Lipid-lowering Agent Gemcabene Down-Regulates Acute Phase C-reactive Protein via C/EBP-δ-mediated Transcriptional Mechanism and Attenuates Inflammation and Osteoarthritis in Animal Models

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ABSTRACT

Background: Inflammation plays a key role in setting the stage as well as causing the progression of atherosclerosis. High sensitivity c-reactive protein (hsCRP), an acute phase reactant released during inflammatory processes, has been recognized as a predictor of cardiovascular risk. **Experiments & Results**: Since gemcabene reduced hsCRP in humans, we investigated the mechanism of hsCRP reduction and efficacy of anti-inflammatory activity in animal models of arthritis and pain. In human hepatoma cell line, PLC/PRF/5, gemcabene showed dose-dependent inhibition of IL-6+ IL-1β-induced CRP production by -70% inhibition at 2 mM. In TNF-α-stimulated primary human coronary artery endothelial cells, both CRP and IL-6 productions were inhibited by gemcabene in a dose-dependent manner (-70% at 2mM). Transfection studies with human CRP regulatory sequences in luciferase/ β-gal system showed a 25-fold increase in IL-6- as well as IL-6+ IL-1β -stimulated CRP transcription, which was reduced by gemcabene (-50% at 2 mM), suggesting transcriptional down-regulation of CRP. Site-directed mutation of C/EBP, NF-κB, and STAT sites of the human CRP promoter suggested that the overlapping downstream C/EBP and NF-κB binding sites are important for gemcabene-mediated down-regulation of CRP promoter. STAT3 response element, while needed for IL-6-induced expression of CRP, is not required for gemcabene-mediated inhibition. Identification of the protein, in a gel-shift assay, that interacts with C/EBP binding sites revealed it to be C/EBPδ. Anti-inflammatory efficacy of gemcabene was evaluated in a rat model of monosodium iodoacetate (MIA)-induced osteoarthritis (OA) and carrageenan-induced thermal hyperalgesia (CITH). Gemcabene improved joint comfort (-50% at 30 mg/kg/d for 2 wk) in MIA and attenuated paw withdrawal latency (60% at 30 mg/kg/d and 97% at 100 mg/kg/d, compared to untreated control) in the CITH model. These findings were further confirmed by an IL-6/IL-6sR knee injection model showing 63 and 71% reduction in hind paw weight distribution at 10 and 30 mg/kg/d doses, respectively. **Conclusions**: Gemcabene decreases CRP by C/EBPδ and NF-κB mediated transcriptional mechanism, and attenuates inflammation-induced OA and hyperalgesia

INTRODUCTION

A number of studies for the past 15 years suggest that atherosclerosis, the main cause of coronary artery disease (CAD), is an inflammatory disease in which inflammation plays a key role in setting the stage as well as causing the progression of atherosclerosis (1). C-reactive protein (CRP), an acute phase reactant released during inflammatory processes (2,3), has been recognized as a powerful predictor of traditional markers of cardiovascular risk (4). Additional support for the role of inflammation comes from the JUPITER trial, demonstrating that patients with normal LDL-C levels but elevated CRP levels showed highly significant (-44%) reduction in adverse cardiovascular events following rosuvastatin treatment (5). Gemcabene, like rosuvastatin and other statins, showed significant reductions in CRP in clinical studies (6). We carried out a number of in vitro studies in a variety of cell-types to address gemcabene-mediated inhibition of CRP secretions. The mechanism of action of gemcabene was further investigated using CRP promoter and site-directed mutagenesis. We found that gemcabene inhibits CRP secretion via C/EBP-δ. Antiinflammatory activity of gemcabene was further established in animal models in which we demonstrated attenuation of osteoarthritis and paw swelling in a dose-dependent manner. Thus, our studies show for the first time the mechanism of action of cholesterol-lowering drug via modulation of the CRP promoter.

HYPOTHESES

1. Gemcabene lowers CRP in humans by a mechanism that involves direct effect on CRP gene regulation leading to reduced secretion.
2. The antiinflammatory effect of gemcabene attenuates osteoarthritis and hyperalgesia in animal disease models.

EXPERIMENTAL DESIGN AND ANALYSES

Cell-based Studies: Cell-based studies in PLC/PRF/5 (Alexander) and, HCAEC cells were carried out to investigate gemcabene-medated inhibition of CRP secretion. This was carried out both in the uninduced and proinflammtory cytokine-induced conditions (7). CRP promoter with cis-acting sequences of C/EBP, NF-kB, and STAT were employed to examine the mechanism of action of gemcabene in down-regulating CRP transcriptional activity (8). The WT promoter (900 bp) was subjected to site-directed mutagenesis and the engineered constructs with different regions of promoter sites (C/EBP, Nf-kB, STAT) were transfected to cells and CRP promoter activity quantitated. In a separate study both WT and mutated oligos corresponding to C/EBP, Nf-kB and STAT sites were used for competition binding activity in gel-shift assays in the presence or absence of gemcabene.

In vivo Studies: The effect of gemcabene on weight bearing and cartilage structure was studied in the rat monosodium iodoacetate (MIA) model of osteoarthritis (OA) (9) and on weight bearing in an IL-6-induced change in hind paw weight distribution model. The effect of gemcabene on carrageenan-induced thermal hyperalgesia was studied in the rat (10), and mouse model of collagen-induced arthritis was used to study gemcabene-mediated attenuation of arthritis.

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RESULTS

Figure 1: Human CRP Promoter

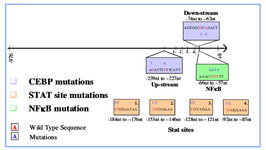


Figure 2: Oligos Used for Gel-Shift Assay

Table 1. Gel Shift Oligonucleotides for C/EBP, NF-κB and STAT3/CRP

Gel Shift Oligonucleotide	Sequences
C/EBP Consensus/WT	5'-TGGAGA**TTGCGCAA**CTGCA-3' **Binding site for CCAAT enhancer binding protein**
C/EBP Consensus/Mut	5'-TGGAGA**GACTAGTC**CTGCA-3' **8 base pair substitution**
C/EBPup/WT	5'-CCTGAGA**GATTTCTTA**TTTTC- **(-245 to -223)**
C/EBPup Mut	5'-CCTGAGA**TATTTAGTC**ATTTTTC- **-77** G→T / C→A
C/EBPdown/WT	5'-CATA**GTGGCTCCAAC**TCCCC-3' **(-77 to -59)**
C/EBPdown Mut 1	5'-CATA**GTGGCTCCAAC**TCCCC-3' G→T / A→C
C/EBPdown Mut 2	5'-CATA**GTGGCTCCAAC**GCCC-3' G→T / A→C / T-G
NFkB/Consensus	5'-AGTTGAGGGGACTTTCCCAGGC-3'
STAT3/CRP-WT	5'-CCCCTCTTCCCGAAGCTCTG-3'

Fig 3: Gemcabene Inhibits IL-6 + IL1-β Induced CRP Secretion in Hepatoma (Left) and HCAE Cells

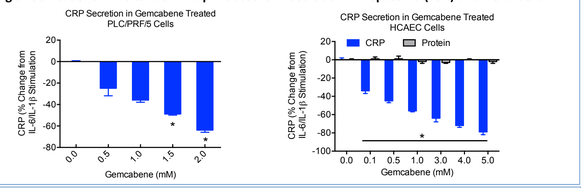


Fig 4: Gemcabene Inhibits IL-6 + IL1-β Induced CRP Promoter Activity in PLC/PRF/5 Hepatoma Cells

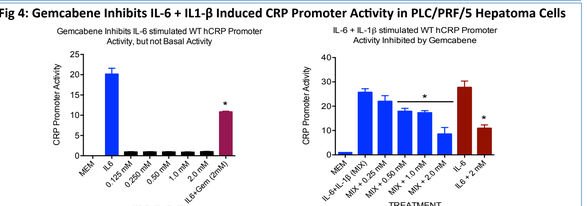


Fig 5: Effect of Gemcabene on WT and Mutated C/EBP, NF-kB, and STAT Sequences in the CRP Promoter Activity in Hepatoma Cells & in Gel-shift Assay

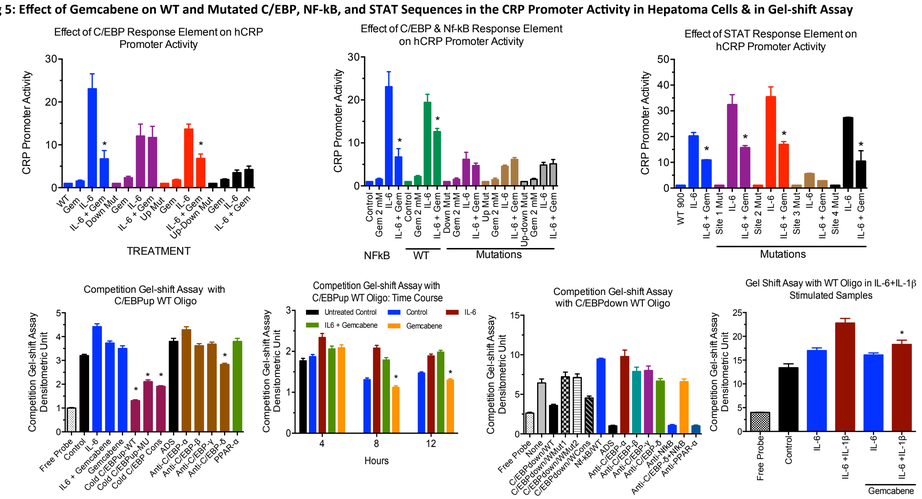


Fig 6: Effect of Gemcabene on Rat Model of MIA-Induced Osteoarthritis

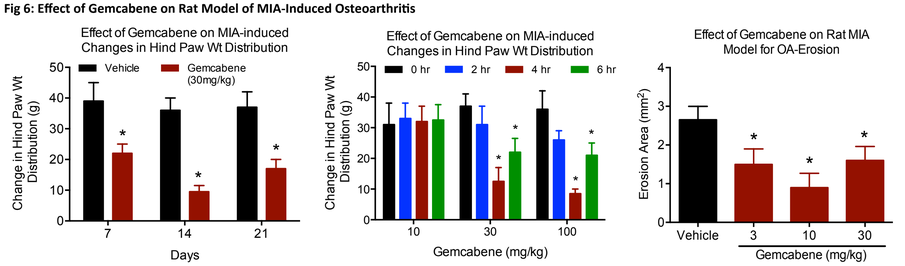


Fig 7: Effect of Gemcabene on Rat Model IL-6/IL-6sR-Induced Osteoarthritis

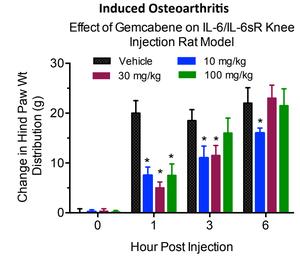


Fig 8: Effect of Gemcabene on Mouse Model on Collagen-Induced Arthritis

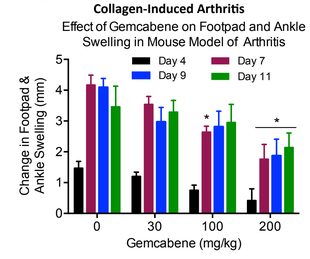
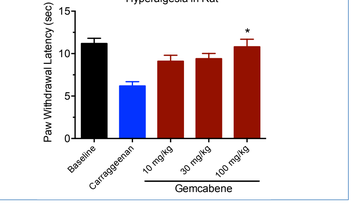
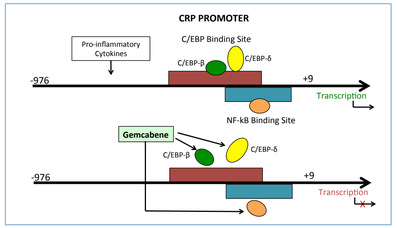


Fig 9: Effect of Gemcabene on Rat Model of Carrageenan-Induced Hyperalgesia



PROPOSED WORKING HYPOTHESIS



SUMMARY OF RESULTS

- Gemcabene inhibited cytokine-induced CRP secretion in hepatoma and endothelial cells, and IL-6+ IL1-β induced human CRP transcription in hepatoma cell line
- Gemcabene inhibits IL-6-induced CRP promoter activity via the CRP protein binding site located from -162 to -150 in the human CRP promoter
- The Nf-kB site overlapping the downstream C/EBP may be involved in the gemcabene-induced human CRP promoter activity
- STAT site 3 in the proximal CRP promoter influenced IL-6-induced promoter activity, but failed to influence gemcabene-induced inhibition
- Gemcabene-induced inhibition of CRP promoter activity occurs primarily via binding to C/EBP-δ site
- Gemcabene attenuated MIA, IL-6/IL-6sR, collagen, and carrageenan-induced osteoarthritis and hyperalgesia in animal models

CONCLUSIONS & CLINICAL IMPLICATIONS

- Gemcabene inhibits CRP secretion by down-regulating CRP transcription via interacting with the C/EBP-δ and Nf-κB binding sites in the promoter of CRP gene.
- Gemcabene attenuates osteoarthritis and hyperalgesia in a number of rat and mouse models
- Results presented demonstrate that the reductions in blood hsCRP levels in gemcabene-treated patients possibly occurs via gemcabene-mediated inhibition of CRP transcription

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