligate receptor for capsaicin and a key receptor for noxious physical heat (Caterina MJ, *et al.* 1997). Transient Receptor Potential Ankyrin 1 (TRPA1) is expressed on unmyelinated nociceptors, suggesting a role in nociception. Consistent with this posit, TRPA1 is activated by burning, irritating compounds, such as allyl isothiocyanate and formalin, and can also transduce noxious cold or mechanical force stimuli (Corey DP, *et al.* 2004; McNamara CR, *et al.* 2007; Story GM, *et al.* 2003).

Intraplantar injection of RvE1 is effective in reducing nocifensive behavior induced with CAP, a TRPV1 agonist, but not by mustard oil, a TRPA1 agonist. (Xu ZZ, *et al.* 2010). Patch clamp experiments in lamina II neurons *ex vivo* in isolated spinal cord slices and in dissociated mouse DRG neurons, showed that RvE1 inhibited capsaicin-induced TRPV1-dependent sEPSCs (Figure 14A) (Park C-K, *et al.* 2011), but not mustard oil allyl isothiocyanate (AITC)-induced TRPA1-dependent sEPSCs (Figure 14B) (Park C-K, *et al.* 2011). Taken together, these results suggest that RvE1 reduces CAP-induced pain inhibiting TRPV1-dependent sEPSCs. RvE1 does not directly bind TRPV1 (Xu ZZ, *et al.* 2010), but there is evidence that RvE1's known receptor, ChemR23, is co-localized on TRPV1+ neurons (Figure 15) (Xu ZZ, *et al.* 2010).

## RvE1 and ChemR23 in Analgesia

ChemR23, the receptor for RvE1, is co-expressed DRG neurons that express TRPV1 (Figure 15) (Xu ZZ, *et al.* 2010). Other lines of evidence also point to a possible role for ChemR23 in RvE1 analgesia for formalin-induced second phase TRPV1-mediated pain. First, RvE1's analgesic action is abolished with pertussis toxin, a G<sub>i</sub>-coupled GPCR inhibitor, but not with naloxone, an opioid receptor antagonist. ChemR23 is a G<sub>i</sub>-coupled GPCR. Second, ChemR23 specific siRNA knockdown inhibited RvE1's analgesic effects. And finally, chemerin, another endogenous ligand for ChemR23, also shows analgesic effects in this model (Xu ZZ, *et al.* 2010). Potentially, RvE1-binding to ChemR23 may inhibit TRPV1 downstream signaling resulting to suppress TRPV1-dependent sEPCs and pain.

## *RvE1 Analgesic Activity in RA*

RvE1 has multiple potential routes to providing analgesia in RA, by acting on the immune system to reduce inflammatory pain, or by directly acting on the nervous system to quiet sEPSCs. By directly inhibiting TNF- $\alpha$ , RvE1 reduces TNF- $\alpha$ -mediated edema. RvE1 can also down-regulate TNF- $\alpha$  expression from immune cells. Reduction in circulating TNF- $\alpha$  would translate to less activation of TNFR1- and TNFR2-mediated hyperalgesic sEPSCs responses on neurons. RvE1 can also act through the TRPV1 pathway to quiet hyperalgesic sEPSCs. It is not known if RvE1 can cross the blood-brain barrier (BBB); however, *i.t.* or locally administered RvE1 has been shown to act in the CNS- and PNS-mediated analgesia respectively. This suggests that even if an RvE1-

based therapeutic cannot cross the BBB to affect CNS- mediated pain; it has the potential to act locally to inhibit PNS-mediated pain.

### **RvE1 and Bone Homeostasis**

Compared to understanding of the role of RvE1 in inflammation resolution, and the role of RvE1 in analgesia, the evidence of a role for RvE1 in bone homeostasis is relatively recent. However in both a physical bone injury model, and a bacterially-induced injury model, RvE1 reverses bone loss.

#### *RvE1 and Uniform Craniotomy*

The uniform craniotomy mouse model is a bone-healing model for physical bone injury. In this model, a 1-mm craniotomy defect is inflicted. Over two weeks, the injury is allowed to heal. The endpoint is a histological measure of the bone recovery as a percentage of the area of the original defect. New bone formation was significantly increased in mice that were treated with 100 ng RvE1 in PBS, compared with PBS, subperiosteal injections over the defect every other day for the 2-week healing period. In this model CD11b<sup>+</sup> monocytes infiltrate the injured tissue, and osteoblasts form new bone. Both CD11b<sup>+</sup> monocytes and osteoblasts endogenously express ChemR23, the receptor for RvE1. To determine which of these cell types was responsible for the RvE1-accelerated bone regrowth; this model was performed on a CD11b promoter-driven ChemR23 transgenic mouse. In these transgenic mice, CD11b<sup>-</sup> osteoblasts express only endogenous levels of ChemR23 while CD11b<sup>+</sup> monocytes overexpress ChemR23.

CD11b promoter-driven ChemR23 transgenic showed the same rate of regrowth as wild type mice with either RvE1-treatment or PBS treatment (Figure 16) (Gao L, *et al.* 2013). This unexpected result suggested that RvE1-mediated signaling through ChemR23 on infiltrating CD11b+ monocytes was not responsible for RvE1-accelerated bone regrowth because overexpression of ChemR23 does not further enhance this effect.

To further address the possibility that RvE1 directly acts on ChemR23 on osteoblasts to promote bone growth, investigators applied RvE1 to *ex vivo* neonatal calvarial osteoblast cultures and measured the RANKL/OPG ratio, an indicator osteoclast/osteoblast activity. If the RANKL expression is favored, osteoclastogenesis is favored, leading to net bone loss. If OPG expression is favored, osteoblastogenesis is favored, leading to net increase in bone volume and density. In the presence of RvE1, the bone cultures showed increased expression of OPG, favoring osteoblast action (Gao L, *et al.* 2013). Addition of the pro-inflammatory cytokine IL-6 suppressed RvE1-mediated increase in OPG. Expression of RANKL was not altered with RvE1 in the presence or absence of IL-6 in calvarial osteoblast cultures (Figure 17) (Gao L, *et al.* 2013). The calvarial osteoblast culture data is consistent with the *in vivo* uniform craniotomy data: both sets of data suggest RvE1 directly acts through ChemR23 on osteoblasts to favor osteoblast activity and bone growth.

#### *RvE1 and Periodontitis*

RvE1's effects on bone damage in animal models of arthritis have not been reported; however, RvE1's effects on bone damage have been reported in animal models

of periodontitis. Periodontitis is constellation of inflammation-mediated bone loss diseases and has pathological features that are similar to arthritis. The etiology of periodontitis is known to be specific gram-negative bacteria; however, bone loss is directed by host inflammatory response through osteoplastic resorption. In the *Porphyromonas gingivalis* ligature rabbit model of periodontitis, *P. gingivalis*-treated ligatures on the second molars results in neutrophil infiltration and osteoclast-mediated bone loss over a 6-week course. Although RvE1 has no antibacterial activity against *P. gingivalis*, topical application of RvE1 to the ligature inhibited alveolar bone loss by 95% (Figure 18) (Hasturk H, *et al.* 2007).

#### *RvE1 and Bone Homeostasis in RA*

In RA, as in the uniform craniotomy model and the periodontitis model, the bone homeostatic equation is skewed toward net bone loss. If RvE1 can up-regulate OPG in RA-affected bone, as it does in the bone culture experiments, this would serve to skew the bone homeostasis equation back toward bone regeneration.

## **Rodent models of Rheumatoid Arthritis**

Animal models have been proven to be an essential tool in understanding the pathogenic mechanisms of RA. Several rodent models have been developed, each with its limitations and insights. Broadly, RA animal models can be divided into two categories: (1) exogenously induced models of RA, and (2) genetically modified spontaneous models of RA (Asquith DL, *et al.* 2009).

### *Exogenously Induced Models of RA*

The first category, exogenously induced models of RA, includes the collagen-induced arthritis model (CIA) (Courtney JS, *et al.* 1980; Trentham DE, *et al.* 1977), the collagen antibody-induced arthritis model (CAIA) (Rowley MJ *et al.*, 2008; Stuart JM and Dixon FJ. 1983), the antigen-induced arthritis model (AIA) (Brackertz D, *et al.* 1977a; Brackertz D, *et al.* 1977b), the zymosan-induced arthritis model (Keystone EC, *et al.* 1977), and arthritis models induced with non-immunogenic adjuvants (Holmdahl R, *et al.* 2001).

#### Collagen-induced Arthritis (CIA)

Collagen-induced arthritis is considered the gold standard in arthritis animal models. As with human arthritis, this model features breach of tolerance to an autoantigen against collagen. While the model was first described in rats (Trentham DE, *et al.* 1977), it can also be performed in mice that carry the MHC class II haplotype I-A<sup>q</sup> or I-A<sup>r</sup> (Courtenay JS, *et al.* 1977; Wooley PH, *et al.* 1985) and in non-human primates

(Cathcart ES, *et al.* 1986) making it a useful model for allometric scaling and human dose projection. Immunohistochemical analysis of articular joints through the CIA time course show that while T-lymphocytes are present and secreting IFN- $\gamma$  and IL-17A during initiation of disease, these cells become scarce during clinical disease. In contrast, macrophages infiltrate into the synovial lining and pannus early in disease initiation and appear to persist through all stages of disease. By immunohistochemical staining, these inflammatory macrophages secrete TNF- $\alpha$ , IL-6 and TGF- $\beta$  through the disease course (Table 5) (Mussener A, *et al.* 1997). Maximal expression of macrophage-derived IL-1 $\beta$  and IL-10 transcripts in synovial tissue correlate with peak CIA disease score (Stasiuk LM, *et al.* 1996). These observations suggest that CIA initiation is orchestrated by TH1 lymphocytes recruiting macrophages into the synovia, and that these macrophages sustain disease by producing TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (Luross JA, *et al.* 2001).

#### Collagen Antibody-induced Arthritis (CAIA)

Serum from collagen-immunized susceptible strains of mice can cause arthritic disease when transferred to either susceptible or resistant strains of mice. Immunohistochemical characterization of this serum transfer model showed that donor immunoglobulins were localizing on recipient joints and other cartilage (Stuart JM, *et al.* 1983). Collagen antibody-induced arthritis (CAIA) can be induced in most strains of mice by immunizing with purified anti-collagen type II (anti-CII) antibodies and results in a fast 48-hour onset severely erosive arthritis. Macrophages and PMN cells have been

implicated in disease, while T- and B-lymphocytes do not appear to play a role (Rowley MJ, *et al.* 2008).

#### Antigen-induced Arthritis (AIA)

In the antigen-induced arthritis model, mice are immunized with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant with B pertussis vaccine, and challenged with an intra-articular injection of mBSA in saline in the knee joint on day 21 post-immunization. Susceptible strains of mice develop a chronic, antigen-specific, T-cell driven arthritis. While C57BL/6 and BALB/c mice are susceptible, the CBA strain is relatively resistant. Differences in MHC class II haplotype only partially explain this disparity, as larger immunogenic doses could overcome lower humoral responses. *In vivo* radioisotopic assay showed that delayed-type hypersensitivity (DTH) responses to mBSA differed between the strains. CBA mice had a quick, weak response that dissipated quickly, while C57BL/6 mice had a later, more sustained DTH response. In this model, arthritis susceptibility depends on two factors, MHC class II haplotype and the strength of T-lymphocyte dependent DTH response (Brackertz D, *et al.* 1977a; Brackertz D, *et al.* 1977b).

#### Zymosan-induced Arthritis

Zymosan, a glucan of repeating glucose units, binds to macrophages via Toll-like receptor 2 (TLR2) to activate complement via the alternative pathway, up-regulate IL-1 $\beta$  and TNF- $\alpha$  cytokines, promote NF- $\kappa$ B translocation to the nucleus, and mobilize

arachidonate to generate sterile inflammation (Sato M, *et al.* 2003). Zymosan also increases cyclin D2 to induce macrophage proliferation. Intra-articular (i.a.) injection of zymosan particles into mouse knee joints results in chronic inflammatory arthritis characterized by mononuclear cell infiltration and synovial hypertrophy. In contrast, i.a. injection of latex particles elicits a mild, transient inflammatory reaction (Keystone EC, *et al.* 1977).

#### Arthritis Models Induced with Non-immunogenic Adjuvants

Finally, chronic relapsing arthritis can be induced in rats using non-immunogenic adjuvants such as mineral oil, pristane and squalene (Holmdahl R, *et al.* 2001). These arthritogenic adjuvants do not contain MHC binding peptides, yet disease is both MHC-dependent and T cell-dependent. While this suggests that an endogenous peptide or peptides must be involved, none have been discovered to date (Holmdahl R, *et al.* 2001). Mice or rats that are repeatedly injected i.p. with pristane, a natural saturated terpenoid alkane derived from shark liver oil, develop some biomarkers of human RA such as high levels of circulating RF and anti-CII antibodies. However, these animals also develop plasmacytomas in the peritoneal cavity, which are not common in human RA, suggesting a possible non-concordant mechanism (Wooley PH, *et al.* 1989). In rats, arthritis induced with single subdermal injection of squalene, a triterpene adjuvant derived from shark liver oil, generates T-lymphocytes that express IL-1 $\beta$  and IFN- $\gamma$ . By day 8 post-injection, these T-lymphocytes are arthritogenic and can transfer disease when adoptively transferred to naïve rats (Holm BC, *et al.* 2004).

### *Genetically-modified spontaneous models of RA*

The second category, genetically-modified spontaneous models of RA, includes the TNF- $\alpha$  transgenic mouse (Keffer J, *et al.* 1991), the K/BxN mouse (Kouskoff V, *et al.* 1996), the human DR4-CD4 transgenic mouse (Eming R, *et al.* 2002; Fugger L, *et al.* 1994), the IL-1 receptor antagonist<sup>-/-</sup> mouse (Koenders ML, *et al.* 2008), and the DNase II<sup>-/-</sup> IFN-IR<sup>-/-</sup> mouse (Kawane K, *et al.* 2006).

#### The TNF- $\alpha$ transgenic mouse

The 3' untranslated region of the human TNF- $\alpha$  gene plays a role in regulating transcription of TNF- $\alpha$ . Transgenic mice expressing intact 3' untranslated region with the full human TNF- $\alpha$  gene have low constitutive TNF- $\alpha$  expression, but can respond to endotoxin challenge with macrophage-specific TNF- $\alpha$  secretion. However, when the 3' untranslated region is replaced with the 3' untranslated region from human  $\beta$ -globulin gene, transgenic mice with this modified 3' region constitutively express high levels of TNF- $\alpha$ , and develop chronic inflammatory arthritis with 100% penetrance by between weeks 3 and 4 post-birth (Keffer J, *et al.* 1991). This disease is characterized by severe, symmetric swelling of joints, and PMN and lymphocytic inflammatory infiltration of the synovial space, which are consistent with human RA. However, these mice never develop detectable levels of circulating anti-RF. Treatment of arthritic mice with monoclonal anti-TNF- $\alpha$  completely eliminates disease (Keffer J, *et al.* 1991).

#### The K/BxN mouse

The K/BxN model is a complex model of rheumatoid arthritis that was serendipitously discovered when the TCR transgenic KRN line was crossed with MHC class II A<sup>g7</sup>-transgenic mice (Kouskoff V, *et al.* 1996). K/BxN mice develop severe, erosive arthritic disease characterized by high serum anti-glucose-6-phosphate isomerase (GPI) antibodies, IL-1 $\beta$ - and TNF- $\alpha$ -mediated complement cascade activation, recruitment of neutrophils and macrophages to joints, and mast cell degranulation (Matsumoto I, *et al.* 2003; van Gaaleen, *et al.* 2004). Serum from these mice can transfer disease to several strains of naïve mice. Other known RA-associated autoantibodies, such as anti-RF, anti-CCP and anti-CII, target the cartilage, so the connection to the resulting joint inflammation is easy to understand. GPI, on the other hand, is ubiquitously expressed and yet in this model, inflammation is still limited to joints. Subsequent to the publishing of the K/BxN model, human RA patients were tested for serum anti-GPI antibodies. In one study, as high as 64% of human RA patients not only have serum anti-GPI antibodies, but highly somatically mutated anti-GPI antibodies associated with synovial endothelium and joint-infiltrating neutrophils (Schaller M, *et al.* 2001). Hypermutation suggested affinity maturation that repeatedly drove higher affinity to the target GPI. However, unlike autoantibodies specific for cartilage components, which bound to cartilage via antigen-specific hypermutated regions, anti-GPI antibodies bound to synovial endothelium and neutrophils via their F<sub>c</sub> regions. This suggested that antigen specificity was not targeting these autoantibodies to the joints. Transfer of K/BxN serum to mice deficient in F<sub>c</sub>R $\gamma$ , complement 5a receptor (C5aR) or CD11a/LFA-1 resulted in protection from disease relative to transfer into a wild type host. Transfer of K/BxN

serum into mice with a neutrophil-specific deficient for IL-1 $\beta$  or LTB4 resulted in a diminution of disease. Together these results suggest that F $_c$ R $\gamma$ , C5aR and CD11a/LFA-1 function are essential, and IL-1 $\beta$  and LTB4 function contribute to anti-GPI-mediated arthritis (Monach PA, *et al.* 2010).

#### The Human DR4-CD4 Transgenic Mouse

In human RA, genetic susceptibility to disease is increased with the HLA class II allele HLA-DR\_0401. To create a mouse model to mimic this, four separate transgenes are required: (1) HLA-DR\_0401, the human HLA class II allele, (2) HCgp-39, an RA-related human auto antigenic protein, (3) the human T cell receptor (TCR) specific for the HCgp-39 epitope in the context of HLA-DR\_0401, and (4) human CD4, the human T helper cell TCR-accessory molecule. This model is used to study the breach of tolerance mechanisms that initiate disease (Eming R, *et al.* 2002; Fugger L, *et al.* 1994).

#### The IL-1R antagonist<sup>-/-</sup> Mouse

In IL-1 receptor antagonist (IL-1Ra)<sup>-/-</sup> BALB/c mice, unopposed IL-1 receptor signaling, in combination with environmental stimuli, drive progressive arthritis. IL-1Ra-deficient mice develop arthritic disease, with an onset at 3 weeks of age, characterized by inflamed hind limbs, high levels of IL-1 $\beta$ , TNF- $\alpha$  and IL-17A in serum and synovial fluid, increased numbers of TH17 cells in serum and synovial fluid, and accelerated bone erosion (Koenders ML, *et al.* 2008).

#### The DNase II<sup>-/-</sup>IFN-IR<sup>-/-</sup> Mouse

DNase II / IFN-IR double deficient mice and induced DNase II-deficient mice develop a severe polyarthritis. These mice have TNF- $\alpha$ -dependent inflammation, high levels of circulating anti-CCP antibodies and rheumatoid factor. It is hypothesized that inappropriate cytokine release is precipitated by DNase II-deficiency-mediated incomplete DNA disposal in macrophages (Kawane K, *et al.* 2006).

#### *RvE1 Evaluation in an RA Animal Model*

Collagen-induced arthritis is an appropriate animal RA model for RvE1-based therapeutic testing. This is an established and well-studied model with clinical, cellular and molecular features that mimic human RA disease course. Unlike genetic models like the TNF- $\alpha$  transgenic or the IL-1 $\beta$  antagonist <sup>-/-</sup> mouse, this model has a full spectrum of immune mediators contributing to disease progression. Unlike the DR4-CD4 transgenic mouse that focuses on disease initiation, DNase II <sup>-/-</sup> IFN-IR <sup>-/-</sup> mouse that focuses on macrophage dysfunction (Kawane K, *et al.* 2006), or the AIA model that focuses on post-antibody disease progression (Brackertz D, *et al.* 1977a), the CIA model looks at the full time course of disease progression (Luross JA, *et al.* 2001).

The disease score in the CIA model is based on the number and severity of swollen joints, and is roughly similar to the disease activity score (DAS) used in assessment of RA in human patients. While this measure is subjective, it can be used to assess whether RvE1 treatment can reduce the overall clinical disease score. Pilot experiments looking at CIA disease score over disease course for animals treated *i.p.* with PBS vehicle or RvE1 should determine if an RvE1 therapeutic is viable for the treatment

of RA. The dose and the timing of dose administration would have to be optimized. Inflammatory markers, such as serum cytokine expression, NF- $\kappa$ B translocation and kinetics of immune cell infiltration into the joints, are well-characterized in this model. The kinetics and severity of bone erosion as measured by x-ray radiography have also been well studied in CIA.

### **Pharmacological Challenges of RvE1 Therapeutics**

#### *The Synthesis Challenges of RvE1*

Multiple possible routes for the synthesis of bioactive RvE1 have been reported in the literature. Early isolation of RvE1 was achieved through isolation of biogenic RvE1 from plasma (Serhan CN, *et al.* 2000). Biogenic RvE1 also can be isolated from *Candida albicans* (Haas-Stapleton EH, *et al.* 2007). And finally, RvE1 can be produced through stereospecific chemical synthesis (Arita M, *et al.* 2005)

Early biogenic preparation methods of RvE1 were low yield as concentrations of RvE1 are only 0.1-0.4 ng/ml in healthy human or mouse plasma (Mas E, *et al.* 2012; Serhan CN, *et al.* 2000). Although RvE1 concentrations have not been reported in conventional farm animals, it is difficult to imagine biogenic purification from plasma could be commercially viable. However, in human milk, RvE1 precursor 18R-HEPE and RvE1 were measured at between 4-12 ng/ml, at least 100 times plasma levels, and were stable in milk for 330 days (Weiss GA, *et al.* 2013). Again, RvE1 levels in milk from conventional farm animals have not been reported. However, dietary methods of

increasing  $\omega$ -3 fatty acid concentrations in goat, sheep and cow milk are known (Moghadasian MH. 2012), although whether this also leads to increases in RvE1 is not known. Currently, isolation of biogenic RvE1 from plasma or milk is not possible on a commercial scale. However, in conjunction with transgenic COX or 5-LO expression, it may be feasible in the future to harvest drug-manufacturing quantities of biologically synthesized RvE1 from animal milk. Perhaps more importantly, evidence that RvE1 is stable in milk suggests that oral administration of RvE1 may be viable.

Biological synthesis of RvE1 is another potential route. *Candida albicans* is a fungus that resides commensally on human epithelial surfaces, but can cause pathogenic inflammation. RvE1 synthesized by EPA-fed *C. albicans* or by human cells is chemically identical. *C. albicans*-derived RvE1 also has the same anti-inflammatory activities as human-derived RvE1, including decreased interleukin-8 (IL-8)-induced neutrophil chemotaxis, increased neutrophil phagocytosis and production of reactive oxygen species (ROS) (Haas-Stapleton EH, *et al.* 2007). It is thought that RvE1-induced clearance of *C. albicans* may represent a symbiotic system by which *C. albicans* can induce the host's immune system to return fungal homeostasis to commensal status. Haas-Stapleton *et al* do not reveal the yield of RvE1 from *C. albicans* cultured in the presence of EPA, but they note that RvE1 is the most abundant of many pro-resolution mediators discovered in these cultures.

The traditional manufacturing of a small molecule drug is through chemical synthesis. The chemical synthesis of bioactive RvE1 is a well-established protocol that requires sequential coupling of three stereochemically pure isomers to form an acetylenic

intermediate which can be selectively hydrogenated to form a stereochemically pure RvE1 (Figure 19) (Arita M, *et al.* 2005).

### *The Stability Challenges of RvE1*

RvE1 is structurally metabolically unstable because it contains many reactive double bonds and hydroxyl groups (Arita M, *et al.* 2005; Hong S, *et al.* 2008). The RvE1 metabolome, metabolically derived products of RvE1 can reduce and even eliminate the pro-resolution potency of RvE1 (Figure 20) (Hong S, *et al.* 2008). 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalysis of NAD<sup>+</sup>-linked oxidation of the 15 (S)-hydroxyl group of eicosanoids, such lipoxins and prostaglandins, is the major route of inactivation for these COX pathway pro-inflammatory mediators, (Ensor CM and Tai HH. 1995). 15-PGDH can also inactivate RvE1 through a similar reaction to oxo metabolites (Arita M, *et al.* 2006). In theory chemical modification to block dehydrogenation of RvE1 and prevent reduction of its conjugated double bonds may reduce 15-PGDH-catalyzed metabolism resulting a more stable pro-resolution agonist.

### *Route of Administration*

The scientific research on the many biological actions of RvE1 is based on animal models, in which RvE1 is injected, or *in vitro* experiments, in which RvE1 is applied directly to cells. Traditionally, the best route of administration for a commercially viable drug would be oral. However, the RA patient population has accepted biologic therapies

that require *i.v.* or *s.c.* injection. In these cases, the therapeutic benefit of the biologic outweighed the discomfort of injection. RvE1 may offer an even greater therapeutic benefit than current biologic therapies. Therefore, a non-oral route of administration may be commercially feasible.

#### *Toxicity Challenges for RvE1*

There have been no reported toxicities with RvE1 or RvE1 analogs tested in animal models or in humans. This is potentially because the efficacious dose of RvE1 is very low, in the nanomolar range, because as a pro-resolving mediator it is not expected to be toxic. Indeed, in a therapeutic dosing scheme, RvE1-based therapeutics could potentially be used as a short-term therapy that resets homeostasis. Although the lack of over toxicity is an encouraging pharmacological property, this presents a challenge for pre-clinical toxicology studies. In a typical toxicology study, the dose is escalated beyond the therapeutic dose until toxic effects can be observed. Although a synthetic RvE1 (Rx-10001) and a proprietary synthetic RvE1 mimetic (Rx-10045) have completed Phase 1 (NCT009401018) (Resolvyx Pharmaceuticals, Inc. 2009) and Phase 2 (NCT00799552) (Resolvyx Pharmaceuticals, Inc. 2008) clinical trials, respectively, the results were not reported. However, Rx-10045 entering Phase 2 suggests that Phase 1 findings were favorable.

#### *Current Clinical Development Activity for RvE1*

Resolvyx Pharmaceuticals, in cooperation with Celtic Therapeutics, reported development of two therapeutics based on RvE1: (1) Rx-10001, a synthetically produced

RvE1, (2) Rx-10005, a methyl ester of RvE1, and Rx-10045, a proprietary RvE1 mimetic.

Resolvix chose to target in a mouse dry eye model, as their first target disease indication. Dry eye is a good first indication to test a RvE1 therapeutic for several reasons: (1) the target tissue, the cornea, is accessible to topical application, by-passing the short half-life issue; (2) inflammation can be assessed via corneal permeability; and (3) this model of dry eye is predicative of human dry eye. C57BL/6 mice treated with systemic scopolamine and exposed to air draft and low humidity for 16 hours per day for 5 days develop increased corneal permeability to Oregon Green Dextran and reduced conjunctival goblet cell density. Mice treated topically with RX-10001 showed reduced corneal Oregon Green Dextran staining and maintenance of goblet cell density when compared to vehicle controls. The pro-drug form of RvE1, RX-10005, enhanced drug delivery into the cornea, suggesting that modification to the methyl ester form of RvE1 may have increased drug utility for RvE1 to treat dry eye (Figure 21) (de Paiva CS, *et al.* 2012). Resolvix completed a Phase I clinical trial with Rx-10001, a synthetic RvE1, in healthy volunteers in 2009 (NCT009401018) (Resolvix Pharmaceuticals, Inc. 2009), and a Phase II clinical trial with RX-10045, the RvE1 mimetic, in dry eye patients in 2010 and 2012 (NCT00799552, NCT01675570) (Resolvix Pharmaceuticals, Inc. 2009, C.T. Development America, Inc., 2012). Currently, Auventx, the re-branded incarnation of Celtic Therapeutics, reports on its website that RX-10045, a proprietary RvE1 mimetic has successfully completed the Phase II study in allergic conjunctivitis patients in 2012 (NCT01639846) (C.T. Development America, Inc., 2012) and will soon

enter a Phase III randomized, placebo-controlled, multicenter dry eye study ([www.auventx.com](http://www.auventx.com)). The Resolvyx ([www.resolvyx.com](http://www.resolvyx.com)) and Auventx websites also suggest that RvE1 therapeutics RX-10001 and RX-10005 will be tested in clinical trials in a myriad of autoimmune diseases such as asthma, IBD, RA, and various other indications. Clinical trial dose, formulation, and trial outcome data has not been disclosed publically for any RvE1 therapeutic.

## DISCUSSION

RvE1 is an endogenously produced lipid mediator that activates the resolution of inflammation, analgesia and bone homeostasis, making it an attractive potential therapeutic for RA. Although well-established animals of RA exist, current research has not directly tested RvE1 in these models. However, RvE1 administration is efficacious in inhibiting other *in vivo* models of autoimmunity, models of pain and models of joint destruction. *In vitro* assays have also confirmed that RvE1 is an effective inhibitor of inflammatory cytokine production, inflammatory cell migration, pain receptor-mediated cation channel signaling, and osteoblast activation. Extensive research into RvE1 molecular mechanisms provides a rich platform of *in vivo* and *in vitro* drug development.

Several RvE1 production methods are known, but scaling these methods to drug production quantities may be challenging. Protocols for detection of RvE1 and its metabolites in serum are established. In most animal models, nanomolar RvE1 administration elicits pro-resolving, analgesic and osteogenic effects with no apparent toxicity in animals. Although drug discovery efforts will be complicated by evaluating

three RA mechanisms *in vitro* and *in vivo*, the resulting therapeutic has the potential to treat all three mechanisms of RA and reverse disease damage without toxicity.

### **RvE1 Drug Development Strategies**

As a potential therapeutic, endogenous RvE1 has many favorable characteristics: nanomolar binding affinity for its target ChemR23 ( $K_d = 11.3$  nM) (Arita M, *et al.* 2005), and a nanomolar potency in animal models of inflammation (Arita M, *et al.* 2005; Flesher, RP *et al.*, 2013; Ishida T, *et al.* 2010; Seki H, *et al.* 2010), pain (Xu ZZ, *et al.* 2010) and bone repair (Gao L, *et al.* 2013). Endogenous RvE1 also has significant drug development challenges that need to be addressed in a RvE1-based mimetic: RvE1 is metabolized to a biologically inactive oxo-18-RvE1 within 2 hours *in vivo* (Figure 22) (Arita M, *et al.* 2006), as a lipid mediator it is highly lipophilic and unstable in aqueous solutions, the pharmacokinetics of RvE1 are not well understood especially in joints, the RvE1 pathway is not validated as target in rheumatoid arthritis, and the translation between mouse and human biology is not known.

Traditional drug discovery uses high throughput screening (HTS) of large chemical libraries to identify initial drug candidate hits and structure-activity relationship (SAR) and structure-property relationship (SPR) driven optimization to improve drug-like properties. However, in this case, an endogenous molecule, RvE1, could be used directly as the drug product or a starting point for drug development.

*Proof of Pharmacology: RvE1 in CIA*

Before an RvE1 pathway drug development can be considered, endogenous RvE1 should be tested in a mouse model of rheumatoid arthritis as a proof of pharmacology. Although the efficacy of RvE1 has been shown in other animal models of inflammation, pain and bone damage, there has not been a published study of RvE1 in an animal model of RA. In addition, because there are no approved RA therapies targeting RvE1 or ChemR23, there is no proof of pharmacology in humans.

Of the three mechanisms of RA, inflammation and bone erosion are routinely quantitatively measured in animal models of RA, but pain is not. CIA has a full course of RA disease that models the human RA time course. Inflammation can be assessed through disease progression via measurement of inflammatory cytokines IL-6 and TNF- $\alpha$  in serum, or as an endpoint measure by histologically assessing inflammatory cytokine expression and inflammatory cell infiltration into joints. Joint damage can be measured by x-ray radiography in the hind limbs of anesthetized animals through disease course, or as a very precise endpoint measure by microscopically quantitating bone loss in x-ray radiographs of dissected forelimb and hind limbs. Pain is not typically measured in this or any RA model.

To determine analgesia in animals, a pain stimulus, such as heat, is given and the pain response, such as duration of nocifensive behavior, is measured (Figure 10) (Xu ZZ, *et al.* 2010). In the presence of an analgesic, the response should be significantly diminished. Arthritis should cause hyperalgesia, so care would have to be taken to

compare naïve animals, CIA-induced PBS vehicle-treated animals, and CIA-induced RvE1-treated animals.

Analysis of proof-of-pharmacology for RvE1 in CIA should include optimization of RvE1 dose and RvE1 dose timing, and should include comparison to methotrexate and TNF- $\alpha$  inhibitors. If proof-of-pharmacology for RvE1 in CIA is achieved, further targeted modifications to RvE1 may be pursued to improve potency and pharmacological properties such as stability and manufacturability.

#### *Drug Development Screening Funnel*

Although RvE1 activity has been characterized in many *in vitro* and *in vivo* assays, many are time-consuming and low throughput. Therefore, strategic chemical modifications to RvE1, rather than a large HTS approach are preferable. The first priority gate is to improve metabolic stability while retaining or maintaining binding to ChemR23. This requires stability assays and binding assays be done in parallel. Secondly, *in vitro* functional assay should be done on a triaged subset of compounds that pass the first gate. Next, compounds that pass this secondary gate should be prioritized for *in vivo* assays: pharmacokinetic studies, followed by efficacy studies in CIA, and toxicity.

Increasing RvE1 stability

The half-life of RvE1 is ~2 hours (Figure 22) (Arita M, *et al.* 2006). The major RvE1 products from human blood were 10,11-dihydro-RvE1, 18-oxo-RvE1, and 20-hydroxy-RvE1. Mouse kidney and liver generated more 18-oxo-RvE1 than other RvE1-derived metabolic products. RvE1 is converted to 18-oxo-RvE1 *in vitro* by human recombinant 15-prostaglandin dehydrogenase (Figure 22) (Arita M, *et al.* 2006). Inhibition of RvE1 catabolism to increase RvE1's half-life could potentially be achieved by making a catabolically resistant RvE1 analog. Inhibition of RvE1 catabolism by modification of RvE1 can be achieved by making a catabolically stable but biologically active analog. Indeed, the RvE1 analog, 19-(*p*-fluorophenoxy)-RvE1 methyl ester appears to fit this profile—it is resistant to 15-PGDH catabolism, but retains activity in an *in vivo* zymosan A-induced inflammation model (Arita M, *et al.* 2006). Thus, 19-(*p*-fluorophenoxy)-RvE1 show that it is possible to design a potent anti-inflammatory, pro-resolution mediator that is more metabolically stable than endogenous RvE1.

An easy, *in vitro* enzymatic assay to measure the major RvE1 catabolic assay exists. 15-PGDH-catalyzed catabolism of RvE1 requires NAD<sup>+</sup> as a co-factor. As NAD<sup>+</sup> is converted to NADH, the 340nm absorbance increases and can be measured easily by spectrophotometer (Figure 22) (Arita M, *et al.* 2006). As the first step, chemical modifications to RvE1 can be easily assessed for resistance to 15-PGDH catabolism.

ChemR23 Binding Assay

$^3\text{H}$ -RvE1 bound to ChemR23-transfected HEK with a high affinity ( $K_d$  of 11.3 nM) (Arita M, *et al.* 2005). Unlabeled RvE1 mimetics can be assayed for ability to compete for ChemR23 by measuring the decrease in  $^3\text{H}$ -RvE1.  $^3\text{H}$ -RvE1 and human ChemR23-transfected HEK cells are commercially available.

Both the stability assay and the binding assay can be used as the first line assays to assess the largest number of RvE1 mimetics. Compounds that have improved 15-PGDH resistance and improved or maintained ChemR23 binding should be further assessed for *in vitro* function.

#### *In vitro* Functional Assays

RvE1 can target three different functional mechanisms; therefore, at least three functional assays should be developed to fully assess potency. RvE1 function on promotion of inflammation resolution, inhibition of pain, and inhibition of bone loss should all be explored as follows. While these readouts may be best assessed through *in vivo* models, for initial screening, *in vitro* assays offer a less complex assay, higher throughput, and shorter assay times.

#### *In vitro* NF- $\kappa$ B activation in ChemR23-transfected HEK

In immune cells, the anti-inflammatory, pro-resolving activity of RvE1 is mediated through ChemR23. Human ChemR23-transfected HEK cells that have an NF- $\kappa$ B-luciferase reporter will luminesce when NF- $\kappa$ B is activated by TNF- $\alpha$ . TNF- $\alpha$ -

induced luciferase activity in these cells is inhibited by RvE1 (Figure 4) (Arita M, *et al.* 2005). RvE1 mimetics can be compared to native RvE1 in this assay to determine if they are likely to inhibit inflammation *in vivo*.

#### *In vitro* RANKL/OPG Assay

The *in vitro* activity of RvE1 analogs on bone can be measured using neonatal mouse calvarial primary bone cultures grown in the presence of ascorbic acid,  $\beta$ -glycophosphate and vitamin D<sub>3</sub>. At day 10, cultures are stimulated with IL-6 and IL-6 receptor in the presence of RvE1 analog or vehicle. Native RvE1 can be used as a positive control. 48 hours post-stimulation, supernatants can be analyzed for RANKL and OPG via ELISAs that are commercially available (Figure 17) (Gao L, *et al.* 2013). Compared to supernatants from vehicle control-treated cultures, supernatants treated with RvE1 or analogs with RvE1 activity should have the same concentration of RANKL and a higher concentration of OPG (Gao L, *et al.* 2013).

#### *In vitro* sEPSC Assay

The TRPV1 agonist capsaicin can evoke nocifensive behavior *in vivo* (Xu ZZ, *et al.* 2010) and can increase sEPSC frequency in DRG neuron *in vitro* (Park C-K, *et al.* 2013) which can be inhibited with RvE1. This *in vitro* capsaicin-elicited sEPSC patch clamp assay can be used as a surrogate readout to test RvE1 analogs for analgesic activity. Dorsal root ganglion cells isolated from CD1 mice can be cultured on glass cover slips in neurobasal defined medium with 2% B27 supplement for 24 hours. Inward currents can be measured with room temperature whole-cell current-clamp recordings

holding the potential at -60mV (Figure 14) (Park C-K, *et al.* 2013). Capsaicin-elicited increases in sEPSC frequency should be unaffected in the presence of PBS vehicle, but should be diminished with RvE1 or with analogs with RvE1 activity. Due to the time-intensive nature of this assay, it should only be used to assess compounds that showed positive results in the other *in vitro* activity assays.

#### *In vivo* Functional Assays

Compounds that show *in vitro* functional activity as potent as or more potent than endogenous RvE1 should be further tested in *in vivo* assays: pharmacokinetic studies, followed by efficacy studies in CIA, and toxicity.

#### Pharmacokinetics

A liquid chromatography-ultraviolet-tandem mass spectrometry (LC-UV-MS-MS) method has been developed to detect RvE1 and other lipid mediators in biological fluids (Lu Y, *et al.* 2005). Initially compounds should be tested *i.v.* to determine pharmacokinetic characteristics. Compounds with increased *in vivo* half-life, improved joint tissue penetration, or decreased clearance should be used to make further compound structure improvements and a limited number of representatives of compound classes should be further assessed in CIA. As testing progresses, the bioavailability of compounds given *s.c.* or *p.o.* should be evaluated as these are more attractive treatment options.

## CIA

CIA is a well-established mouse model of rheumatoid arthritis. Initial disease studies should assess compounds for decreased clinical disease score, delayed onset of disease. Inflammation can be evaluated by measuring serum IL-6 and TNF- $\alpha$  throughout, or through endpoint histology to assess inflammatory cytokine expression and inflammatory cell infiltration into joints. Joint damage can be assessed through x-ray radiography. Although pain is not typically measured in CIA, efficacy of compounds in analgesia could potentially be assessed by decrease in heat-induced hyperalgesia.

As candidates progress, CIA studies can be modified. Initially, compounds should be tested prophylactically and dosed *i.v.* to allow the highest bioavailability and best chance at efficacy in this model. As compounds are further studied, *s.c.* and *p.o.* administration, therapeutic dosing, and short term dosing should be explored. Ultimately, a once-a day *p.o.* short-term therapeutic dose that allows animals to achieve remission and reversal of disease would be optimal.

A short term dosing schedule would also help answer some of the questions about effects of chronic RvE1 mimetic dosing.

## CONCLUSION

A RvE1-based therapeutic has the potential to be a first-in-class therapeutic that targets the three main mechanisms of rheumatoid arthritis: inflammation, pain and joint damage. Endogenous RvE1 has three biological activities: an anti-inflammatory pro-

resolving activity, a potent analgesic activity and enhancement of osteoblasts. These activities would not only alleviate symptoms of RA, they have the potential to reverse disease, resolve inflammation, and restore damaged joints.

Although there are no direct reports of RvE1 or RvE1-based therapeutics in RA or animal models of RA, there are reports of RvE1 used therapeutically in animal models of inflammation, pain, and bone damage as well as clinical applications of RvE1 derivatives. These studies have provided a foundation of data that supports the efficacy of RvE1 in inhibiting and reversing inflammation, pain and bone damage *in vivo*. In addition, there are *in vitro* assays that confirm RvE1's actions on these three mechanisms on a molecular and cellular level. Well-studied animal models of RA, particularly collagen-induced arthritis, could be used as proof-of-pharmacology for an RvE1-based therapeutic in RA. Further chemical structural changes could be assessed in established *in vitro* assays for 15-PGDH stability, ChemR23 binding, and *in vitro* potency. Compounds that showed improved compound stability and *in vitro* functional activity can be further assessed *in vivo* to determine pharmacokinetic characteristics, and efficacy in CIA with inflammatory, bone damage and pain readouts.

RA is an autoimmune disease driven by chronic inflammation that results in joint damage and debilitating pain. Although there are many RA therapies available, most are focused providing analgesia or inhibiting inflammation. A RvE1-based therapeutic has potential to not only inhibit inflammation, but may also coordinate a pro-resolution program that may return the immune system to a normal homeostatic state. A RvE1 mimetic also has the potential to restore joints by favoring osteoblastogenesis rather than

osteoclastogenesis. Finally, a RvE-1 therapeutic has the potential to provide analgesia, not just through inhibiting TNF- $\alpha$ -mediated inflammatory pain, but by acting on central and peripheral nociceptive neurons to inhibit TRPV1-mediated pain. Thus, a RvE1 mimetic with increased stability and potency relative to endogenous RvE1 should result in an RA therapeutic that can decrease inflammation, increase resolution of inflammation, regenerate joints and decrease pain.

**Table 1: Rheumatoid Arthritis Therapies**

Drug Characteristics			Mechanisms of RA			Major Side Effects
Class	OTC /Rx	Name	Anti-inflammatory	Analgesia	Bone regrowth	
Analgesic	OTC	Acetaminophen	No	Yes	No	Liver tox., Multiple drug interactions
	Rx	Narcotics	No	Yes	No	Risk of dependence
NSAIDs	OTC	Aspirin	Yes	Yes	No	GI irritation, Reyes' syndrome, kidney tox.
	OTC	Ibuprofen, Aleve®	Yes	Yes	No	GI irritation, liver or kidney tox.
	Rx	Vioxx®, Celebrex®	Yes	Yes	No	Cardiovascular risk, liver or kidney tox.
Steroid	Rx	Prednisone	Yes	Yes	Thins bones	Weight gain, cataracts, diabetes
DMARD	Rx	Methotrexate	Yes	Yes	Slows Joint Damage	Increased risk of infections, nausea, low blood count
	Rx	TNF- $\alpha$ blockers	Yes	Yes	No	ADA, increased risk of infections, TB, low blood count
	Rx	Rituximab®	Yes	Yes	Slows joint damage	Increased risk of infections, nausea, low blood count
	Rx	Kineret ®	Yes	Yes	No	Malignancy, TB, increased risk of infections, low blood count
	Rx	Actemra®	Yes	Yes	No	increased risk of infections, GI risk, low blood count, cancer
	Rx	Xeljanz®	Yes	Yes	No	Increased risk of infections, cancer, and leukemia.

<b>RvE1</b>	Rx	Putative RvE1-based Therapeutic	Yes	Yes	Yes	Unknown
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OTC = over the counter medication, Rx = prescription medication

**Table 2: Lipid mediators and their anti-inflammatory and pro-resolution actions in inflammatory animal models.**

<b>Mediators</b>	<b>Species/disease model</b>	<b>Action(s)</b>	
Lipoxin A4/ATL	Rabbit/Periodontitis	Reduce neutrophil infiltration; prevent connective tissue and bone loss	
	Mouse/Peritonitis	stops neutrophil recruitment and lymphatic removal of phagocytes	
	Mouse/Dorsal air pouch	stops neutrophil recruitment	
	Mouse/Dermal inflammation	stops neutrophil recruitment and vascular leakage	
	Mouse/Colitis	Attenuate pro-inflammatory gene expression and reduce severity of colitis. Inhibit weight loss, inflammation and immune dysfunction	
	Mouse/Asthma	Inhibit airway hyper-responsiveness and pulmonary inflammation	
	Mouse/Cystic fibrosis	Decrease neutrophil inflammation, pulmonary bacterial burden and disease severity	
	Mouse/Ischemia-reperfusion		Attenuate hind-limb I/R-induced lung injury
			Detachment of adherent leukocytes in mesenteric I/R
	Mouse/Cornea	Accelerate cornea re-epithelialization, limit sequela of thermal injury and promote host defense	
	Mouse/Angiogenesis	Reduce angiogenic phenotype: endothelial cell proliferation and migration	
	Mouse/Bone-marrow transplant (BMT)	Protect against BMT-induced graft-versus-host diseases	
	Murine/Glomerulonephritis	Reduce leukocyte rolling and adherence Decrease neutrophil recruitment	
	Rat/Hyperalgesia	Prolong paw withdraw latency, reducing hyperalgesic index Reduce paw edema	
Rat/Pleuritis	Shorten the duration of pleural exudation		
Resolvin E1	Rabbit/Periodontitis	Reduces neutrophil infiltration; prevents connective tissue and bone loss; promotes healing of diseased tissues; regeneration of lost soft tissue and bone	
	Mouse/Peritonitis	Stops neutrophil recruitment; regulates chemokine/cytokine production	

		Promotes lymphatic removal of phagocytes
	Mouse/Dorsal air pouch	Stops neutrophil recruitment
	Mouse/Retinopathy	Protects against neovascularization
	Mouse/Colitis	Decreases neutrophil recruitment and pro-inflammatory gene expression; improves survival; reduces weight loss
Resolvin D1	Mouse/Peritonitis	Stops neutrophil recruitment
	Mouse/Dorsal skin air pouch	Stops neutrophil recruitment
	Mouse/Kidney ischemia-reperfusion	Protects from ischemia-reperfusion-induced kidney damage and loss of function; regulates macrophages
	Mouse/Retinopathy	Protects against neovascularization
Protectin D1	Mouse/Peritonitis	Inhibit neutrophil recruitment
		Regulate chemokine/cytokine production
		Promote lymphatic removal of phagocytes
		Regulate T-cell migration
	Mouse/Asthma	Protect from lung damage, airway inflammation and airway hyper responsiveness
	Human/Asthma	Protectin D1 is generated in humans and appears to be diminished in asthmatics
	Mouse/Kidney ischemia-reperfusion	Protect from ischemia-reperfusion-induced kidney damage and loss of function; regulate macrophages
	Mouse/Retinopathy	Protect against neovascularization
	Rat/Ischemic stroke	Stop leukocyte infiltration, inhibit NF- $\kappa$ B and cyclooxygenase-2 induction
Human/Alzheimer's disease	Diminished protectin D1 production in human Alzheimer's disease	

Adapted from Table 2 of Serhan CN, *et al.* 2008

**Table 3: Resolvins and their anti-nociceptive and anti-inflammatory actions in animal models of pain.**

<b>Resolvin</b>	<b>Animal</b>	<b>Pain model</b>	<b>Anti-nociceptive and anti-inflammatory action</b>
RvE1	Mouse	Formalin	Reduce inflammatory pain
		CRG	Decrease inflammatory pain
			Reduce IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and CCL2 expression
			Reduce edema and neutrophil infiltration
		CFA	Reduce inflammatory pain
		CAP	Reduce spontaneous pain
		TNF- $\alpha$	Abolishes heat hyperalgesia and mechanical allodynia
		Incision	Reduce postoperative pain
RvD1	Mouse	CRG	Reduce inflammatory pain
		CFA	Reduce inflammatory pain
		Formalin	Reduce inflammatory pain
		Mustard oil	Reduce spontaneous pain
		PGE <sub>2</sub>	Reduce inflammatory pain
	Rat	Incision	Reduce postoperative pain
		SMIR	Reduce postoperative pain
AT-RvD1	Rat	CFA	Reduce inflammatory pain
			Reduce TNF- $\alpha$ , IL-1 $\beta$ , COX-2, and NF- $\kappa$ B expression

Adapted from Table 2 of Ji RR, *et al.* 2012

**Table 4: Step-wise cleavage of prochemerin**

<b>Protease</b>	<b>C-terminal sequence</b>	<b>Amino Acid Order</b>	<b>Activity</b>
Prochemerin	...YFPGQFAFSKALPRS	21-163	Inactive
Tryptase	...YFPGQFAFSK	21-158	Chemotaxis
Plasmin	...YFPGQFAFSK	21-158	Chemotaxis
Plasmin/CPB	...YFPGQFAFS	21-157	Chemotaxis, strong
Plasmin/CPN	...YFPGQFAFS	21-157	Chemotaxis, strong
Staphopain B	...YFPGQFAFS	21-157	Chemotaxis, strong
Elastase	...YFPGQFAFS	21-157	Chemotaxis
Cathepsin G	...YFPGQFAF	21-156	Chemotaxis
PR3	...YFPGQFA	21-155	Less chemotaxis
Chymasin	...YFPGQF	21-154	No chemotaxis
Cathepsin S	...FLPGQFA	23-154	Anti-inflammatory
Calpains	...FLPGQFA	23-154	Anti-inflammatory

Step-wise cleavage of pro-chemerin results in altered biological activity.

Adapted from Table 1 from Du XY and Leung LL. 2009.

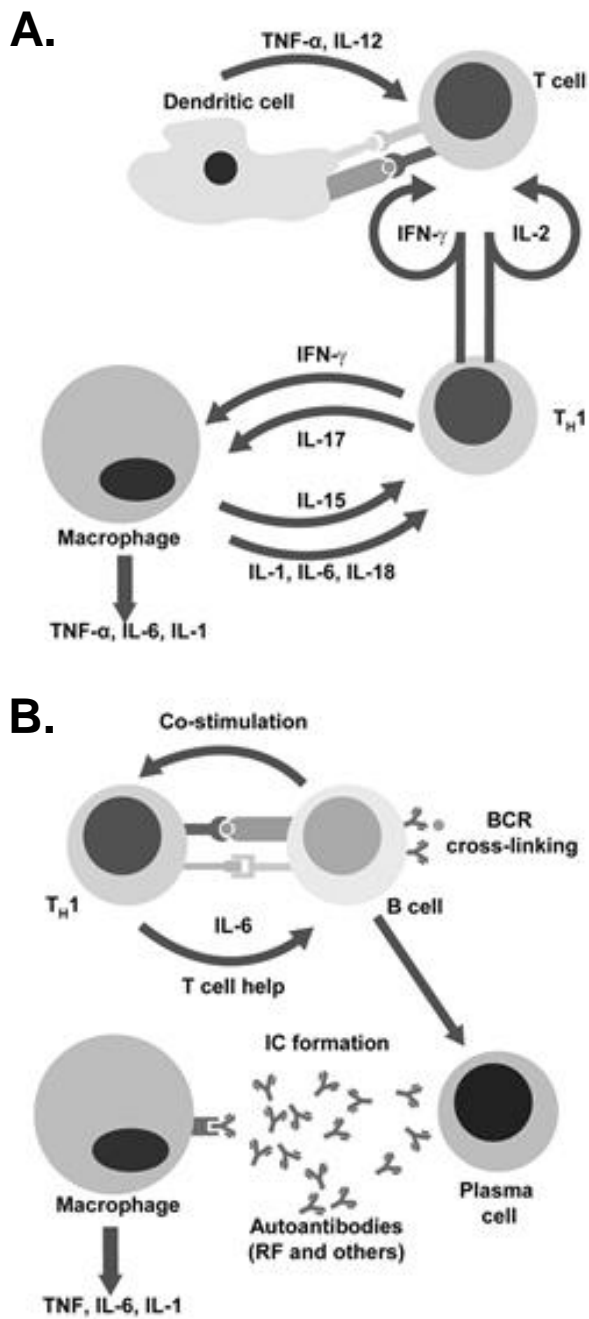
**Table 5: Frequency of cells that stain positive for cytokine and immune cell surface markers by immunohistochemistry through CIA disease course.**

	Days post onset			
	Day 0	Day 2-3	Day 11-12	Day 23-25
<b>TNF-<math>\alpha</math></b>	0	+++	+++	++
<b>IL-6</b>	0	++	+++	++
<b>IFN-<math>\gamma</math></b>	0	+	+	0
<b>CD4</b>	0	+	++	+
<b>I-A<sup>q</sup></b>	(+)	++	++	+
<b>Mac-1</b>	+++	+++	++++	+++

Frequency of cells in arthritic and non-arthritic limbs recognized immunohistochemistry by antibodies for cytokine or cell surface marker shown.

\*0 = 0%, (+) <1%, + = 1-10%, ++ = 10-25%, +++ = 25-50%, ++++ = 50-75%

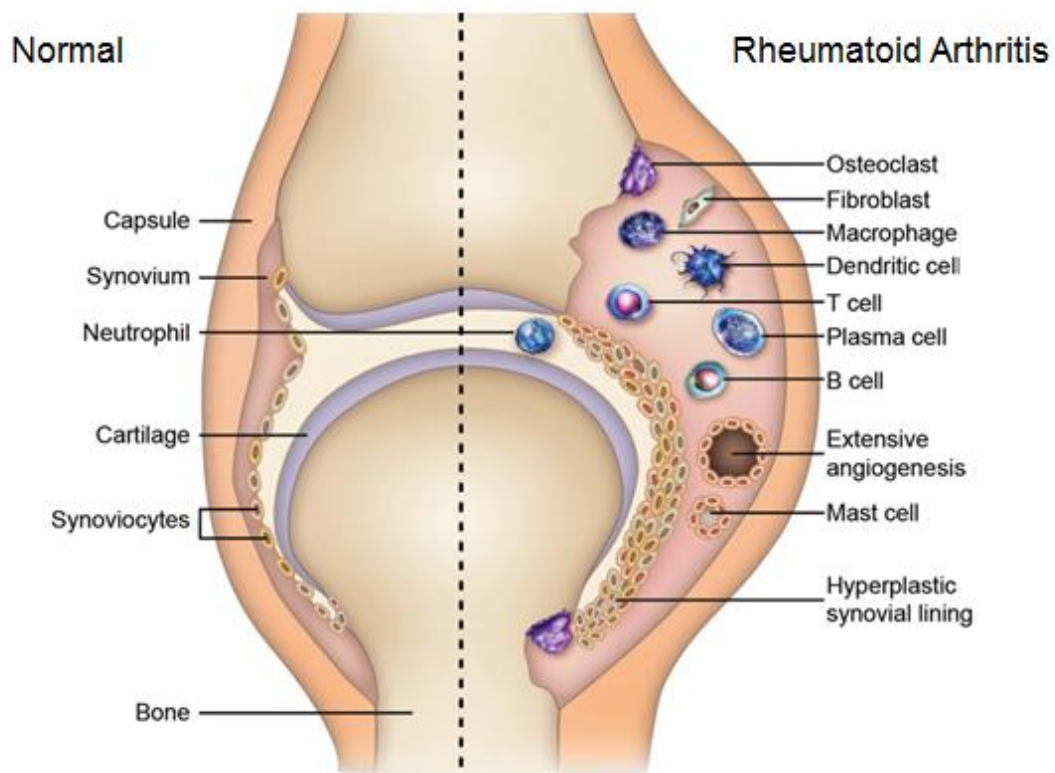
Adapted from Table 1 of Müssener A, *et al.* 1997.



**FIGURE 1: Mechanisms of Rheumatoid Arthritis: Inflammation**

Macrophages participate in pro-inflammatory cytokine (TNF- $\alpha$ , IL-6, IL-1 $\beta$ ) production in both (A.) T-cell driven and (B.) B-cell driven phases of RA progression.

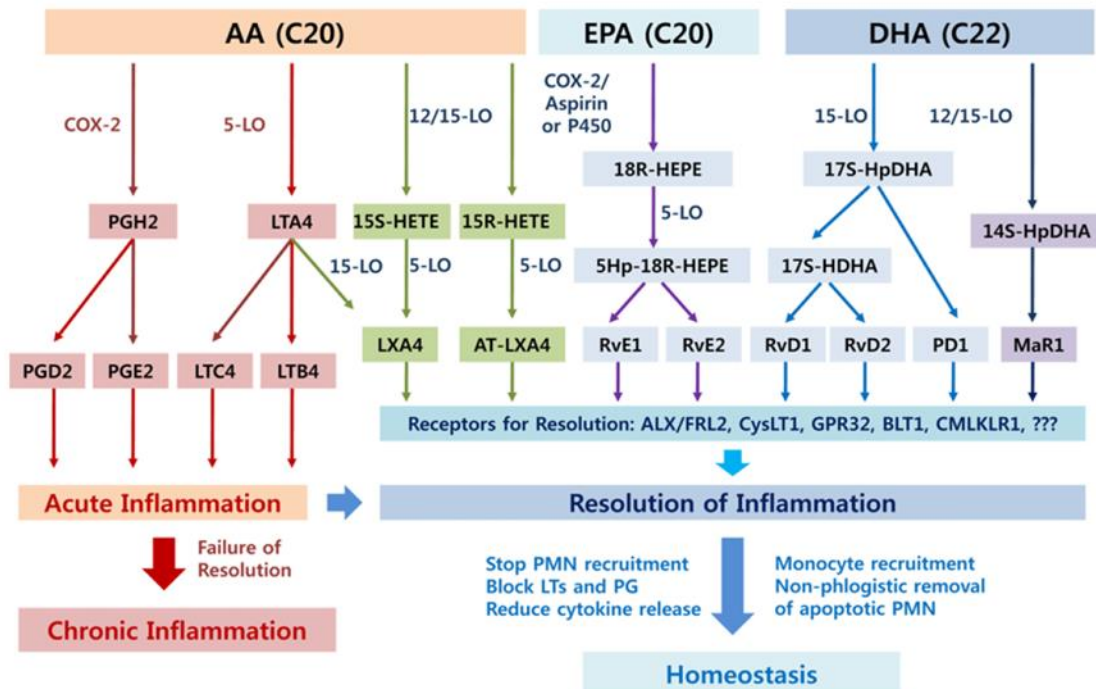
Figure 2 from Choy E. 2012



**Figure 2: Mechanisms of Rheumatoid Arthritis: Bone Damage**

Schematic view of (left) a normal joint and (right) a joint affected by RA.

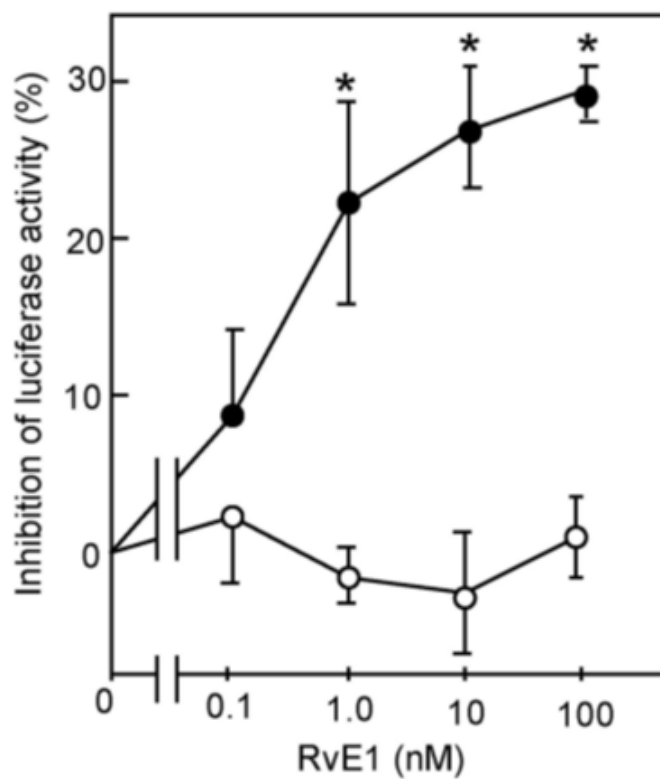
Figure 1 from Choy E. 2012.



**FIGURE 3: Biosynthetic Cascade, Actions and Receptors for Lipid Mediators.**

Biosynthetic cascades of selected lipid mediators derived from arachadonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). COX-2 (cyclooxygenase-2), 5-LO (arachidonate 5-lipoxygenase), 12/15-LO (12/15-lipoxygenase), COX-2/aspirin (COX-2 in the presence of aspirin), P450 (cytochrome P450), 15-LO (15-lipoxygenase) catalytically convert these precursors into 15S-hydroxyeicosatetraenoic acid (15S-HETE), 15R-hydroxy-eicosatetraenoic acid (15R-HETE), 18R-hydroxyeicosapentaenoic acid (18R-HEPE), 5S-hydroperoxy-18R-hydroxyeicosapentaenoic acid (5Hp-18R-HEPE), 17S-hydroperoxy docosahexaenoic acid (17S-HpDHA), 17S-hydroxydocosahexaenoic acid (17S-HDHA), 14S-hydroperoxydocosahexaenoic acid (14S-HpDHA), as well as PG (prostaglandins), including PGH2, PGD2, and PGE2, and LT (leukotrienes), including LTA4, LTC4 and LTB4) participate in chronic inflammation if acute inflammation fails to resolve. Resolvins including RvE1, RvE2, RvD1 and RvD2, along with LipoxinA4 (LXA4), aspirin triggered LX4 (AT-LXA4), Protectin D1 (PD1), and Maresin 1 (MaR1) promote resolution of inflammation by binding to cell surface receptors such as lipoxin A4 receptor/formyl peptide receptor 2 (ALX/FPR2), cysteinyl leukotriene receptor 1 (CysLT1), G-coupled receptor 32 (GPR32), LTB4 receptor 1 (BLT1), Chemokine-like Receptor 1 (CMLKR1, also called ChemR23) and other unidentified receptors.

Figure 2 from Serhan CN and Petasis NA. 2011.



**Figure 4: RvE1 inhibits TNF- $\alpha$ -induced NF- $\kappa$ B activation in ChemR23-transfected HEK cells.**

Dose-dependent RvE1 inhibition of TNF- $\alpha$ -induced NF- $\kappa$ B activation with an EC<sub>50</sub> of 1.0 nM in ChemR23-transfected (filled circles) but not in mock-transfected (open circles) NF- $\kappa$ B-luciferase reporter HEK cell.

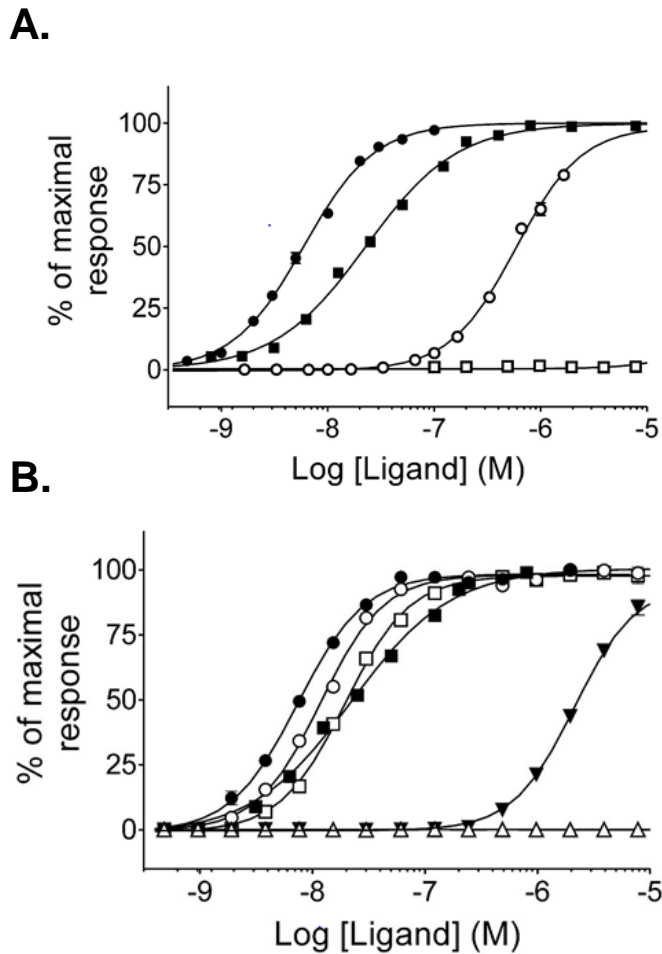
Adapted from Figure 3D of Arita M, *et al.* 2005.

	Signal peptide							
Human	MRRLLIPLAL	WLGAVGVG..VA	ELTEAQRRGL	QVALEEFHKK	PPVQWAFQET	SVESAVDTPF		60
Pig	MWQLLLPLAL	WLGTMGLG..RA	ELTAAQLRGL	QVALEEFHKK	PPVQWAFRET	GVNSAMDTPF		60
Cattle	MWQLLLPLAL	GLGTMGLG..RA	ELTTAQRHGL	QVALEEFHKK	PPVLWAFQVT	SVDNAADTLF		60
Rat	MKCLLISLAL	WLGTTADHGTGL	ELSETQRRGL	QVALEEFHRH	PPVQWAFQEI	GVDSADDLFF		62
Mouse	MKCLLISLAL	WLGTVGTRGTEP	ELSETQRRSL	QVALEEFHKK	PPVQLAFQEI	GVDRAEVLF		62
Human	PAGIFVRLEF	KLQQTSCRKR	DWKKPECKVR	PNGRKRKCLA	CIKLGSEDKV	LGRLVHCPIE		120
Pig	PAGTFVRLEF	KLQQTSCRKR	DWKKAECKVK	PNGRKRKCLA	CIKLNSDKV	LGRMVHCPIE		120
Cattle	PAGQFVRLEF	KLQQTSCRKK	DWRKEDCKVK	PNGRKRKCLA	CIKLDSKDQV	LGRMVHCPIQ		120
Rat	SAGTFVRLEF	KLQQTSCCLK	DWKKPECTIK	PNGRKRKCLA	CIKLDPKGKV	LGRMVHCPII		122
Mouse	SAGTFVRLEF	KLQQTNCPKK	DWKKPECTIK	PNGRRRKCLA	CIKLDPKGKI	LGRIVHCPII		122
					↓ Chemotaxis			
Human	TQVLRAEEH	QETQCLRVQR	AGEDPHSYFF	PGQFAFSKAL	PRS			163
Pig	TQVQREPEER	QEAQCSRVER	AGEDPHSYFF	PGQFAFFKAL	PPS			163
Cattle	TQVQRELDDA	QDAQCSRVER	AGEDPHSYLL	PGQFAFIKAL	SP.			162
Rat	KQGPQQ..EP	QESQCSKIAQ	AGEDSRIYFF	PGQFAFSRAL	QSK			163
Mouse	KQGPQ...DP	QELQCIKIAQ	AGEDPHGYFL	PGQFAFSRAL	RTK			162
					↑ Anti-inflammation			

**Figure 5. Comparison of prochemerin across species.**

Domains of signal peptide, chemotaxis, and anti-inflammation are indicated.

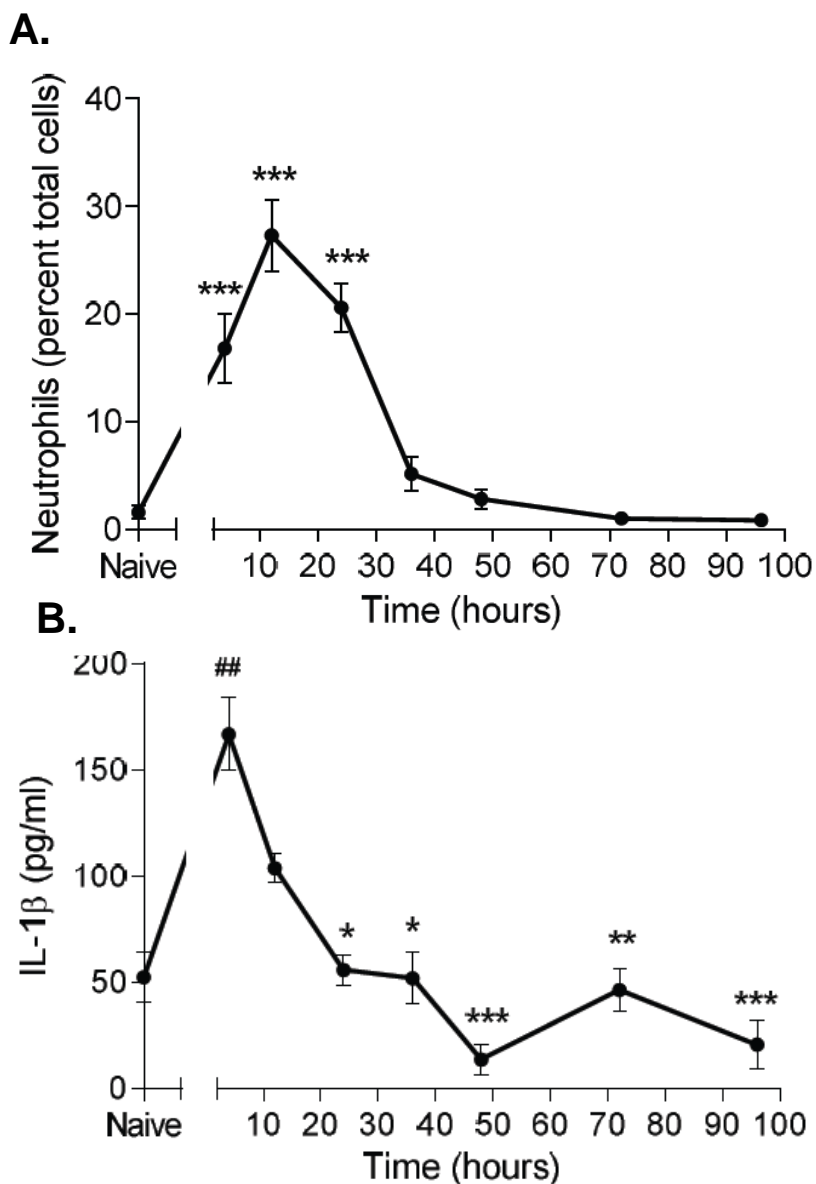
Figure 1 from Du XY and Leung LL. 2009.



**Figure 6: Proteolytic processing of prochemerin results in altered biological activity**

Calcium release induced by prochemerin, chemerin, and peptides derived from their C terminus on ChemR23 transfected cells. (A.) The biological activity of recombinant prochemerin (○), recombinant processed chemerin (●), the 25-amino acid C-terminal peptide of prochemerin (prochemerin<sup>139-163</sup>, □), and the corresponding 19-amino acid C-terminal peptide of processed chemerin (chemerin<sup>139-157</sup>, ■), using the aquaporin-based intracellular Ca<sup>2+</sup> release assay. (B.) Effects of N-terminal modifications of chemerin-derived peptides chemerin<sup>139-157</sup> (■) and N-terminally truncated peptides: chemerin<sup>151-157</sup> (Δ), chemerin<sup>150-157</sup> (▼), chemerin<sup>149-157</sup> (●), chemerin<sup>148-157</sup> (○), and chemerin<sup>146-157</sup> (□). The curves represent the mean ± S.E. of duplicate data points and are representative of at least three independent experiments.

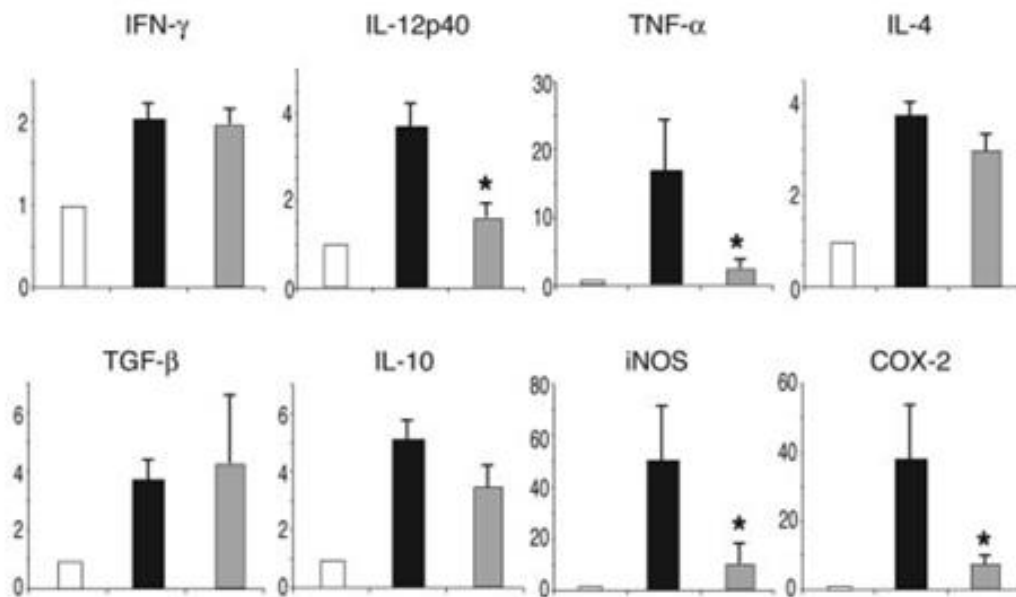
Adapted Figures 1 and 3A from Wittamer V, *et al.* 2004.



**Figure 7: RvE1 pretreatment reduced inflammatory neutrophils and inflammatory IL-1 $\beta$  in BALF of OVA-treated mice**

(A.) Percentage of neutrophils in bronchoalveolar lavage fluid (BALF) as an indicator of numbers of neutrophils in lung tissues. Significant differences compared to naïve \*\*\* $P < 0.001$ . (B.) Concentrations of the pro-inflammatory cytokines IL-1b in BAL fluid after induction of an OVA-induced experimental acute inflammatory lung exacerbation. Data are mean  $\pm$  SEM ( $n=7$ ). Significant differences compared to naïve ##  $P < 0.01$ , compared to the peak \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

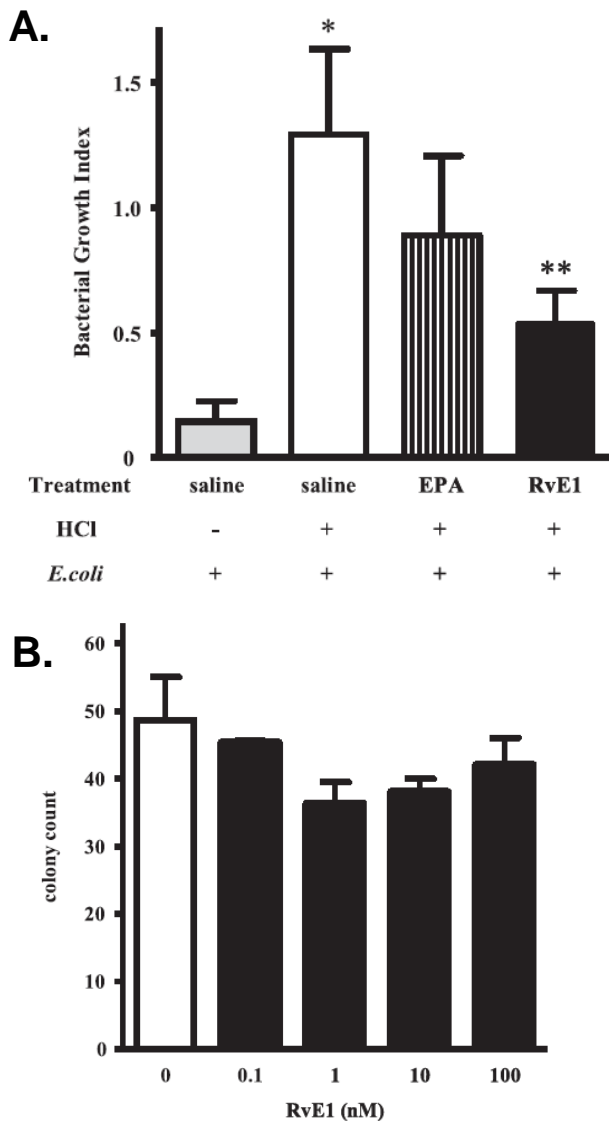
Adapted from Figure 2A and 3C of Flesher RP, *et al.* 2013.



**Figure 8: RvE1 reduces pro-inflammatory mediators in TNBS-induced colitis.**

Quantitative real-time PCR analysis of mRNA expression of inflammatory mediators in colons obtained on day 4 from control mice (white column), mice treated with TNBS alone (black column), or mice receiving TNBS plus pre-treatment with RvE1 (gray column). \* P < 0.01 (n=6).

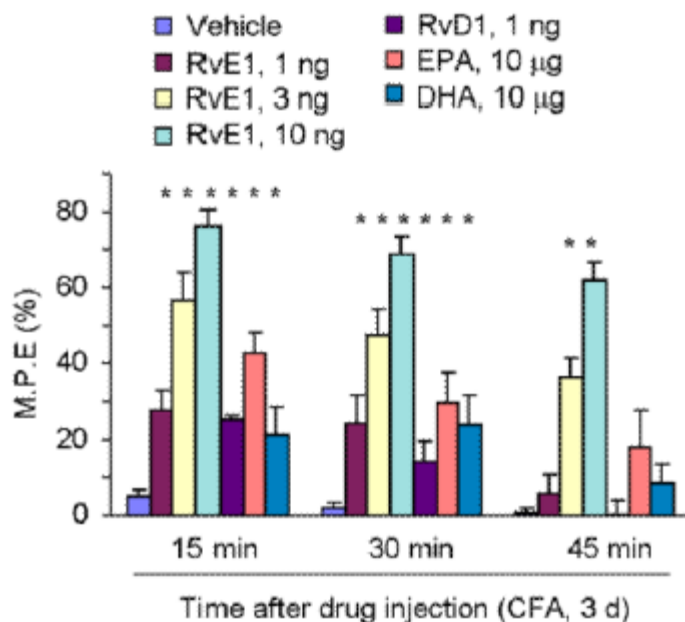
Figure 4C, Arita M, *et al.* 2005.



**Figure 9. RvE1 enhances bacterial clearance in *E. coli*-induced pneumonia mouse model.**

(A.) Mice treated as follows: t=0: saline, EPA, or RvE1 i.v.; t=30min: intratracheal left lung instillation of HCl or PBS; t=12 hrs: left lung inoculation with  $\sim 10^6$  CFU of *E. coli*; t= 24 hrs: the left lungs collection, homogenization, and a BGI calculation. \*, p<0.05 for saline/HCl/*E. coli* vs saline/PBS/*E. coli*; \*\*, p<0.005 for RvE1/HCl/*E. coli* vs saline/HCl/*E. coli*. Mean  $\pm$  SEM (n =12). (B.) RvE1 does not directly impair the growth of *E. coli* *in vitro*. *E. coli* was plated on blood-agar with 0, 0.1, 1, 10, 100 nM RvE1, incubated for 24h at 37°C, and colonies were counted. Mean $\pm$ -SEM (n = 3).

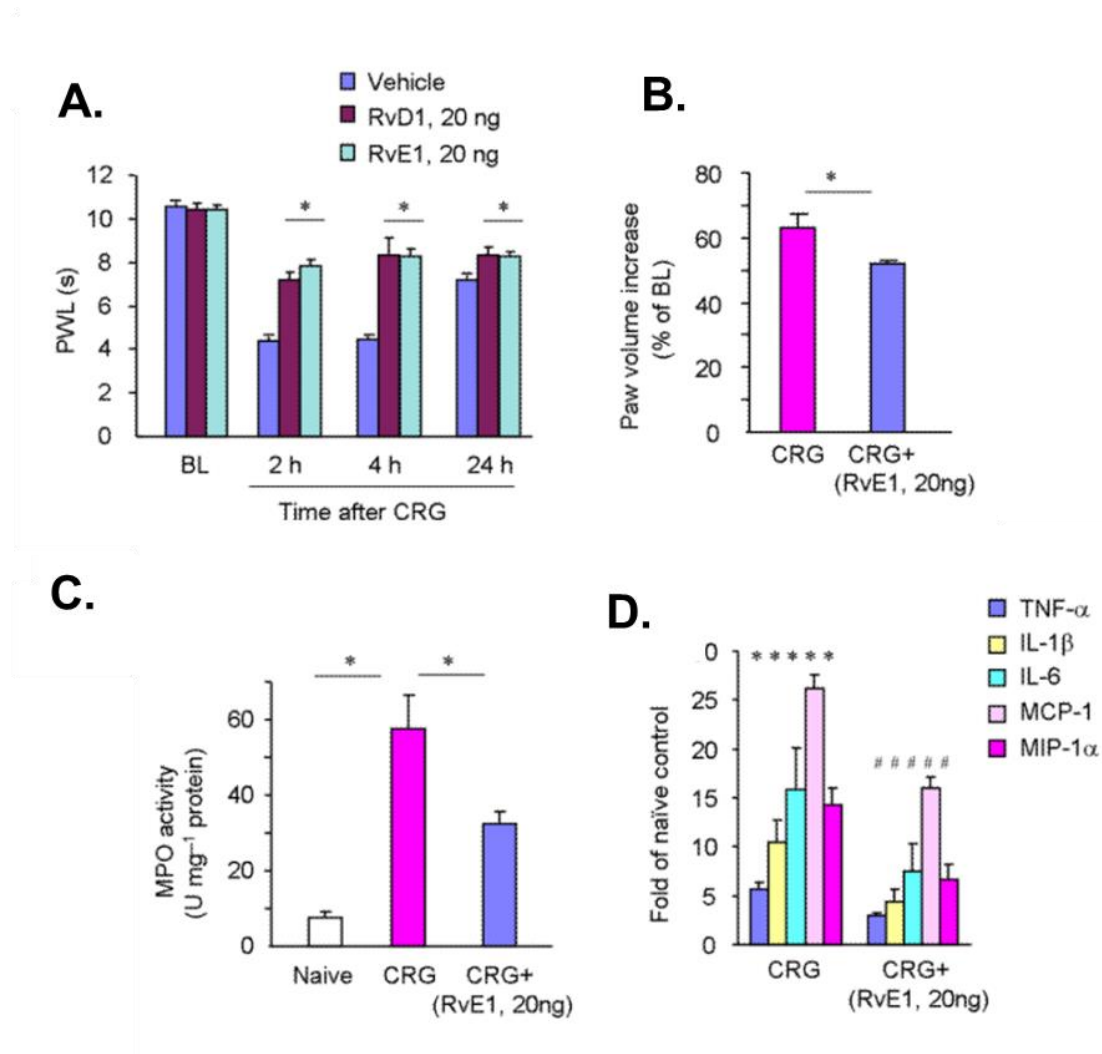
Adapted from Figure 2 and 3 from Seki H, *et al.* 2010.



**Figure 10. RvE1 is anti-hyperalgesic at doses 1000-fold lower than EPA in the acute CFA-evoked heat hyperalgesia model**

Acute actions of RvE1, RvD1, DHA and EPA compared with 1% ethanol (Vehicle) in the CFA-evoked heat hyperalgesia model. # $P < 0.05$ , (n=4-7). For the CFA model, thermal paw withdrawal latency (PWL) and data were presented normalized for each animal as the maximum possible effect of hyperalgesia (M.P.E.) (%) in terms of the change in the PWL ipsilateral to CFA. These values were calculated as follows:  $M.P.E.(\%) = \frac{Time_x PWL - Time_0 PWL}{(baseline - Time_0 PWL)}$ .  $Time_0$  is the PWL 24 hrs. following CFA, before drug ministration.

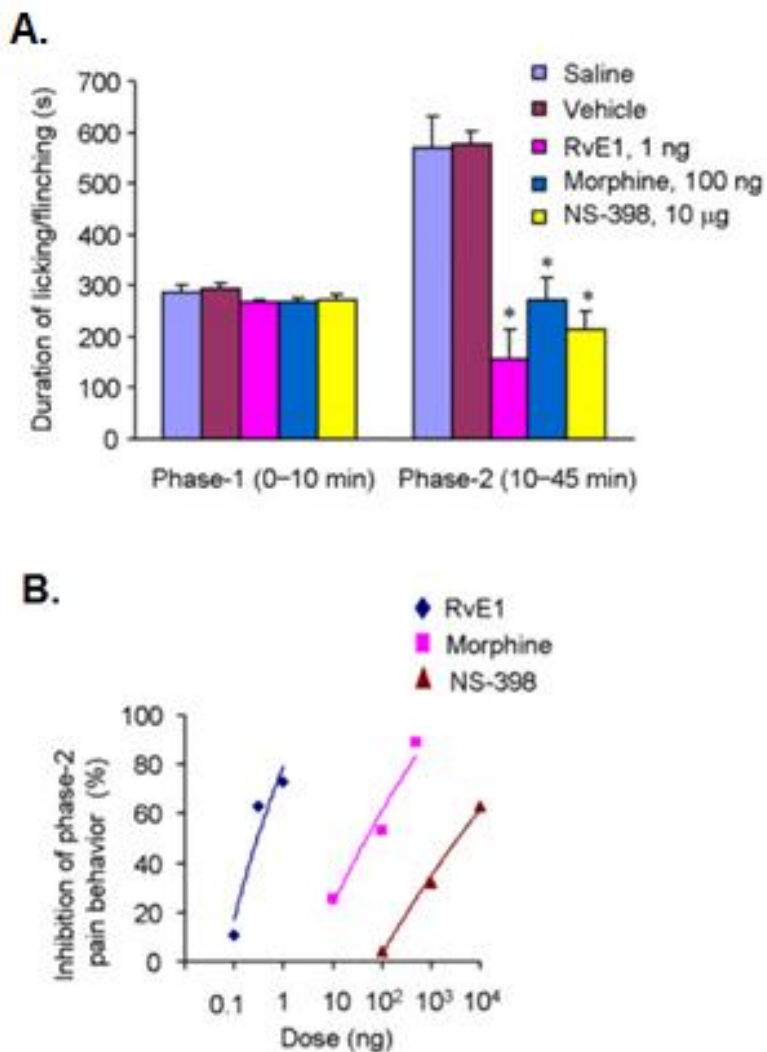
From Figure 2B, Xu ZZ, *et al.* 2010.



**Figure 11. RvE1 reduces of carrageenan (CRG)-elicited heat hyperalgesia, even as inflammatory responses remain increased.**

(A.) Paw withdrawal latency (PWL), (B.) paw edema, (C.) neutrophil infiltration, and (D.) expression of pro-inflammatory cytokines and chemokines in the inflamed paw, following intraplantar pretreatment of resolvins. Edema, neutrophil infiltration, and cytokine expression at protein levels were examined by paw volume (B.), myeloperoxidase (MPO) activity (C.), and cytokine array (D.), respectively, at 4 or 2 hours after CRG injection. \* $P < 0.05$ , vs. vehicle (A., B.) or naïve (C., D.), # $P < 0.05$ , vs. CRG,  $n = 3-6$ .

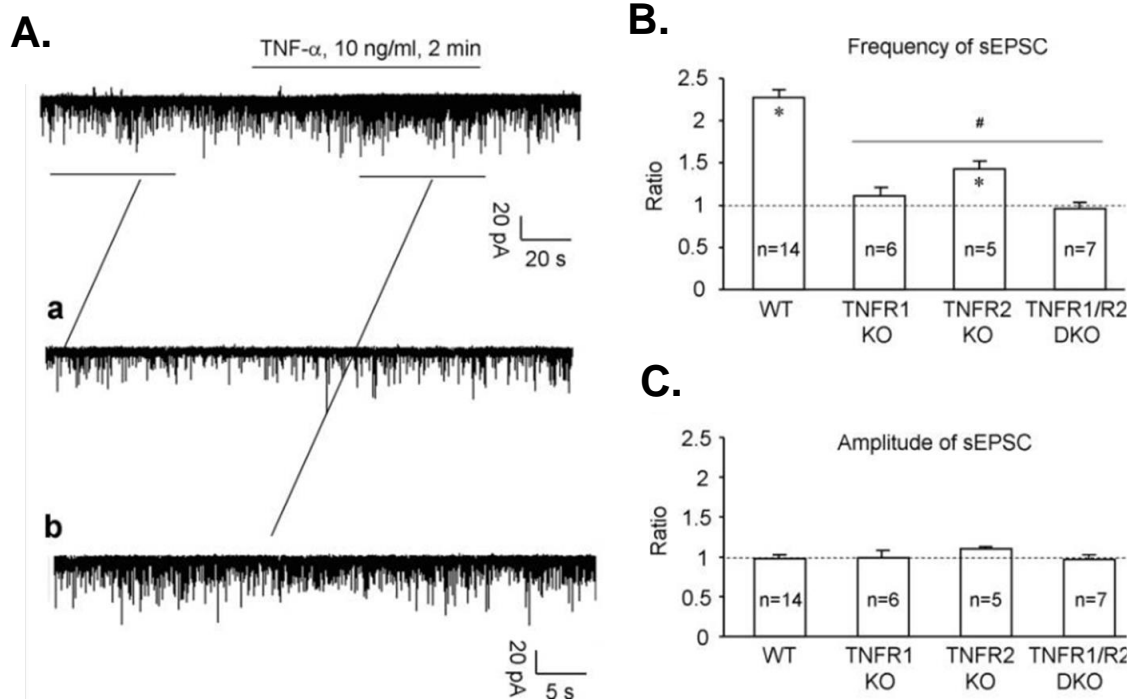
Adapted from Figure 2E-H, Xu ZZ, *et al.* 2010.



**Figure 12. RvE1 pretreatment reduces formalin induced phase 2, but not phase 1 pain response.**

(A.) Preemptive *i.t.* RvE1 reduced second phase formalin-induced pain. (B.) Dose response curve of % inhibition of formalin-induced second phase pain (vs. vehicle) of pre-emptive *i.t.* RvE1, morphine and NS-398, a selective COX-2 antagonist (n=5-8 mice).

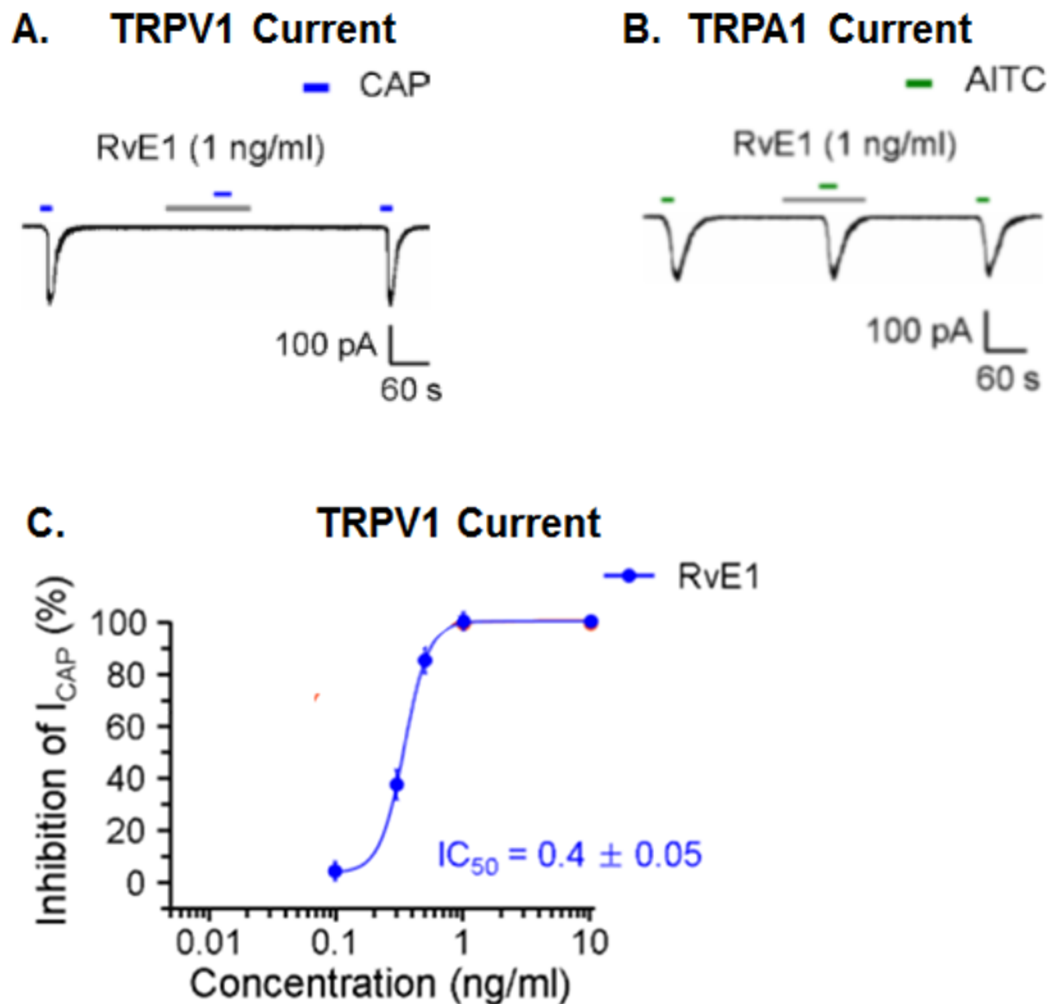
Figures 1B-C from Xu ZZ, *et al.* 2010.



**Figure 13. TNF- $\alpha$  enhances excitatory synaptic transmission, sEPSC in spinal lamina II neurons via both TNFR1 and TNFR2.**

(A.) Patch-clamp recording in spinal cord slices shows an increase in the frequency of sEPSC after bath application of TNF- $\alpha$  (10 ng/ml, 2 min). (Aa.) and (Ab.) are enlarged recordings before and after TNF- $\alpha$  treatment, respectively. (B.) TNF- $\alpha$  increases the frequency of sEPSC in WT mice. This frequency increase is abolished in TNFR1-KO and TNFR1/2 -DKO mice and partially reduced in TNFR2-KO mice. \* $P$ <0.05, compared to pretreatment baseline; # $P$ <0.05, compared to WT. The number of recorded neurons is indicated inside each column. (C.) TNF- $\alpha$  fails to increase the amplitude of sEPSC in WT mice, in TNFR1-KO and TNFR2-KO mice, as well as in TNFR1/2-DKO mice. All the data are expressed as the ratio of corresponding baseline. The number of recorded neurons is indicated inside each column.

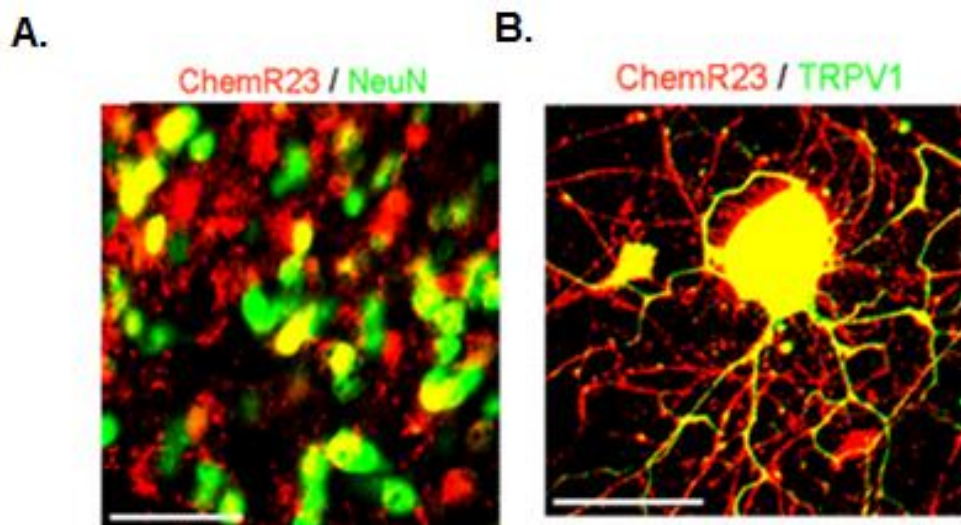
Figure 4 from Zhang L, *et al.* 2011.



**Figure 14. RvE1 inhibits TRPV1, but not TPRA1 currents on DRG cells.**

(A.) RvE1 inhibits the capsaicin-induced inward TRPV1 current on a DRG cell. (B.) RvE1 does not inhibit the AITC-induced inward TRPA1 current on a DRG cell. (C.) RvE1 inhibits capsaicin-induced inward current on DRG cells with an  $IC_{50}$  of  $0.4 \pm 0.05$  ng/ml ( $n=5-8$  neurons).

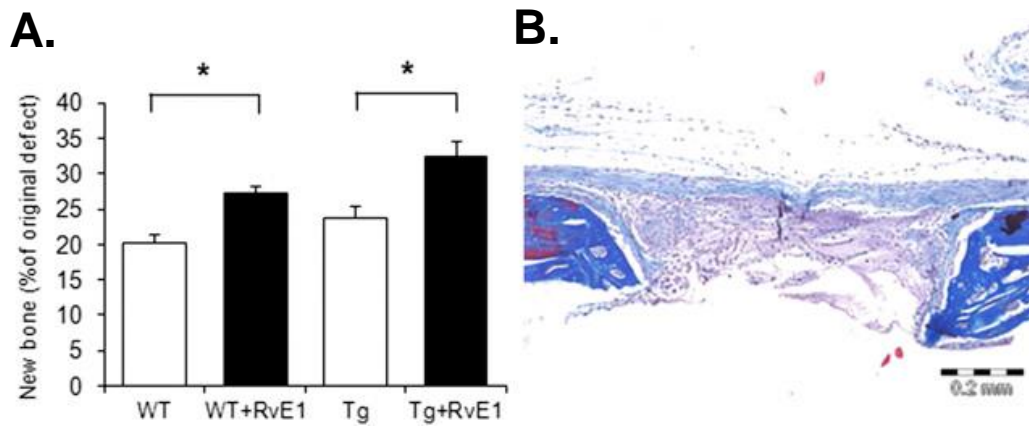
Adapted from Figure 1B, C, F of Park C-K, *et al.* 2013.



**Figure 15: ChemerinR23 co-localizes with TRPV1 on cultured neurons and with NeuN on the superficial dorsal horn.**

Co-localization of ChemR23 (A.) with TRPV1 in cultured DRG neuron and (B.) with NeuN, a neuron-specific marker, in the superficial dorsal horn. Scale bars, 25  $\mu\text{m}$ . All data are means  $\pm$  SEM. \* $P < 0.05$ . (n=5-8 mice).

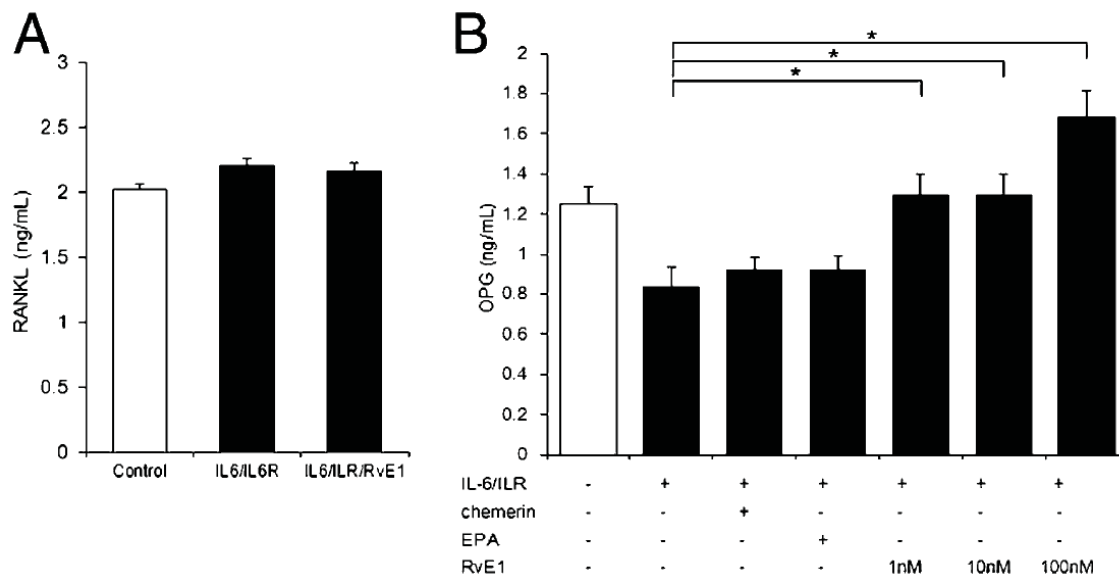
Figures 1G from Xu ZZ, *et al.* 2010



**Figure 16: RvE1 significantly enhances bone healing *in vivo*.**

(A.) 1 mm wide circular bone defect was created in the parietal bone of WT and ChemR23 Tg mice and treated with subperiosteal injections of RvE1 (100ng in 20  $\mu$ L) every other day for 2 weeks. Bone healing is expressed as percentage of original defect. RvE1 significantly enhanced bone healing in both WT and ChemR23Tg (Tg) mice (Mean + SEM,  $P < 0.05$ , t-test,  $n = 16$  for each group). No significant difference between WT and Tg was found. (B.) Histological section across a healing calvarial bone defect (Masson's trichrome staining).

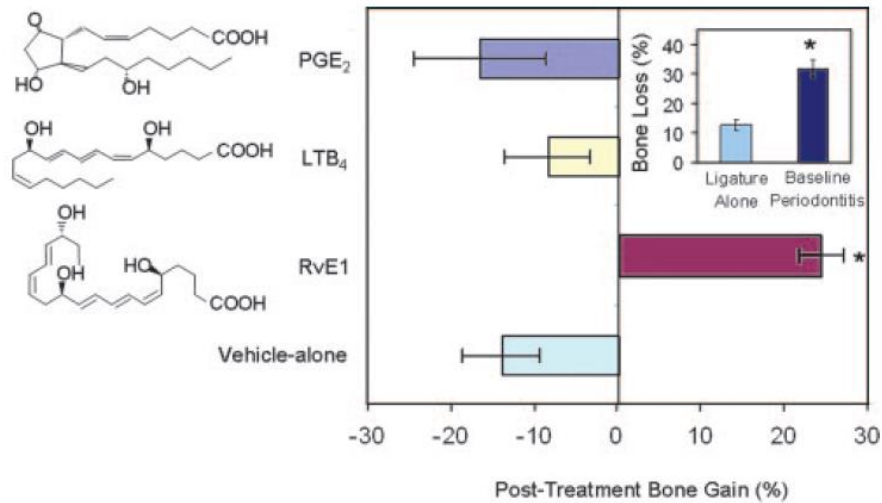
From Figure 4 of Gao L, *et al.* 2013



**Figure 17: RANKL and OPG measurements in primary osteoblast cultures.**

IL-6 and IL-6 receptor (IL-6/ILR) treatment of primary osteoblast induces an inflammatory phenotype. 48 hrs. culture supernatants were assayed for RANKL and OPG by ELISA. (A.) RANKL expression was not significantly altered by RvE1. (B.) However, OPG expression was rescued in an RvE1 dose-dependent manner, while EPA and chemerin was not. All experiments show mean +/- SEM. \*P<0.05 ANOVA. (n=4)

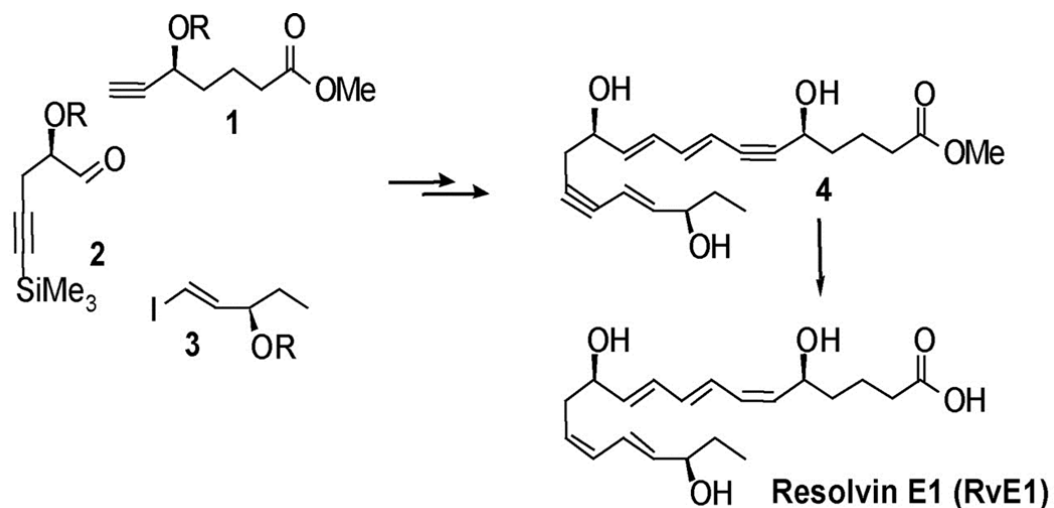
From Figure 6 of Gao L, *et al.* 2013.



**Figure 18: RvE1 induces restoration of lost bone in *P. gingivalis*-induced ligature model of periodontitis in rabbits.**

Radiographic bone loss was quantified. Baseline periodontal disease (6 wks.) resulted in 30% bone loss (*inset*). Radiographic analyses revealed that RvE1 restored the lost bone (95%), whereas the vehicle alone, LTB<sub>4</sub>-, and PGE<sub>2</sub>-treated groups showed 13, 9, and 18% more bone loss, respectively.

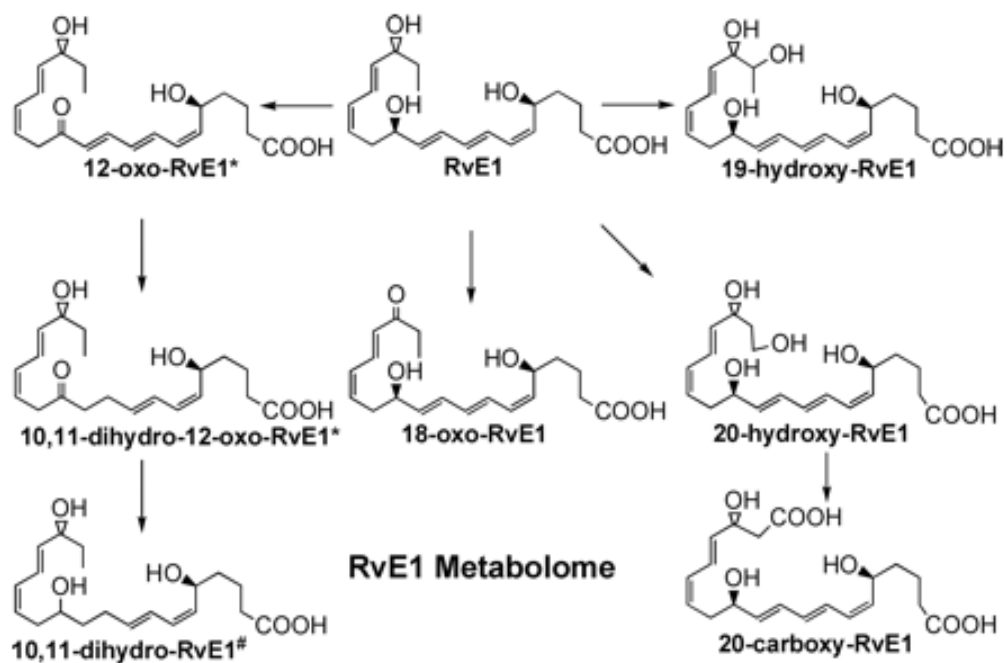
Figure 3 from Hasturk H, *et al.* 2007.



**Figure 19: Total organic synthesis of RvE1.**

Precursors 1–3 were prepared in isomerically pure form from starting materials with known stereochemistry and coupled sequentially to form acetylenic intermediate compound 4, which was selectively hydrogenated to form isomerically pure RvE1.

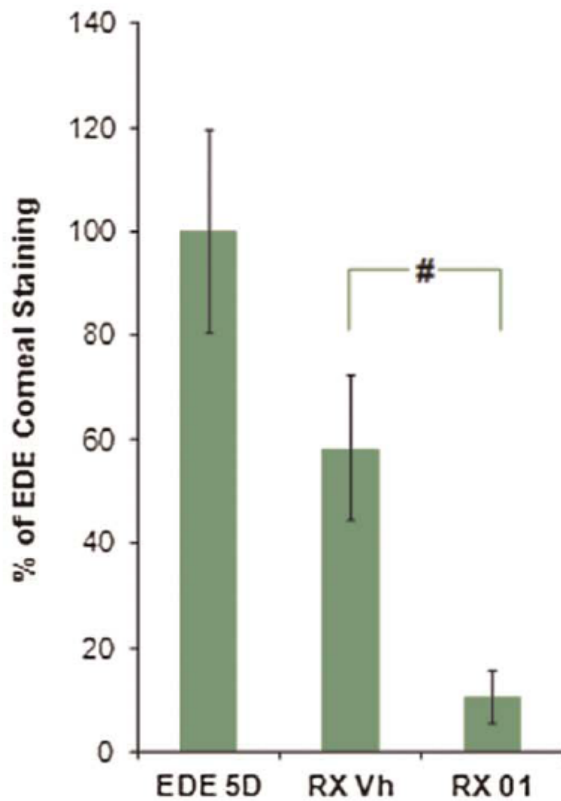
From Figure 2A of Arita M, *et al.* 2005



**FIGURE 20: The Proposed RvE1 Metabolome.**

Human and murine tissues convert RvE1 to the illustrated products. The asterisk (\*) denotes identified proposed intermediates. The stereochemistry of the alcohol at carbon-12 position in the 10,11-dihydro-RvE1 metabolite remains to be determined.

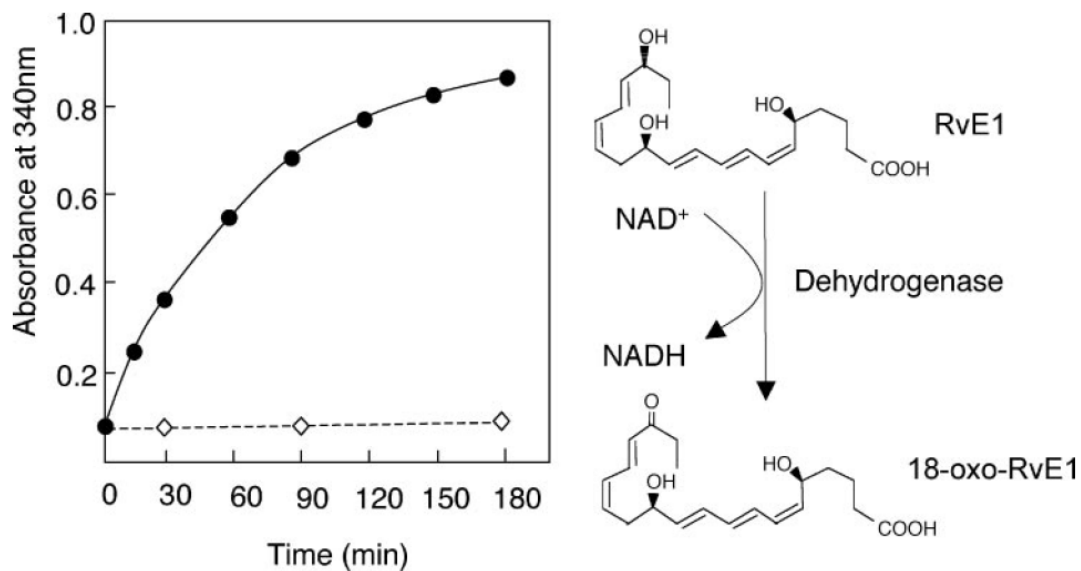
Figure 4 from Hong S *et al*, 2008



**Figure 21. RX-10001, a synthetic RvE1, rescues corneal integrity in the scopolamine-induced model of dry eye.**

Corneal permeability measured as the uptake of Oregon Green Dye (OGD) in untreated and experimental dry eye controls (EDE 5D) and the 2 treatment groups: RX Vh (polysorbate vehicle) and RX 01 (synthetic RvE1; RX-10001). Bars indicate mean group changes in OGD in response to treatment with RX Vh or RX 01 and are expressed as percent staining increase with the difference in staining at 5 days of RX Vh compared with normal untreated controls normalized to 100%. #  $P < 0.005$  (n=18–20).

Adapted from Figure 1 of de Paiva CS, *et al.* 2012.



**Figure 22. Time course of RvE1 conversion to 18-oxo-RvE1 via human 15-PGDH and NAD<sup>+</sup>.**

RvE1 is converted to a biologically inactive 18-oxo-RvE1 via dehydrogenation via recombinant human 15-PGDH and NAD<sup>+</sup>. NADH formation was monitored by absorbance at 340 nM. Closed circles: in the presence of 15-PGDH, open diamonds: in the absence of 15-PGDH.

From Figure 3 of Arita M, *et al.* 2006.

**LIST OF JOURNAL ABBREVIATIONS**

Acta Biochim Biophys Sin (Shanghai)	Acta Biochimica et Biophysica Sinica
Ann Rheum Dis	Annals of the Rheumatic Diseases
Annu Rev Immunol	Annual Review of Immunology
Arthritis Care Res (Hoboken)	Arthritis Care & Research
Arthritis Res Ther	Arthritis Research & Therapy
Arthritis Rheum	Arthritis and Rheumatism
Autoimmun Rev	Autoimmunity Reviews
Biochem Biophys Res Commun	Biochemical and Biophysical Research Communications
BJU Int	BJU International
Br J Pharmacol	British Journal of Pharmacology
Brain Behav Immun	Brain, Behavior, and Immunity
Cell Immunol	Cellular Immunology
Clin Chem	Clinical Chemistry
Clin Exp Immunol	Clinical and Experimental Immunology
Clin Exp Rheumatol	Clinical and Experimental Rheumatology
Clin Sci (London)	Clinical science (London, England: 1979)
Crit Rev Food Sci Nutr	Critical Reviews in Food Science and Nutrition
EMBO J	EMBO Journal
FASEB J	FASEB Journal
Histochem Cell Biol	Histochemistry and Cell Biology

Immunol	Immunology
Immunol Rev	Immunological Reviews
Inflamm Bowel Dis	Inflammatory Bowel Diseases
J Appl Physiol	Journal of Applied Physiology
J Biol Chem	Journal of Biological Chemistry
J Clin Invest	Journal of Clinical Investigation
J Clin Rheumatol	Journal of Clinical Rheumatology
J Dent Res	Journal of Dental Research
J Exp Med	Journal of Experimental Medicine
J Immunol	Journal of Immunology
J Lipid Mediat Cell Signal	Journal of Lipid Mediators and Cell Signalling
J Lipid Res	Journal of Lipid research
J Neurosci	Journal of Neuroscience
J Pharmacol Exp Ther	Journal of Pharmacology and Experimental Therapeutics
J Rheumatol	Journal of Rheumatology
Joint Bone Spine	Joint, Bone, Spine: Revue du Rhumatisme
Lab Invest	Laboratory Investigation
Lipids Health Dis	Lipids in Health and Disease
Med Clin (Barc)	Medicina Clínica
Mod Rheumatol	Modern Rheumatology
Mol Pain	Molecular Pain
Nat Clin Prac Rheumatol	Nature Clinical Practice. Rheumatology

Nat Med	Nature Medicine
Nat Neurosci	Nature Neuroscience
Nat Rev Drug Discov	Nature Reviews. Drug Discovery
Nat Rev Immunol	Nature Reviews. Immunology
Neurochem Res	Neurochemical Research
Pharmacol Rev	Pharmacological Reviews
Proc Natl Acad Sci USA	Proceedings of the National Academy of Sciences of the United States of America
Sci STKE	Science's STKE: Signal Transduction Knowledge Environment

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**CURRICULUM VITAE**

