The novel flavone tetramethoxyluteolin is a potent inhibitor of human mast cells

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Background: Mast cells (MCs) are hematopoietic cells that mature in tissues and are involved in allergy, immunity, and inflammation by secreting multiple mediators. The natural flavone luteolin has anti-inflammatory actions and inhibits human mast cells (MCs).

Objective: We sought to investigate the ability of luteolin and its novel structural analog 3′,4′,5,7-tetramethoxyluteolin (methlut) to inhibit human MC mediator expression and release *in vitro* and *in vivo*.

Methods: Human LAD2 cells and umbilical primary human cord blood–derived cultured mast cells were stimulated with substance P (SP) or IgE/anti-IgE with or without preincubation with luteolin, methlut, or cromolyn (1-100 μmol/L) for 2 or 24 hours, after which mediator secretion was measured. The effect of the compounds on MC intracellular calcium levels and nuclear factor κB activation was also investigated. Pretreatment with methlut was also studied in mice passively sensitized with dinitrophenol–human serum albumin and challenged intradermally.

Results: Methlut is a more potent inhibitor than luteolin or cromolyn for β-hexosaminidase and histamine secretion from LAD2 cells stimulated by either SP or IgE/anti-IgE, but only methlut and luteolin significantly inhibit preformed TNF secretion. Methlut is also a more potent inhibitor than luteolin of *de novo*–synthesized TNF from LAD2 cells and of CCL2 from human cord blood–derived cultured MCs. This mechanism of action for methlut might be due to its ability to inhibit intracellular calcium level increases, as well as nuclear factor κB induction, at both the transcriptional and translational levels in LAD2 cells stimulated by SP without affecting cell viability. Intraperitoneal treatment with methlut significantly decreases skin vascular permeability of Evans blue dye in mice passively sensitized to dinitrophenol–human serum albumin and challenged intradermally.

Conclusion: Methlut is a promising MC inhibitor for the treatment of allergic and inflammatory conditions. (J Allergy Clin Immunol 2015;135:1044-52.)

Key words: *Allergy, inflammation, mast cells, luteolin, tetramethoxyluteolin, calcium, nuclear factor κB*

Mast cells (MCs) are immune cells derived from hematopoietic precursors that mature in tissue microenvironments.[1-3] In addition to allergic triggers, MCs can be stimulated by neuropeptides, such as substance P (SP).[3,4] On stimulation, MCs release preformed mediators stored in their numerous secretory granules; these include β-hexosaminidase, histamine, TNF, and tryptase through rapid degranulation, as well as newly synthesized prostaglandin D_2 (PGD_2), TNF, and CCL2 (monocyte chemoattractant protein 1).[5-7]

MC-derived histamine induces bronchoconstriction and mucus secretion, contributing to asthma.[8,9] MCs are probably the only cell type that stores preformed TNF,[10] which is rapidly released and influences T-cell recruitment and activation.[11,12] MC-derived CCL2[13] and CXCL-8 (IL-8) enhance recruitment of immune cells to the site of inflammation.[6,7,14] The ability to release multiple mediators allows MCs to actively interact with other cell types in their surrounding environment and participate in the induction, propagation, or both of various immune and inflammatory responses, including mastocytosis,[15] asthma,[16] atopic dermatitis,[17] and psoriasis.[3,16,18,19] Therefore inhibition of MC activation has clear therapeutic potential.

Disodium cromoglycate (cromolyn; Fig 1, *A*) is the only clinically available MC stabilizer because it was reported to reduce gastrointestinal effects in patients with mastocytosis.[20] Even though cromolyn inhibits rat peritoneal MC histamine secretion,[21] it does not inhibit rat mucosal MCs[22,23] or mouse MCs.[24] A recent study concluded that the beneficial effect of cromolyn in reducing pruritus in human subjects might be mediated through inhibition of sensory nerve endings instead of MCs.[25-28] In addition, poor intestinal absorption of cromolyn severely limits its clinical efficacy. Consequently, there is an urgent need to develop effective inhibitors of human MCs.

While searching for potential MC inhibitors, we noticed that part of the structure of cromolyn is similar to the backbone of flavones (see Fig E1, highlighted areas, in this article's Online Repository at www.jacionline.org), which are naturally occurring compounds with potent antioxidant, anti-inflammatory, and MC-blocking activities.[29] The flavone luteolin (see Fig E1) inhibits the release of histamine and PGD_2 from human cultured MCs.[30] Luteolin also inhibits mercury-induced vascular endothelial growth factor release from human MCs[31] and activated T cells.[32] The structural analog of luteolin, 3′,4′,5,7-tetramethoxyluteolin (methlut; see Fig E1) is more lipid soluble than luteolin, more likely to penetrate cells, and less metabolized.[33] However, the action of methlut on MC activation has not been investigated.

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Abbreviations used
CCL2: Chemokine (C-C motif) ligand 2
DMSO: Dimethyl sulfoxide
DNP-HSA: Dinitrophenol–human serum albumin
hCBMC: Human cord blood–derived mast cell
IκBα: Nuclear factor κB inhibitor α
MC: Mast cell
Methlut: 3′,4′,5,7-Tetramethoxyluteolin
NF-κB: Nuclear factor κB
PGD2: Prostaglandin D2
rhSCF: Recombinant human stem cell factor
SP: Substance P

In this study we examined whether methlut could inhibit mediator release from human cultured MCs stimulated by an allergic or a neuropeptide trigger and compared it with luteolin and cromolyn. We also studied the effect of methlut in mice passively sensitized to dinitrophenol-human serum albumin (DNP-HSA) and challenged intradermally.

METHODS

Reagents

Recombinant human stem cell factor (rhSCF) was kindly donated by Orphan Biovitrum AB (Stockholm, Sweden). Cromolyn, luteolin, and SP were purchased from Sigma-Aldrich (St Louis, Mo). Methlut was obtained from Pharmascience Nutrients (Clear Water, Fla) and was 100% pure, as determined by means of HPLC–mass spectroscopy (see Fig E2 in this article's Online Repository at www.jacionline.org). Cromolyn, luteolin, and methlut were dissolved in dimethyl sulfoxide (DMSO). SP was prepared in distilled water. Working dilutions for all compounds were prepared in culture medium immediately before use. The final concentration of DMSO was less than 0.1% and had no effect on cell viability.

Human MC culture

The limited number of MCs obtained from normal human tissues has led to the increased use of human LAD2 cells derived from a human patient with MC leukemia[34] or primary human cord blood–derived MCs (hCBMCs).[35] Human LAD2 cells (kindly supplied by Dr A. Kirshenbaum, National Institutes of Health, Bethesda, Md) were cultured in StemPro-34 medium (Life Technologies, Carlsbad, Calif) supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 ng/mL rhSCF (Orphan Biovitrum AB).

Human umbilical cord blood was obtained after normal deliveries in accordance with established institutional guidelines to culture primary hCBMCs.[36] Mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, Ohio). $CD34^+$ progenitor cells were isolated by means of positive selection of AC133 ($CD133^+/CD34^+$) cells by using magnetic cell sorting (CD133 Microbead Kit; Miltenyi Biotech, Auburn, Calif). For the first 6 weeks, $CD34^+$ progenitor cells were cultured in Iscove modified Dulbecco medium (Life Technologies) supplemented with 0.1% BSA, 1% insulin-transferrin-selenium, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. After 6 weeks, the cells were cultured in Iscove modified Dulbecco medium supplemented with 10% FBS, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. hCBMCs cultured for at least 15 weeks were used for experiments, and cell purity was greater than 98%. Cell viability was determined by means of trypan blue (0.4%) exclusion.

Degranulation assays

β-Hexosaminidase release was assayed as an index of MC degranulation. LAD2 cells (0.5×10^5) were preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L for 30 minutes). LAD2 cells were treated with cromolyn (100 μmol/L) and SP (2 μmol/L) at the same time to test the effect of cromolyn. Control cells were treated with 0.1% DMSO. Supernatant fluids were collected, and cell pellets were lysed with 1% Triton X-100. Supernatants and cell lysates were incubated in the reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1.5 hours, and then 0.2 mol/L glycine was added to stop the reaction. Absorbance was measured at 405 nm. Results are expressed as the percentage of β-hexosaminidase released over the total amount present in LADs cells.

MC degranulation was also assessed by measuring histamine release. After the same treatment, LAD2 cells were pretreated with cromolyn, luteolin, or methlut and subsequently stimulated with SP. Supernatant fluids were collected, and histamine release was measured with a Histamine EIA Kit (Cayman Chemical, Ann Arbor, Mich).

hCBMCs (0.5×10^5) were first primed with human IgE (1 μg/mL; Millipore, Billerica, Mass) overnight and preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L) for 30 minutes before stimulation with anti-IgE (10 μg/mL for 30 minutes, Life Technologies) to assay degranulation in primary MCs.

TNF and CCL2 ELISA

For TNF release, LAD2 cells (1×10^5) were preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L) and subsequently stimulated with SP (2 μmol/L) for 30 minutes to measure preformed TNF release. LAD2 cells were also stimulated for 24 hours to measure *de novo*–synthesized TNF release, which occurs in addition to rapid preformed TNF release. TNF levels were measured in supernatant fluids by using a TNF ELISA assay kit (R&D Systems, Minneapolis, Minn).

For CCL2 release, primary hCBMCs (1×10^5) were primed with human IgE (1 μg/mL, Millipore) overnight and preincubated with luteolin or methlut (50 μmol/L) for 30 minutes before stimulation with anti-IgE (10 μg/mL for 2 hours, Life technologies). CCL2 was measured with a CCL2 ELISA assay kit (R&D Systems).

RNA isolation and quantitative real-time PCR

LAD2 cells and hCBMCs (5×10^5) were treated with luteolin or methlut (10-100 μmol/L for 30 minutes) before stimulation with either SP (2 μmol/L) or anti-IgE (10 μg/mL) for 6 hours. Total RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, Calif). An iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif) was used for reverse transcription of each sample. Quantitative real-time PCR was performed with TaqMan gene expression assays (Applied Biosystems, Foster City, Calif) for TNF (Hs99999043_m1) and CCL2 (Hs00234140_m1) and the 2 genes encoding different subunits of the nuclear factor κB (NF-κB) protein complex, NFKB1 (NF-κB p50 subunit, Hs00765730_m1) and RELA (NF-κB p65 subunit, Hs00153294_m1). Samples were run at 45 cycles by using a real-time PCR system (7300, Applied Biosystems). The mRNA gene expressions were normalized to human GAPDH endogenous control (4310884E, Applied Biosystems).

Intracellular calcium measurements

LAD2 cells were incubated in Tyrode buffer with the calcium indicator Fura-2 AM (30 nmol/L for 20 minutes, Life Technologies). Cells were washed and resuspended in plain Tyrode buffer and incubated for another 20 minutes. Cells were then transferred to 96-well plates (1×10^5 cells per well) and pretreated with luteolin or methlut (10 and 50 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L). Changes in Fura-2 AM fluorescence was immediately read with the MDC FlexStation II (Molecular Devices, Sunnyvale, Calif) at an excitation wavelength of 340 nm/380 nm and an emission wavelength of 510 nm. Results were processed according to the Life Technologies Fura-2 AM protocol and reported as the relative ratio.

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