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Identification and pharmacological characterization of a novel inhibitor of autotaxin in rodent models of joint pain

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SUMMARY

Objective: Autotaxin is a secreted lysophospholipase that mediates the conversion of lysophosphatidyl choline (LPC) to lysophosphatidic acid (LPA), a bioactive lipid mediator. Autotaxin levels in plasma and synovial fluid correlate with disease severity in patients with knee osteoarthritis (OA). The goal of this study was to develop and characterize a novel small molecule inhibitor of autotaxin to inhibit LPA production *in vivo* and determine its efficacy in animal models of musculoskeletal pain.

Design: Compound libraries were screened using an LPC coupled enzyme assay that measures the amount of choline released from LPC by the action of autotaxin. Hits from this assay were tested in a plasma assay to assess inhibition of endogenous plasma autotaxin and subsequently tested for their ability to lower plasma LPA levels upon oral dosing of rats. The best compounds were then tested in animal models of musculoskeletal pain.

Results: Compound screening led to the identification of compounds with nanomolar potency for inhibition of autotaxin activity. Studies in rats demonstrated a good correlation between compound exposure levels and a decrease in LPA levels in plasma. The leading molecule (compound-1) resulted in a dose dependent decrease in joint pain in the mono-sodium iodoacetate (MIA) and meniscal tear models and a decrease in bone fracture pain in the osteotomy model in rats.

Conclusion: We have identified and characterized a novel small molecule inhibitor of autotaxin and demonstrated its efficacy in animal models of musculoskeletal pain. The inhibitor has the potential to serve as an analgesic for human OA and bone fracture.

Introduction

Although non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors are the most commonly used medications for osteoarthritis (OA) pain, their side effect concerns and lack of satisfactory pain relief in a large proportion of patients suggest the need for drugs that target other pain mechanisms[1,2]. Inhibition of autotaxin represents a potential novel mechanism for treating OA pain.

Autotaxin is an extracellular enzyme with lysophospholipase D activity whose primary substrate is the lipid lysophosphatidyl choline (LPC). Autotaxin cleaves choline from LPC to generate lysophosphatidic acid (LPA), a bioactive lipid mediator that signals through the activation of six G-protein coupled receptors (GPCRs) (LPA Receptors 1–6)[3–5]. LPA signaling through its receptors results in the induction of several downstream signaling pathways that result in a variety of effects, including cell proliferation, migration, angiogenesis, increase in cytokine signaling and ion channel activation[6–8]. Autotaxin knockout mice have an embryonic lethal phenotype due to defects in blood vessel formation[9]. However, heterozygous mice are viable and show a 50% decrease in autotaxin activity and circulating LPA levels, suggesting that autotaxin is the primary enzyme responsible for the production of LPA in plasma[9]. Autotaxin heterozygous mice show decreased sensitivity to pain evoked via mechanical or thermal stimulation in animals subjected to partial ligation of the sciatic nerve[10]. Intrathecal LPC induced mechanical allodynia (assessed in a paw pressure test) and thermal hyperalgesia (assessed in a thermal paw withdrawal test) are significantly reduced in autotaxin heterozygote animals, suggestive

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of a decreased conversion of LPC to LPA[11] and direct intrathecal administration of LPA has the ability to induce a chronic pain response in rodents[12]. Cyclic phosphatidic acid (cPA) and its stable analog carbacyclic phosphatidic acid (ccPA) are inhibitors of autotaxin, and they have been shown to inhibit acute and chronic pain in animal models[13]. A small molecule inhibitor of autotaxin has been shown to inhibit the local production of LPA and inhibit the vocalization response, a surrogate measure of inflammatory pain in the rat adjuvant induced arthritis model[14]. Mice that lack some of the LPA receptors (LPA1, LPA3 and LPA5) have also been demonstrated to be resistant to neuropathic pain after peripheral nerve injury[12,15,16]. These data provide evidence for a role for autotaxin and LPA in inflammatory and neuropathic pain.

It has recently been reported that plasma and synovial fluid levels of autotaxin correlate with severity of knee OA[17]. Synovial fibroblasts isolated from OA patients express significant amounts of autotaxin mRNA, and this increase in autotaxin expression could lead to increased production of LPA[18], suggesting that autotaxin inhibition could have a therapeutic benefit for OA and other inflammatory states. It is currently not known whether autotaxin inhibition would be efficacious in models of musculoskeletal pain such as those resulting from chemical and surgically induced OA or bone fracture. The goal of this study was to develop novel, highly potent, and orally bioavailable inhibitors of autotaxin with an improved pharmacokinetic profile and determine efficacy in animal models of musculoskeletal pain. In this paper, we describe the development and characterization of compound-1 as a potent inhibitor of autotaxin that shows a dose dependent inhibition of plasma LPA levels in rats and concomitant inhibition of the pain response in various models of musculoskeletal pain, including the mono-sodium iodoacetate (MIA) and meniscal tear models of joint pain and the osteotomy model of bone fracture pain.

Methods

Autotaxin activity assay (choline release assay)

Reagents: His-tagged human and rat autotaxin were expressed in 293E cells and purified using nickel-affinity and size exclusion columns at Lilly. The choline release assay protocol was adapted from previous reports[19,20]. Autotaxin (1 nM) was incubated with LPC 16:0 (Avanti Polar Lipids, 855675P, 30 μM final) in the presence of various concentrations of the compounds for 1 h at 37°C. Assay buffer consisted of 50 mM Tris pH 8.0, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% Triton X-100 and 0.02% Fatty Acid Free bovine serum albumin (BSA) (Sigma A8806). The addition of choline oxidase (Sigma, C5896), horseradish peroxidase (Sigma, P8125) and amplex ultrared (Invitrogen, A36006) to this reaction allows for the measurement of choline liberated by autotaxin. The liberated choline gets oxidized by choline oxidase resulting in betaine and hydrogen peroxide. Hydrogen peroxide reacts with horseradish peroxide and amplex ultrared to form the fluorescent molecule resorufin. The plates were measured on a SpectraMax Gemini EM fluorometer at excitation-emission wavelengths of 530–590 nm using SoftMax Pro 4.8 software. IC50s were calculated using 4-parameter curve fits with the internal Lilly curve fitting software called OLO.

Compounds that non-specifically inhibited the fluorescent signal were screened out with a modified version of the assay using choline chloride (Sigma, C7017) (1.33 μM final) and without the addition of autotaxin.

Plasma autotaxin activity assay

Human, dog and rat plasma collected in sodium-heparin blood tubes, were purchased from Lampire Biologicals (Pipersville, PA). Plasma was incubated for 3 h at 37°C with varying concentrations of compounds. Following the 3 h incubation, EDTA was added to chelate the endogenous autotaxin. The plate was sealed and stored at −80°C. For measuring LPA levels, the plates were thawed on ice and extracted by the addition of 200 μl of extraction buffer (methanol, containing LPA internal standards) and subjected to total LPA analysis by mass spectrometry. IC50 values were calculated using 4-parameter curve fits with the OLO curve fitting software.

LPA mass spectrometry

LPA levels in samples were measured using an LC/ESI/MS/MS method[14]. LPA levels were quantified by the ratio of analyte and internal standard and calibration curves obtained by serial dilution of a mixture of lysophosphatidic acids (LPA C16:0, C18:0, C18:1, C18:2 and C20:4).

Animal studies

For the MIA and meniscal tear studies, male Lewis rats (Harlan, Indianapolis, IN) of approximately 8 and 15 weeks, respectively, were used. For the rat osteotomy model, female rats of 33–34 weeks of age that had been ovariectomized 8 weeks previously were used. The rats were housed in groups of 2 or 3 per cage and maintained in a constant temperature, and on a 12 h light/12 h dark cycle. Animals had free access to food and water at all times except during data collection. All experiments were carried out according to protocols approved by Eli Lilly's Institutional Animal Care and Use Committee. Pain measurements were performed in a blinded fashion using incapacitance testing. This measures the difference in hind paw weight bearing between the MIA and saline injected knees or between the surgical and non-surgical knee (in the meniscal tear and osteotomy models). Each value represents the average of 3 separate measurements acquired over a 1 s period per rat. Dosing was staggered by 10 min for each rat and pain was measured at 2 h post dose (T_{max} for compound-1). Dose volume was 5 ml/kg and animals were randomized to experimental groups based on body weight using the Block Randomized Allocation Tool (BRAT).

MIA model of joint pain

In the standard MIA model, the right knee of each rat was injected with 0.3 mg (low dose) MIA in 50 μl of saline and the left knee with 50 μl of saline. Nine days later, they were dosed once orally with either vehicle (40% 2-hydroxy propyl beta cyclodextrin in water − HPBCD) or compound-1 at doses of 0.1, 0.3, 1, 3 and 10 mg/kg ($n = 5$) and pain measured. Immediately after pain measurements were taken, ethylene diamine tetra acetic acid (EDTA) plasma was collected for measurement of circulating LPA levels.

To test for the duration of action of compound-1, rats were dosed once orally on day 36 post MIA injection with either hydroxy ethyl cellulose (HEC) vehicle (1% HEC plus 0.25% Tween80 plus 0.05% antifoam) or 30 mg/kg compound-1 ($n = 4$) and pain was measured at 2, 4, 6 and 24 h post dose.

Historically, we know that in this low dose MIA model, by day 9, the acute inflammatory response generated by MIA would have resolved, leading to a more chronic phase where the pain has reached a plateau. Therefore the pain response will be similar for the vehicle animals at day 9 and day 36.

Combination study with compound-1 and NSAID diclofenac

Rats were injected with 0.3 mg MIA and 9 days later dosed once orally with either vehicle (HEC), compound-1 (1 mg/kg), diclofenac (0.3 mg/kg), or a combination of the two drugs (in the same dosing solution) and pain was measured ($n = 6$).