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Differential Signaling of Cysteinyl Leukotrienes and a Novel Cysteinyl Leukotriene Receptor 2 ($CysLT_2$) Agonist, N-Methyl-Leukotriene C_4, in Calcium Reporter and Beta Arrestin Assays

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Running Title: N-Methyl Leukotriene C_4 is a potent and selective $CysLT_2$ receptor agonist

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Non-standard Abbreviations: α*, N-terminal β-galactosidase fragment with H31R substitution; AEQ, aequorin; β-arr, beta-Arrestin; β-gal, beta-galactosidase; cysLT, cysteinyl leukotriene; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; hygro, hygromycin; IRES, internal ribosomal entry site; LT, leukotriene; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; $NMLTC_4$, N-methyl-leukotriene C_4; YFP, yellow fluorescent protein

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Abstract

The cysteinyl leukotrienes (cysLTs), LTC_4, LTD_4 and LTE_4, are lipid mediators with physiological and pathophysiological functions. They exert their effects through G protein-coupled receptors (GPCRs), most notably via $CysLT_1$ and $CysLT_2$ receptor. The roles of the $CysLT_2$ receptor are beginning to emerge. Both LTC_4 and LTD_4 are potent agonists for the $CysLT_2$ receptor; however, LTC_4 is rapidly converted to LTD_4, which is also the main endogenous ligand for the $CysLT_1$ receptor. A selective and potent agonist at the $CysLT_2$ receptor would facilitate studies to discern between receptor subtypes. We show here that N-methyl LTC_4 ($NMLTC_4$), a metabolically stable LTC_4-mimetic, is a potent and selective $CysLT_2$ receptor agonist. Two expression systems were used to evaluate the functional activity of $NMLTC_4$ at human and/or mouse $CysLT_1$ and $CysLT_2$ receptors. Through the aequorin cell-based assay for calcium-coupled GPCRs, $NMLTC_4$ was almost equipotent as LTC_4 at $CysLT_2$ receptors, but was the least efficacious at $CysLT_1$ receptors. In a β-galactosidase-β-Arrestin complementation assay the $hCysLT_2$ receptor can couple with β-Arrestin-2 and $NMLTC_4$ is slightly more potent for eliciting β-Arrestin-2 binding when compared to cysLTs. Furthermore LTE_4 is nearly inactive in this assay compared to its weak partial agonist activity in the aequorin system. In a vascular leakage assay, $NMLTC_4$ is potent and active in mice overexpressing $hCysLT_2$ receptor in endothelium while the response is abrogated in $CysLT_2$ receptor knockout mice. Therefore, $NMLTC_4$ is a potent subtype selective agonist for the $CysLT_2$ receptor *in vitro* and *in vivo* and will aid to elucidate its biological roles.

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Introduction

Discovered as a bronchial constrictive agent, slow-reacting substance of anaphylaxis (SRS-A) was later determined to represent a mixture of cysteinyl leukotrienes (cysLTs). Specifically, cysLTs are downstream metabolites of arachidonic acid derived from the 5-lipoxygenase (5-LO) product leukotriene A_4 (LTA_4). Conjugation of LTA_4 with reduced glutathione gives rise to the first of the three cysLTs, LTC_4, by means of LTC_4 synthase (Funk, 2001). LTC_4 is exported from the cell by the multidrug resistance-associated protein-1 (MRP-1) and sequential removal of the γ-glutamic acid moiety, by γ-glutamyl transpeptidase, and then the glycine residue, by aminopeptidases, gives rise to LTD_4 and LTE_4, respectively. CysLTs potentially exert their biological functions through five different GPCRs identified as $CysLT_1$, $CysLT_2$, GPR17, $CysLT_E$ and P2Y12 receptor.

Among these receptors, the $CysLT_1$ receptor is by far the most widely characterized with expression observed in bronchial smooth muscle cells, spleen, leukocytes, and lung macrophages. LTD_4 is the preferred endogenous ligand for $CysLT_1$ receptor and receptor activation elicits elevation in intracellular calcium levels (Lynch et al, 1999; Sarau et al, 1999). $CysLT_1$ receptor is the molecular target of the anti-asthmatic drugs montelukast (Singulair), zafirlukast (Accolate) and pranlukast (Onon), which show efficacy in blocking inflammatory actions in the airways and improving airway function (Grossman et al, 1997; Reiss et al, 1998; Suissa et al, 1997).

The $CysLT_2$ receptor, on the other hand, has been less well characterized. Early pharmacological studies were compatible with $CysLT_2$ receptor expression in guinea pig trachea and ileum (Pong and DeHaven, 1983), ferret trachea and spleen (Aharony et al, 1985), sheep bronchus and in human pulmonary and saphenous vein preparations (Coleman et al, 1995; Labat

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et al, 1992; Walch et al, 1999). Molecular cloning of $CysLT_2$ receptor subsequently revealed expression in the heart, adrenal glands, placenta and brain (Heise et al, 2000; Takasaki et al, 2000; Nothacker et al, 2000; Hui et al, 2001) and more recently, $CysLT_2$ receptor was found selectively expressed in the vasculature of various organs and found to regulate vascular permeability (Moos et al, 2008). Various assays have shown that $CysLT_2$ receptor has a rank order potency of LTC_4=LTD_4>>LTE_4 with the latter leukotriene behaving as a partial agonist at both $CysLT_1$ and $CysLT_2$ receptors. LTE_4 acts as the preferred agonist for the $CysLT_E$ receptor (Maekawa et al, 2008), which has not yet been molecularly characterized. Until recently, the pharmacological inhibitor for $CysLT_2$ receptor has been the non-selective dual antagonist/partial agonist Bay-u9773. However, Wunder *et al* (2010) recently described the first selective $CysLT_2$ receptor antagonist, HAMI3379.

Here, we describe N-Methyl LTC_4 ($NMLTC_4$), as the first selective agonist for $CysLT_2$ receptor, which together with HAMI3379 should yield new insights into $CysLT_2$ receptor function. Moreover, using distinct functional assays we reveal that, besides Ca^{++} activation, the $CysLT_2$ receptor can interact with β-Arrestin-2.

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Methods:

Materials: $NMLTC_4$ was synthesized initially as previously described (Gareau *et al*, 1993) and subsequently was obtained from Cayman Chemical Co. (Ann Arbor, MI). All cysLTs were also obtained from Cayman. Nucleotides ATP, ADP, UTP, and UDP were from Sigma-Aldrich.

Aequorin luminescence assay: The following CysLT receptor constructs were used: for the mouse $CysLT_1$ receptor, the short-FLAG-m$CysLT_1$R-pcDNA3 that contains the influenza hemagglutinin signal sequence (HA) just upstream of the FLAG epitope, as described previously (Martin et al, 2001); for the human $CysLT_1$ receptor, the cDNA (Lynch et al, 1999) was subcloned into the short-FLAG-m$CysLT_1$R-pcDNA3 from which the cDNA for the mouse $CysLT_1$ receptor had been removed resulting in construction of the HA-FLAG-h$CysLT_1$R-pcDNA3; for the mouse $CysLT_2$ receptor, pcDNA3-m$CysLT_2$R, as previously described (Hui et al, 2001); for the human $CysLT_2$ receptor, h$CysLT_2$R-pCR3.1, as previously described (Heise et al, 2000). The AEQ-pCDM plasmid (Molecular Probes) was used to express aequorin in the HEK 293 cells.

Human embryonic kidney (HEK) 293 cells stably expressing the SV40 large T antigen (HEK 293T cells) or stably expressing the Epstein Bar virus nuclear antigen (HEK 293 EBNA cells), were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin-G, 100 µg/ml streptomycin sulphate and 250 µg/ml active Geneticin^{TM} (G418) at 37°C in a humidified atmosphere of 6 % CO_2 in air.

Cells, either HEK 293T or HEK 293 EBNA, were co-transfected with h$CysLT_2$R-pCR3.1, pcDNA3-m$CysLT_2$R, HA-FLAG-h$CysLT_1$R-pcDNA3, or short-FLAG-m$CysLT_1$R-

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pcDNA3 and AEQ-pCDM plasmids (5 µg of each DNA per 75 cm^2 culture flask), using the LipofectAMINE™ PLUS reagent (Invitrogen) according to the manufacturer's instructions. Cells were subsequently prepared for use in the aequorin luminescence functional assay as described previously (Ungrin et al., 1999). Briefly, holo-aequorin was reconstituted in intact cells by charging 85%-confluent cultures for 1 h at 37^{o}C in Ham's F12 (with 0.1 % fetal bovine serum (FBS), 25 mM HEPES, at pH 7.3) (GIBCO BRL, Mississauga, ON) containing 30 µM reduced glutathione (Sigma-Aldrich, St. Louis, MO) and 8 µM of coelenterazine cp (Molecular Probes, Eugene, OR; now Invitrogen, Burlington, ON). After charging, the cells were washed from the growth surface by pipetting up and down, rinsed once and resuspended in Ham's F12 medium (modified as above) at 2-5 x 10^5 cells/mL. Experiments were performed using a Labsystems Luminoskan RS plate reading luminometer (Needham Heights, MA). Leukotrienes, in ethanol, were serially-diluted, in duplicate, in a white 96-well cliniplate FB (Labsystems) using a Biomek in a final volume of 100 µl in PBS (with 1.26 mM $CaCl_2$) so that the final solvent concentration was ≤ 2%. The plate was then loaded into the luminometer, and wells were tested sequentially starting at position A1, by rows. Cells were injected into the well and light emission was recorded over 30 s (Peak 1). The cells were then lysed by injection of 25 µl of 0.9% Triton-X 100 solution in H_2O, and light emission measured for an additional 10 s (Peak 2). Fractional luminescence for each well was determined by dividing the area under peak 1 by the total area under peaks 1 and 2 (P1/(P1+P2)). These calculations were performed using the Lskan controller program, custom software written in LabView (National Instruments, Austin, TX). An Excel compatible data file was generated containing the raw traces, the calculated results for each well (P1, P2, P1+P2 and P1/(P1+P2)) and the drug concentrations. This data file was subsequently

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analyzed using the LDAM software in Excel employing a modified version of the Levenberg-Marquardt four-parameter curve fitting algorithm to calculate EC_{50} values.

Generation and Characterization of Cells for β-Galactosidase-β-Arrestin Complementation Assay: Retroviral constructs (pMFG-YFP-H31Rα*-IRES-CD8 and pWZL-βArrestin2-ω-IRES-Hygro) were generous gifts from Dr. Helen Blau (Stanford University). pMFG vector was previously engineered to include YFP-α* linked with an IRES-CD8 cassette. The α* fragment encodes the N-terminus of β-galactosidase with a point mutation H31R, to decrease affinity between α- and ω-fragments of β-galactosidase. To insert the $hCysLT_2$ receptor sequence into pMFG, *EcoRI* and *XhoI* restriction sites (underlined) were added to the flanking regions by polymerase chain reaction (PCR) with Phusion DNA polymerase (Finnzymes) using oligonucleotide primers: F: 5'-TAGAATTCGCCATGGAGAGAAAATTTATGTCCTTG-3' and R: 5'-TACTCGAGTACTC TTGTTTCCTTTCTCAACC-3' and $hCysLT_2R$ template (Hui et al, 2001) and cloned into the *MfeI-XhoI* site to generate pMFG-$hCysLT_2R$-YFP-H31Rα*-IRES-CD8 (pMFG-$hCysLT_2R$ for short). pWZL-βArrestin2-ω-IRES-Hygro plasmid is denoted as pWZL-βArrestin2 for short. Sequence integrity of each plasmid was verified by sequence analysis at StemCore Laboratories (Ottawa, ON, Canada).

The two retroviral plasmid constructs were introduced separately into Phoenix retroviral packaging cells (generous gift from Dr. Alan Mak, Queen's University) by a standard calcium phosphate method followed 12 h later by sodium butyrate addition (10 mM). The culture medium (DMEM + 10% FBS) was replaced after another 12 h with fresh complete media (DMEM + 10 % FBS + 1 % Pen/Strep) to harvest retroviruses released in the medium. Subsequently, C2C12 myofibroblasts (ATCC, catalog number CRL-1772) at ~70% confluence in 10 cm tissue culture dishes were first transduced with the βArrestin2-ω filtered viral medium

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(0.45 μm, Millipore, Millex) together with 4 μg/mL of Polybrene (Sigma-Aldrich). The retroviral transduction process was repeated twice at 6 h intervals to increase transduction efficiency. After transduction, cells were washed with 1x PBS and cultured in complete media for 24 h, before antibiotic selection with Hygromycin B (HyClone) at initially 500 μg/mL for 1 week then tapered to 100 μg/mL. Hygromycin-resistant cells were then transduced with retroviruses expressing the $CysLT_2R$-α-fragment of β-galactosidase using the same experimental protocols. C2C12 cells transduced with both constructs (C2C12-h$CysLT_2R$-βArr for short) were then subjected to single cell colony selection by serial dilution.

YFP fluorescence, combined with hygromycin resistance, was used to verify expression of both constructs. Coverslips (12 mm #1; Fisher Scientific), were placed in 24-well tissue culture plates and coated with 100 μL of fibronectin (Athena Enzyme Systems). C2C12-h$CysLT_2R$-βArrestin cells (40 to 50,000/coverslip) were grown overnight in a 37^{o}C incubator with 5% CO_2 in complete media. The next day, cells were washed with 1x PBS and fixed in fresh ice-cold paraformaldehyde (2 %; Fisher Scientific) for 15 min before mounting with Mowiol (Biosciences) onto slides for microscope (Leica DMIRB) viewing. The YFP signal was observed with a Green filter (515-560 nm) and analyzed with OpenLab 2D software for Mac. Fluorescence intensity of C2C12-h$CysLT_2R$-βArr cells was monitored as passage number increased. All assay results were produced with cells between passages 9 to 25 as signal intensity decreased after 30 passages.

To examine expression from the two constructs in C2C12 cells, total RNA was extracted with TRI reagent (Sigma-Aldrich). RNA quality was checked with an Agilent 2100 Bioanalyzer using RNA Nano chips before conducting reverse transcription PCR. cDNA was synthesized with an iScript kit (BioRad) according to the manufacturer's protocols. Oligonucleotide primers

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designed to anneal and amplify the junction regions of the fusion proteins were then used to verify viral transduction efficacy. Relative expression level of the two fusion proteins was assessed by qPCR with the same primer sets, normalized to GAPDH gene expression. qPCR was conducted in triplicate for each gene of interest with iTaq Fast SYBR Green Supermix with ROX (BioRad) kits, according to the manufacturer's protocol.

Junction region	Forward Primer	Reverse Primer	Band Size (bp)
hCysLT2R-YFP	5'-TCAGAAAAGGCCATCCACAGA-3'	5'-AACTTGTGGCCGTTTACGTCG-3'	168
YFP-α*	5'-AACGAGAAGCGCGATCACA-3'	5'-ACGTGCTGCAAGGCGATTA-3'	158
β-Arr-ω	5'-TGTTTGAGGACTTTGCCCG-3'	5'-TTCAGGCTGCGCAACTGTT-3'	111
GAPDH	5'-CTGGAGAAACCTGCCAAGTA-3'	5'-TGTTGCTGTAGCCGTATTCA-3'	125

β-Galactosidase- β-Arrestin Assay: C2C12-hCysLT$_2$R-βArr cells were plated in white opaque bottom 96-well tissue culture plates (Corning) at 25,000/well the night prior to assay, with 150 μL complete medium. For dose response stimulation, prior to assay, various concentrations of stimulus in DMEM were prepared in a separate 96-well plate (stimulus plate), allowing for uniform and rapid initiation of the assays. Complete medium was then removed carefully, and 50 μL of the stimulation media was placed in each well also using a multipipette (Eppendorf). Cells were kept in the stimulation media for 1 h at 37^{o}C prior to addition of Tropix Gal-Screen (50 μL; Applied Biosystems) reagent mix to each well. The Gal-Screen reagent was mixed at a substrate-buffer ratio 1:25 v/v (manufacturer protocol), and allowed to incubate for an additional 1 h at 28^{o}C before recording luminescence in a plate reader (FluoStar Optima, Fisher Scientific). For time course assays, cells were stimulated up to 100 min, at 10 min intervals, with various leukotrienes in 100 μL DMEM prior to addition of 100 μL of Tropix Gal-Screen reagent mix for the 1 h incubation. In total, 9 different time course assays were conducted, corresponding to three

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different stimuli at three concentrations each. The concentrations were chosen as 3x EC_{50}, 1x EC_{50}, and 1/3 EC_{50}.

Modeling and Analysis of β-Galactosidase Complementation Dynamics: The modeling was performed using the Berkeley Madonna software package version 8.0.4 (Berkeley Madonna, Berkeley, CA). Time course data were imported for $NMLTC_4$, LTD_4, and LTE_4. GPCR activation was monitored with the β-galactosidase-β-Arrestin assay based on the method of von Degenfeld *et al* (2007). The signal in this assay requires that the receptor to be phosphorylated and interacts with β-Arrestin-2. The receptor is engineered to have the α^* fragment of β-galactosidase incorporated at its C-terminus, while β-Arrestin-2 is engineered to have the ω domain of β-galactosidase incorporated at its C-terminus. As shown by Nichtl *et al* (1998) the α and ω domains initially form a monomer that then dimerizes, after which two dimers form a tetramer to comprise the fully active β-galactosidase. Thus, in this model, the agonist, '*a*', interacts with the engineered receptor '*r*' to form the '*ar*' complex. This then interacts with the engineered β-Arrestin-2, '*b*', to form the '*arb*' monomeric agonist-receptor-β-Arrestin complex. The monomers eventually associate to form the dimer $(arb)_2$ and the corresponding tetramer $(arb)_4$.

$$a+r \underset{j_1}{\overset{k_1}{\rightleftharpoons}} ar+b \underset{j_2}{\overset{k_2}{\rightleftharpoons}} arb \underset{j_3}{\overset{k_3}{\rightleftharpoons}} (arb)_2 \underset{j_4}{\overset{k_4}{\rightleftharpoons}} (arb)_4$$

All on rate constants are denoted by *k*, while the off rate constants are denoted by *j*. The k_1 and j_1 values are determined exclusively by the dynamics of the agonist receptor interactions. The k_2, and perhaps j_2 values for the second step will depend on the phosphorylation state of the ligand-receptor complex. Rate constants for the last two steps of the mechanism, generation of dimer (k_3 *and* j_3) and the functional tetramer (k_4 *and* j_4), should reflect intrinsic association

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properties of β-galactosidase and be independent of agonist used. The rate equation for each species is shown below.

$$\frac{d[a]}{dt} = j_1[ar] - k_1[a][r]$$

$$\frac{d[r]}{dt} = j_1[ar] - k_1[a][r]$$

$$\frac{d[ar]}{dt} = k_1[a][r] + j_2[arb] - j_1[ar] - k_2[ar][b]$$

$$\frac{d[b]}{dt} = j_2[arb] - k_2[ar][b]$$

$$\frac{d[arb]}{dt} = k_2[ar][b] + j_3[(arb)_2] - j_2[arb] - k_3[arb]^2$$

$$\frac{d[(arb)_2]}{dt} = k_3[arb]^2 + j_4[(arb)_4] - j_3[(arb)_2] - k_4[(arb)_2]^2$$

$$\frac{d[(arb)_4]}{dt} = k_4[(arb)_2]^2 - j_4[(arb)_4]$$

The modeling yielded time courses of the concentrations of all species of the model. The time course data of β-galactosidase for the 3 stimulators, LTD_4, LTE_4, and $NMLTC_4$ were fit simultaneously. Separate on and off rate constants for steps 1 and 2 were optimized independently for each agonist, while the rate constants for dimer and tetramer formation were the same for all agonists, since these steps should be independent of the identity of the agonist. In addition, a common scale factor for converting tetramer concentration to luminescence output was used. The fit of the model to the experimental data was optimized by non-linear regression

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using the simplex algorithm with on and off rate constants and the scale factor as best fit parameters (Table 4).

Vascular Ear Permeability Assay: This protocol is based on Hui et al. (2002). In short, mice were anesthetized i.p. with ketamine/xylazine (0.1 mL per 10 g body weight; from100 mg/mL and 20 mg/mL stocks of ketamine (Vetalar) and xylazine (Rompun), respectively). Mice received 200 µL of 2% Evan's Blue dye in 1x PBS via tail vein injection. Immediately thereafter, the right ear was injected intradermally with 5 ng $NMLTC_4$ in 10 µL saline containing 0.5% ethanol vehicle, whereas the left ear was injected with vehicle control. Animals were euthanized 15 min later. A 6 mm ear biopsy was removed and soaked in formamide (750 µL) overnight (~18 h) at 55^{o}C, and absorbance of the extracted Evan's Blue dye was measured at 610 nm with a Beckman DU-600 spectrophotometer. Readouts were averaged for each experimental group and the relative increase was calculated by comparing ears injected with $NMLTC_4$ with ears injected with vehicle. T-tests were conducted with Graph-Pad Prism software using "two-tail, unpaired" conditions.

Results:

$NMLTC_4$ Acts as a Full Agonist for both Human and Mouse $CysLT_2$ Receptors in an Aequorin Assay

$NMLTC_4$ was first synthesized and pharmacologically characterized by Baker *et al* (1990). It was shown to be an LTC_4-mimetic that was stable and was not a substrate for γ-glutamyl transpeptidase and thus could not be converted to LTD_4 (Fig. 1), as occurs rapidly *in vivo* and *in vitro* tissue and cell preparations. $NMLTC_4$ caused the contraction of guinea pig

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tracheal smooth muscle and was equipotent to LTC_4. The response to $NMLTC_4$ was not affected by either pretreatment with acivicin, an inhibitor of γ-glutamyl transpeptidase, or by addition of the $CysLT_1$ receptor antagonists, ICI 204219 (Baker et al, 1990) or MK679.

To test the CysLT receptor subtype selectivity of $NMLTC_4$, the $CysLT_1$ or $CysLT_2$ receptor cDNAs from human or mouse were introduced into a heterologous expression system with an aequorin-based functional assay for calcium-coupled GPCRs (Ungrin et al, 1999). HEK cells were stimulated with various concentrations of leukotrienes, including LTC_4, LTD_4, LTE_4 and $NMLTC_4$. At the human or mouse $CysLT_2$ receptor, LTC_4 was the most potent agonist followed closely by $NMLTC_4$ and LTD_4. LTE_4 was a weak partial agonist at both receptors (Fig. 2 and Table 1). Thus, $NMLTC_4$, in this aequorin assay system, behaves as a full agonist at $CysLT_2$ receptor.

At the $CysLT_1$ receptors, $NMLTC_4$ was a very weak partial agonist (Fig. 3 and Table 2), ≥172-fold and ≥556-fold less potent than LTD_4 for human and mouse $CysLT_1$ receptor, respectively. LTC_4 and LTE_4 were weak agonists for both $CysLT_1$ receptor homologues. The rank orders of $NMLTC_4$ and the cysLTs for the various receptors are shown in Table 3. Thus $NMLTC_4$ is almost as potent as LTC_4 at the $CysLT_2$ receptors and is the least efficacious agonist at the $CysLT_1$ receptors, far less active than any of the other cysLTs tested. LTE_4 is a weak partial agonist for both receptor subtypes in both species tested in the aequorin assay.

$NMLTC_4$ is a Full Agonist for Human $CysLT_2$ Receptor in a β-Galactosidase- β-Arrestin Assay

Due to the inherent promiscuous nature of secondary messengers such as intracellular calcium (von Degenfeld et al., 2007), a β-galactosidase complementation assay involving β-Arrestin-2 binding was developed for the $hCysLT_2$ receptor (Fig. 4). Interactions of the $hCysLT_2$

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receptor with β-Arrestin-2 have not previously been assessed. This assay takes advantage of homologous desensitization to indirectly assess the activation of the hCysLT$_2$ receptor. Results from this assay adds a layer of specificity, either downstream or parallel to the Ca^{++} signaling, for hCysLT$_2$ receptor signaling. C2C12 myofibroblasts transduced with retroviral constructs expressing both hCysLT$_2$ receptor and β-Arrestin-2 were selected based on fluorescence intensity from the YFP fusion of the receptor and by hygromycin resistance. Quantitative real-time PCR was conducted after the first round of transduction, with pWZL-βArrestin2, to determine the colony with the highest expression of β-Arrestin-2 (colony G, data not shown). The colony G cells were used in the second round of transduction, with pMFG-hCysLT$_2$R to establish doubly transduced cells. After completion of both rounds of transduction, single clone isolates were then established and one clonal expansion (colony 2) was used for all data collection based on the YFP signal intensity, referred to hereon as C2C12-hCysLT$_2$R-βArr. YFP signal was observed to decrease over time in culture, so cells were periodically replaced with low passage cell stocks maintained in liquid nitrogen.

C2C12-hCysLT$_2$R-βArr cells were stimulated with various concentrations of leukotrienes, as well as NMLTC$_4$. The results indicate that NMLTC$_4$ is somewhat more potent than both LTC$_4$ and LTD$_4$ in physiologically relevant concentrations (Fig. 4, Table 1). LTE$_4$ in this assay hardly elicits any response above baseline, even with concentrations up to $1x10^{-5}$ M (Fig. 5). LTB$_4$, a non-cysLT product, was devoid of agonist activity as were nucleotides (ATP, ADP, UDP and UTP; data not shown).

In time course assays with constant concentrations of leukotrienes, NMLTC$_4$ and LTD$_4$ display time dependent profiles (Fig. 6). While, consistent with dose response curves, LTE$_4$ did

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not evoke significant β-galactosidase signal output even with stimulation periods up to 100 min with μM concentrations.

Modeling Simulation of CysLT$_2$R-β-Arrestin β-Galactosidase Complementation in Time Course Assays

The β-galactosidase time course data for LTD_4, LTE_4, and $NMLTC_4$, each at concentrations of 1/3 EC_{50}, EC_{50}, and 3x EC_{50}, are shown in Fig. 6. The lines in Fig. 6 are regression lines obtained through modeling. $NMLTC_4$ and LTD_4 gave much higher levels of β-galactosidase activity (as measured by luminescence) and their time courses were also clearly dose dependent. In addition, the model simulated the experimental results reasonably well, especially with $NMLTC_4$ and LTD_4. The fit with LTE_4 was not as good, indicating that the signal output from the assay is nearing the detection limit. The simulation yielded rate constants for each of the steps in the model, as listed in Table 4. Agonist dose responses at 60 minutes were simulated to determine whether the model could yield consistent EC_{50} values as with experimental data (shown in Fig. 5A). The simulated β-galactosidase values at 60 minutes were fitted to the equation:

$$Y = \frac{Y_{\max} \times [Agonist]}{EC_{50} + [Agonist]}$$

where 'Y' is the simulated β-galactosidase activity. The simulations fit the experimental data very well, and yield estimated EC_{50} values of 9.9 nM, 54.8 nM and 2268 nM for $NMLTC_4$, LTD_4 and LTE_4, respectively. These compared favorably with the experimentally measured β-galactosidase-β-Arrestin values of 8.5 nM, and 36 nM for $NMLTC_4$ and LTD_4 respectively. LTE_4 was previously determined to have an EC_{50} of 2300 nM by Ca^{++} functional assay (Heise et al. 2000). The similarity between the "simulated" EC_{50} values and the actual experimental results

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lend to the added confidence towards the computational modeling, and the equilibrium rate equations describing each interaction.

The model indicates that the dynamics of the β-galactosidase response in this assay system is determined primarily by the on rate constant at step two (k_2). The k_2 values for $NMLTC_4$ and LTD_4 are $4.33x10^5$ M^{-1} sec^{-1} and $1.06x10^6$ M^{-1} sec^{-1}, respectively, while the k_2 value for LTE_4 is remarkably smaller at $7.57x10^3$ M^{-1} sec^{-1}. This is worth noting as it might point towards a slower phosphorylation step by G protein receptor kinases (GRKs) after receptor activation, which could not be measured directly in the assay conditions. It is important to point out that this computer modeling is not a mechanistic interpretation, but rather provides a rationale for the low readout for LTE_4 in the β-Arrestin assay. Each of the 8 parameters was independently adjusted by 0.01 % to see the effect on the computed values of output (relative light units). This sensitivity analysis is tabulated in the Supplementary Section (Supplementary Table 1). In general, the final output was most sensitive to the on rate constant for the β-arrestin binding (k_2) for all compounds at all concentrations. The result of the sensitivity analysis hints that k_2 plays a pivotal role in determining the final output of the assay.

$NMLTC_4$ is a selective agonist for the hCysLT$_2$ Receptor in vivo

$NMLTC_4$ was injected into the ears of transgenic mice overexpressing the hCysLT$_2$ receptor in vascular endothelial cells (TG-EC) (Hui *et al* 2002) and in the CysLT$_2$R knockout (KO) mice (Moos *et al* 2008). $NMLTC_4$ elicited a large, statistically significant increase in Evan's Blue dye leakage in the TG-EC mice when compared to vehicle-treated ears (Fig. 7). In KO mice, on the other hand, $NMLTC_4$ did not increase vascular leakage, indicating a CysLT$_2$-receptor mediated leakage and selectivity of the agonist *in vivo*.

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Discussion:

Here we demonstrate that $NMLTC_4$ (Fig. 1) is a $CysLT_2$ receptor subtype-selective, potent agonist using two distinct assays that target Ca^{++} signaling and downstream β-Arrestin 2 binding to the phosphorylated receptor. The β-Arrestin assay offers distinct specificity and low background activation by monitoring a homologous desensitization pathway and this is the first time a specific interaction of the $CysLT_2$ receptor with β-Arrestin is demonstrated. Growing evidence suggests an inherent bias of GPCR signaling towards either trimeric G-protein or β-Arrestin 2, depending on the receptor in question, as well as the agonist (Violin and Lefkowitz, 2007). The data obtained in the present study with these assays enhances our knowledge of the understudied $CysLT_2$ receptor signaling pathways.

$NMLTC_4$ should be a very useful tool to selectively activate the $CysLT_2$ receptor, both *in vitro* and *in vivo*, without affecting the $CysLT_1$ receptor and we have demonstrated its efficacy *in vivo* in an ear vascular permeability assay. Previously, $NMLTC_4$ was tested in the bullfrog, looking at cardiovascular effects (Sun and Herman, 1995) and lung contractions (Herman et al, 1995) prior to discernment of its subtype selectivity at the recombinant $CysLT_2$ vs. $CysLT_1$ receptors as demonstrated in this present study. The role that the $CysLT_2$ receptor plays in normal and diseased states is being examined. Its distribution in the human heart and vasculature, adrenal gland, immune cells, brain and other tissues (Heise et al, 2000; Nothacker et al, 2000; Evans, 2001; Kamohara et al, 2001) suggests that the $CysLT_2$ receptor is potentially involved in both physiological and pathophysiological processes. In animal models, we have demonstrated that transgenic expression of the $hCysLT_2$ receptor in vascular endothelium predisposes to heightened myocardial ischemia-reperfusion injury and increased vascular permeability in certain vascular beds (Jiang et al, 2008; Moos et al, 2008). The $CysLT_2$ receptor has been

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implicated in allergic and inflammatory diseases such as asthma, rhinitis and sinusitis (Pillai et al, 2004; Steinke and Borish, 2004), as well as in cerebral inflammation and edema (Di Gennaro et al., 2004). Leukotrienes are likely to play an important role in the progression of pulmonary hypertension (Jones et al, 2004) and in cardiovascular disease (Helgadottir et al, 2004; Funk, 2005). Probing the role that $CysLT_2$ receptor plays in these disease processes may now be approached using the selective agonist, $NMLTC_4$.

Of the CysLTs, especially pertaining to LTE_4, there was a differential response in the two assays, whereby in the aequorin assay LTE_4 behaved as a partial agonist but was unable to elicit a signal above baseline in the β-Arrestin assay. Through computer modeling, a possible explanation of the observation is that LTE_4 activates Ca^{++} signaling through G_q but is unable to induce a tight enough association between the ligand-bound receptor and β-Arrestin, possibly due to weak receptor phosphorylation induced by this cysLT. The bias nature of agonist signaling through one receptor (Violin and Lefkowitz, 2007) is not without precedence; the μ-opioid receptor (Keith et al., 1996) being one well characterized example. These results with LTE_4 are significant given the recent determination of a (putative) LTE_4-selective receptor ($CysLT_E$) and that LTE_4 can apparently act via non-GPCR pathways (e.g. PPAR-γ) (Paruchuri et al. 2008). LTE_4 signaling via the $hCysLT_2$ receptor apparently displays preference towards trimeric G-protein signaling rather than via β-Arrestin 2 pathways, as evident in the calcium activity but lack of β-Arrestin 2 association. This signaling bias may afford a novel means to study the CysLT receptors.

In summary, we have shown that $NMLTC_4$ is a potent subtype-selective agonist for the $CysLT_2$ receptor both *in vitro* and *in vivo*. Importantly, it can be used as a selective tool together with the recently described (Wunder et al, 2010) first selective $CysLT_2$ receptor antagonist,

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HAMI3379, to identify some of the physiological and pathophysiological roles that the $CysLT_2$ receptor plays in tissues and cells in which it has been identified and in various disease states.

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Author Contributions

Participated in research design: Yan, Stocco, Nesheim, Abramovitz, Funk

Conducted experiments: Yan, Stocco, Sawyer, Nesheim

Performed data analysis: Yan, Stocco, Nesheim

Wrote or contributed to the writing of the manuscript: Yan, Stocco, Nesheim, Abramovitz, Funk

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Footnote

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FIGURE LEGENDS

Fig. 1. Structure of $NMLTC_4$. $NMLTC_4$ is not a substrate for γ-glutamyl transpeptidase, and therefore, the conversion of $NMLTC_4$ to LTD_4 does not occur.

Fig. 2. $NMLTC_4$ is a full and potent agonist at the human (A) and mouse (B) $CysLT_2$ receptors. The fractional luminescence responses to LTC_4, LTD_4, LTE_4, and $NMLTC_4$ are plotted as a function of their concentration. Sigmoidal curves were obtained by plotting the fractional luminescence at each agonist concentration and analyzed using a modified version of the Levenberg-Marquardt four parameter curve fitting algorithm to determine the EC_{50} values. Averages of duplicates for each sample are shown (with error bars).

Fig. 3. $NMLTC_4$ is a very weak partial agonist at the human (A) and mouse (B) $CysLT_1$ receptors. The fractional luminescence responses to LTC_4, LTD_4, LTE_4, and $NMLTC_4$ are plotted as a function of their concentration. Sigmoidal curves were obtained by plotting the fractional luminescence at each agonist concentration and analyzed using a modified version of the Levenberg-Marquardt four parameter curve fitting algorithm to determine the EC_{50} values. Averages of duplicates for each sample are shown (with error bars).

Fig. 4. Depiction of β-galactosidase- β-Arrestin complementation assay. Top, various signaling components in assay system. Bottom, prolonged cysLT agonist stimulation of the $hCysLT_2$ receptor leads to receptor phosphorylation by G protein receptor kinases (GRKs) with subsequent recruitment of β-Arrestin that allows for complementation of the two β-galactosidase fragments α and ω for enzyme activity assay

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Fig. 5. $NMLTC_4$ is a full and potent agonist at the human $CysLT_2$ receptor in the β-galactosidase-β-Arrestin assay. (A) The fractional luminescence response to LTC_4, LTD_4, LTE_4 and $NMLTC_4$ were plotted as a function of their concentration. Results were analyzed with GraphPad Prism 4, with 'dose response curve' settings, upper constrain set to "equal to 100" and bottom constrain set to "less than 10". Average of duplicate assays with multiple cell preparations (n=6-8) for each sample are shown.

Fig 6. Computer simulation data using β-galactosidase mechanism to model the β-Arrestin assay dynamics *in silico*. The output of tetramer concentration, from Berkeley Madonna, converted to luminescence readout by the scale factor was overlaid with experimental results in GraphPad Prism. (A) The experimental data of $NMLTC_4$, depicted by ■, ▼, and ○, as concentrations of 3x EC_{50}, 1x EC_{50}, and 1/3x EC_{50}, respectively. The modeled data are depicted by solid (—), dashed (---) and dotted lines (···) at the three respective concentrations. The same legend is used in panels B and C, as overlays of experimental and computer modeled data for LTD_4 and LTE_4, respectively. The corresponding residual plots, using the same time axis as the overlay, are shown above the individual graphs.

Fig 7. $NMLTC_4$ is a potent selective agonist at the $CysLT_2$ receptor *in vivo*. TG-EC mice showed a 22-fold increase in vascular leakage upon $NMLTC_4$ administration compared to vehicle control (set to 1.0) as measured by O.D. at 610 nm. Mice lacking the $CysLT_2$ receptor showed no statistically significant response to $NMLTC_4$ compared to vehicle-treated ears. Average absorbencies were: 0.012±0.01 (TG-EC, vehicle; n=8), 0.26±0.13 (TG-EC, $NMLTC_4$; n=8), 0.063±0.07 (KO, vehicle; n=3), and 0.034±0.01 (KO, $NMLTC_4$; n=3)

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Table 1 Potency and efficacy of leukotrienes at $CysLT_2$ receptors.

	Mouse $CysLT_2$ Receptor Aequorin Assay			Human $CysLT_2$ Receptor Aequorin Assay			Human $CysLT_2$ Receptor β-Arrestin Assay		
	Potency (nM) EC_{50} ± SEM (n = 3)	Efficacy % Max @ [μM] (Avg., n = 3)	Fold Diff. EC_{50} Agonist/ EC_{50} LTC4	Potency (nM) EC_{50} ± SEM (n = 3)	Efficacy % Max @ [μM] (Avg., n = 3)	Fold Diff.: EC_{50} Agonist/ EC_{50} LTC_4	Potency (nM) EC_{50} ± SEM (n = 6 - 8)	Efficacy % Max @ [μM] (Avg., n = 6 - 8)	Fold Diff.: EC_{50} Agonist/ EC_{50} LTC_4
LTC_4	38.6 ±6.1	100 @ 1.5	1	94.6 ± 19.9	100 @ 1	1	54.4±9.6	100 @ 0.8	1
$NMLTC_4$	46.1± 8.7	89 @ 1.5	1.2	122.3 ± 27.1	98 @ 3	1.3	8.7±4.5	90 @ 0.8	0.16
LTD_4	49.2 ± 12.6	85 @ 1.5	1.3	144.9 ± 66.1	89 @ 3	1.5	34.7±5.7	90 @ 0.9	0.64
*LTE_4	146 ± 16.7	29 @ 1.5	3.8	1208 ± 554.2	45 @ 10	12.8	-	-	-

* Partial agonist

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Table 2 Potency and efficacy of leukotrienes at $CysLT_1$ receptors.

	Mouse $CysLT_1$ Receptor			Human $CysLT_1$ Receptor		
	Potency (nM) EC_{50} ± SEM (n = 3)	Efficacy % Max @ [μM] (Avg., n = 3)	Fold Diff.: EC_{50} Agonist/ EC_{50} LTD_4	Potency (nM) EC_{50} ± SEM (n = 3)	Efficacy % Max @ [μM] (Ave., n = 3)	Fold Diff.: EC_{50} Agonist/ EC_{50} LTD_4
LTD_4	5.4 ± 0.8	100 @ 0.3	1	11.6 ± 5.5	100 @ 0.4	1
*LTC_4	183 ± 27.8	84 @ 1	34	1483 ± 516	62 @ 1	128
*LTE_4	231 ± 1.3	73 @ 3	43	391 ± 48.7	66 @ 5	34
*NMLTC4	≥ 3,000	28 @ 10	≥ 556	≥ 2,000	60 @ 40	≥ 172

* Partial agonist

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Table 3. Rank order of agonist potencies at the CysLT receptors

$hCysLT_2R$:	$LTC_4 \geq NMLTC_4 \geq LTD_4 >> LTE_4$	$^*NMLTC_4 \geq LTC_4$, $LTD_4 >> LTE_4$
$mCysLT_2R$:	$LTC_4 \geq NMLTC_4 \geq LTD_4 > LTE_4$	
$hCysLT_1R$:	$LTD_4 >> LTE_4 >> LTC_4 > NMLTC_4$	
$mCysLT_1R$:	$LTD_4 >> LTC_4 > LTE_4 >> NMLTC_4$	

from aequorin and *β-Arrestin assays

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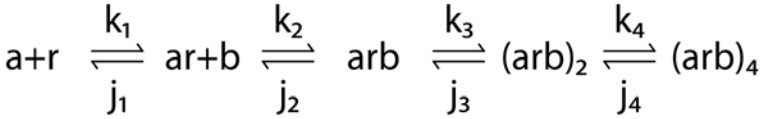
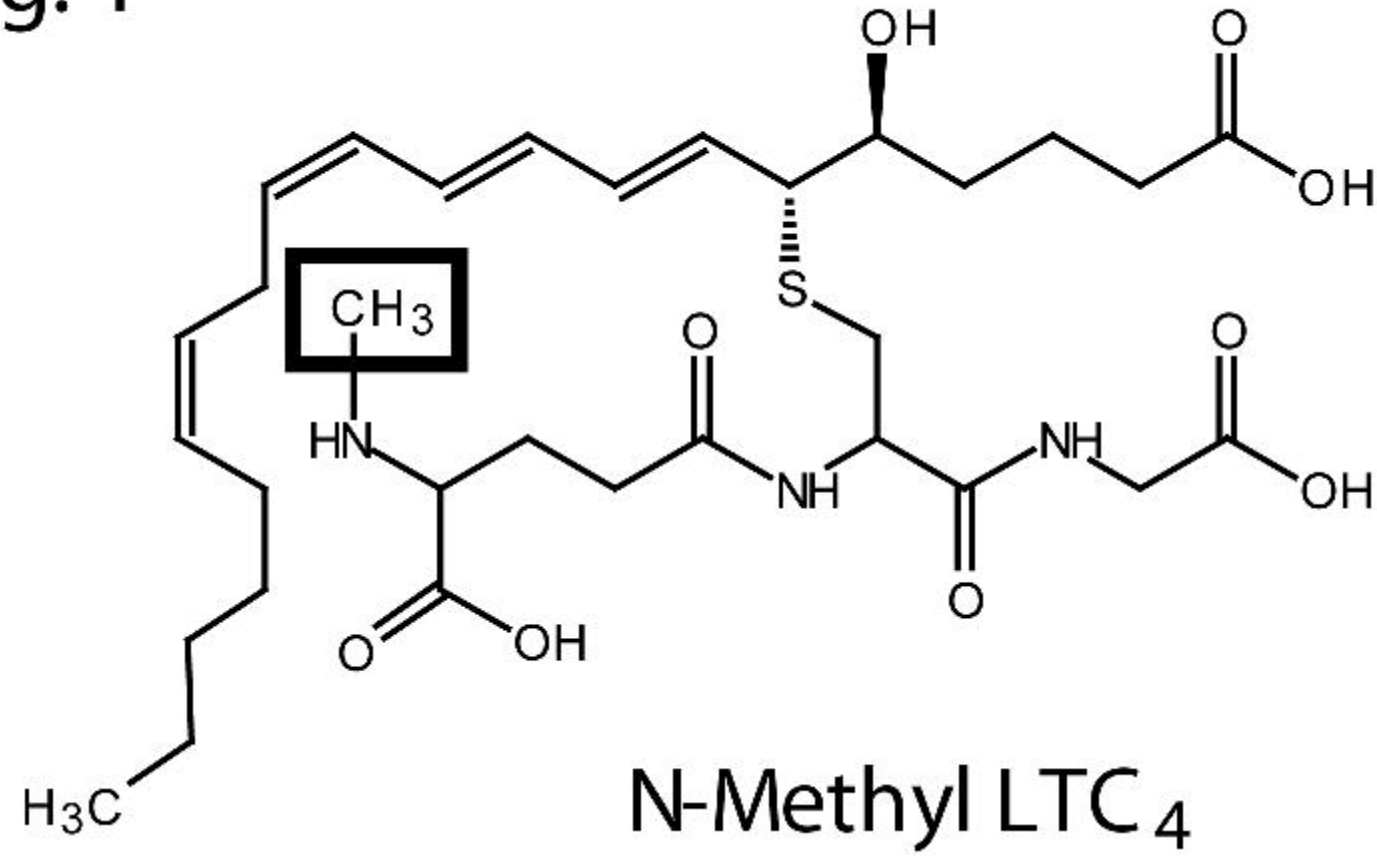
Table 4. Rate constants fitted to experimental data *in silico* by fixing the *k3*, *j3*, *k4*, and *j4* values between data sets.



	NMLTC$_4$	**LTE$_4$**	**LTD$_4$**
k_1 $(M^{-1}\,\mathrm{sec}^{-1})$	1.06E+06	1.06E+04	8.28E+05
j_1 (sec^{-1})	9.51E-02	9.47E-03	1.12E-01
Kd_1 (nM)	89.76	891.9	135.0
$\boldsymbol{k_2}$***** $(M^{-1}\,\mathrm{sec}^{-1})$	**4.33E+05**	**7.57E+03***	**1.06E+06**
j_2 (sec^{-1})	1.00E-04	1.41E-03	7.39E-02
Kd_2 (nM)	0.2	186.2	69.8
k_3 $(M^{-1}\,\mathrm{sec}^{-1})$	2.72E+07	2.72E+07	2.72E+07
j_3 (sec^{-1})	1.04E-05	1.04E-05	1.04E-05
Kd_3 (M)	3.82E-13	3.82E-13	3.82E-13
k_4 $(M^{-1}\,\mathrm{sec}^{-1})$	2.98E+07	2.98E+07	2.98E+07
j_4 (sec^{-1})	2.83E-05	2.83E-05	2.83E-05
Kd_4 (M)	9.52E-13	9.52E-13	9.52E-13
Scale Factor	58801.8	58801.8	58801.8

Fig. 1



N-Methyl LTC_4

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Fig. 2A

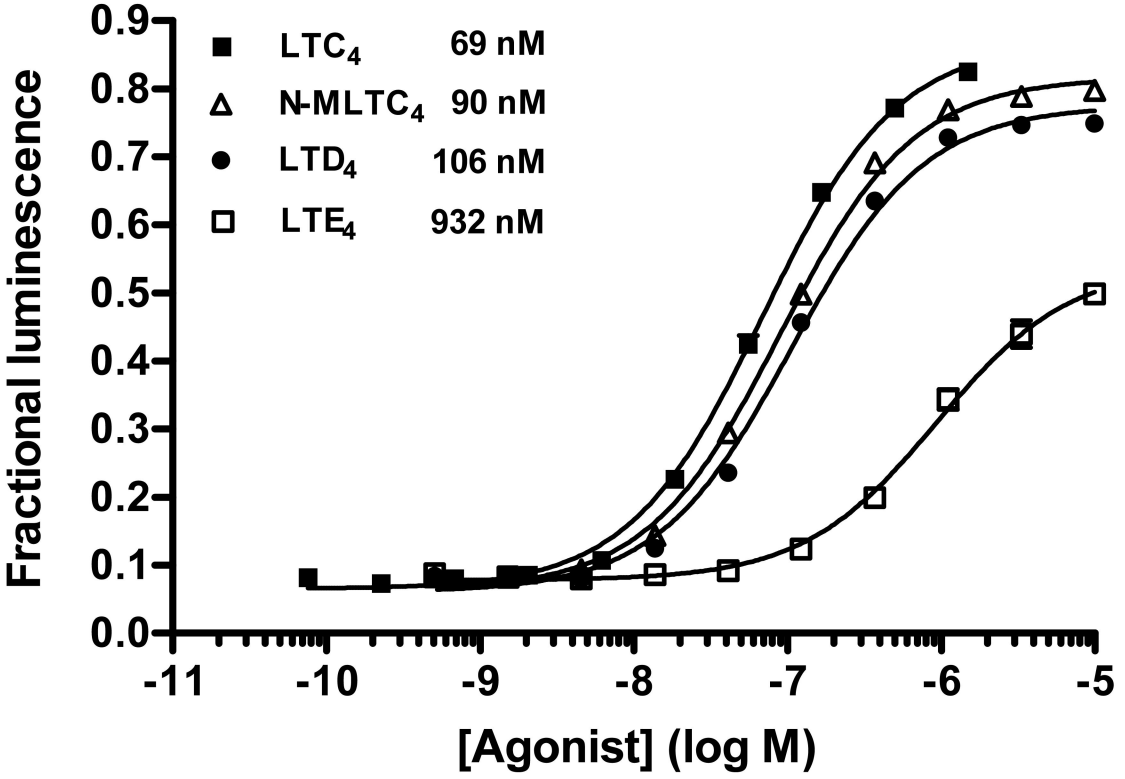
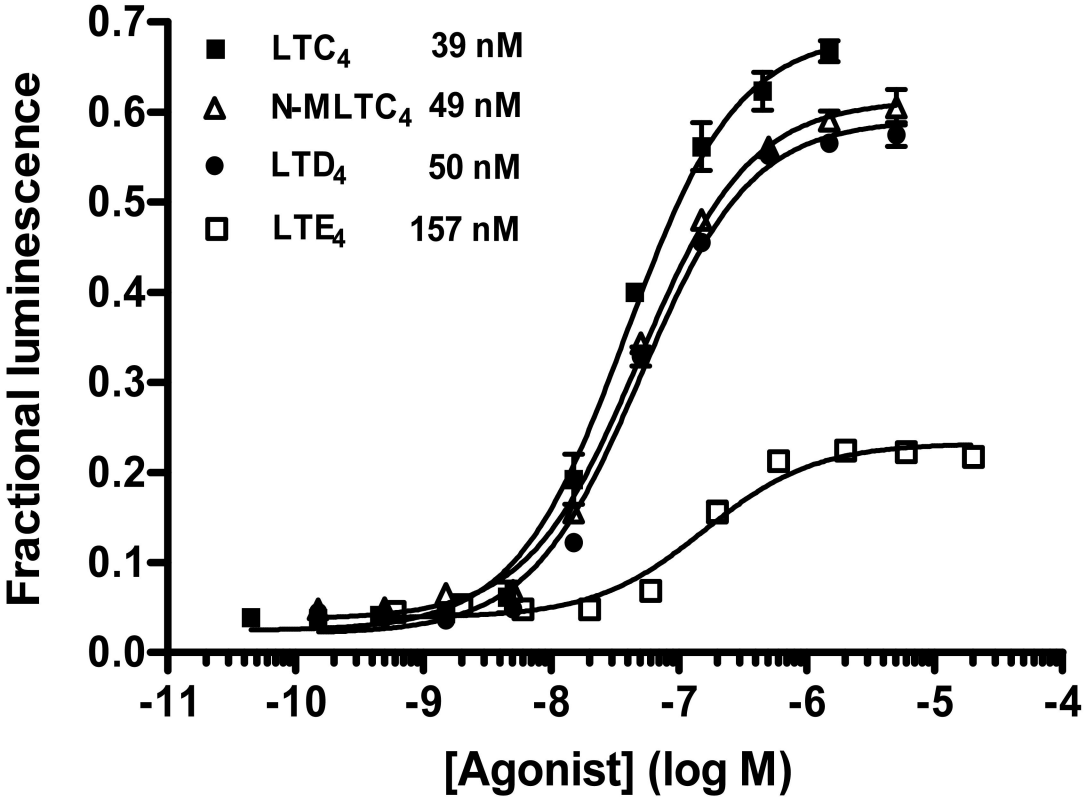


Fig. 2B



Molecular Pharmacology Fast Forward. Published on November 15, 2010 as DOI: 10.1124/mol.110.069054
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3A

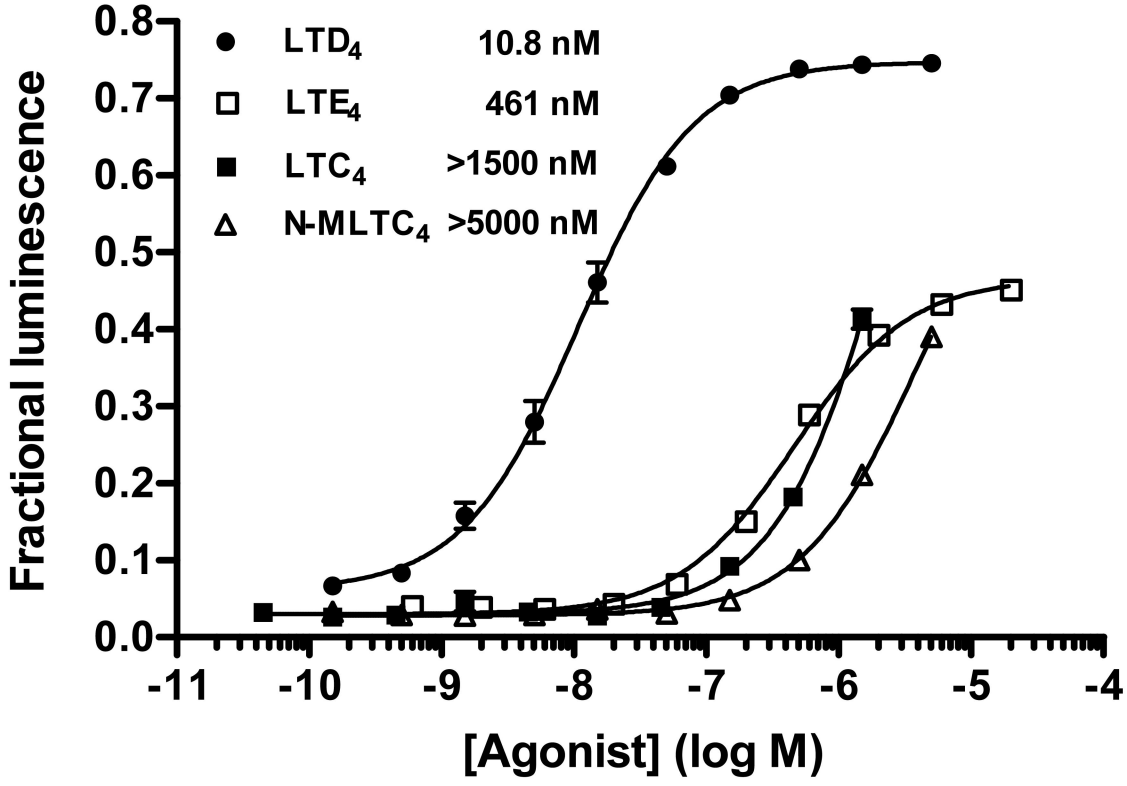


Fig. 3B

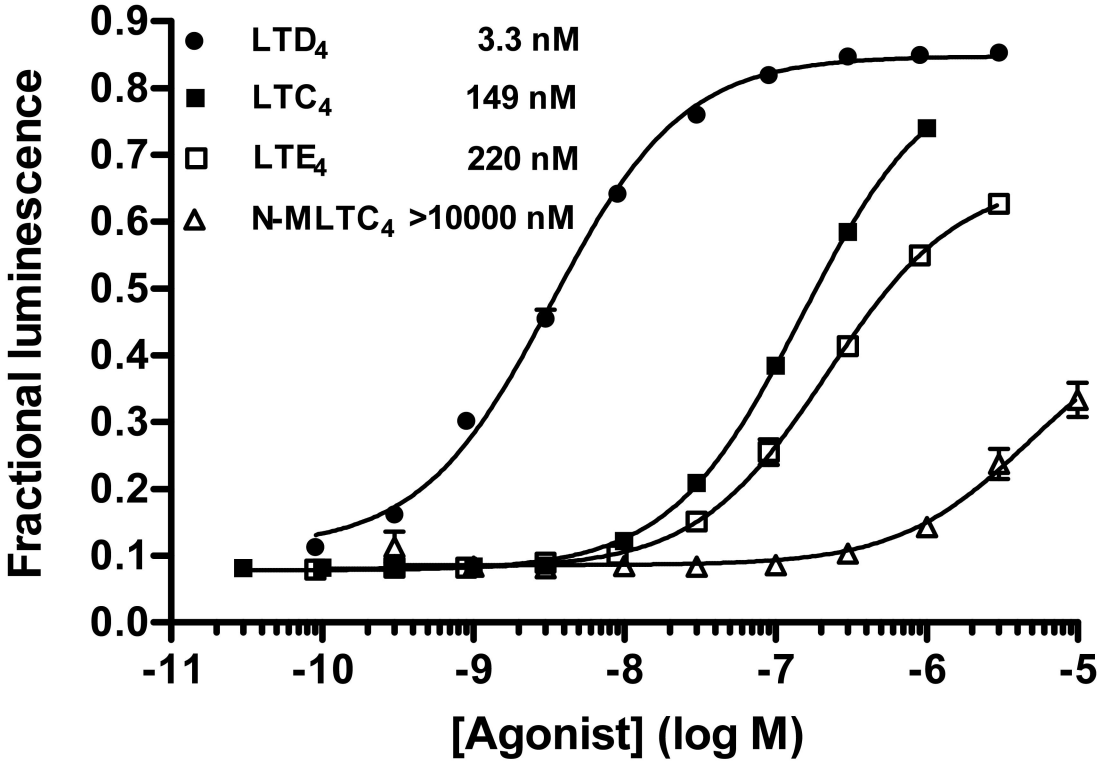


Fig. 4

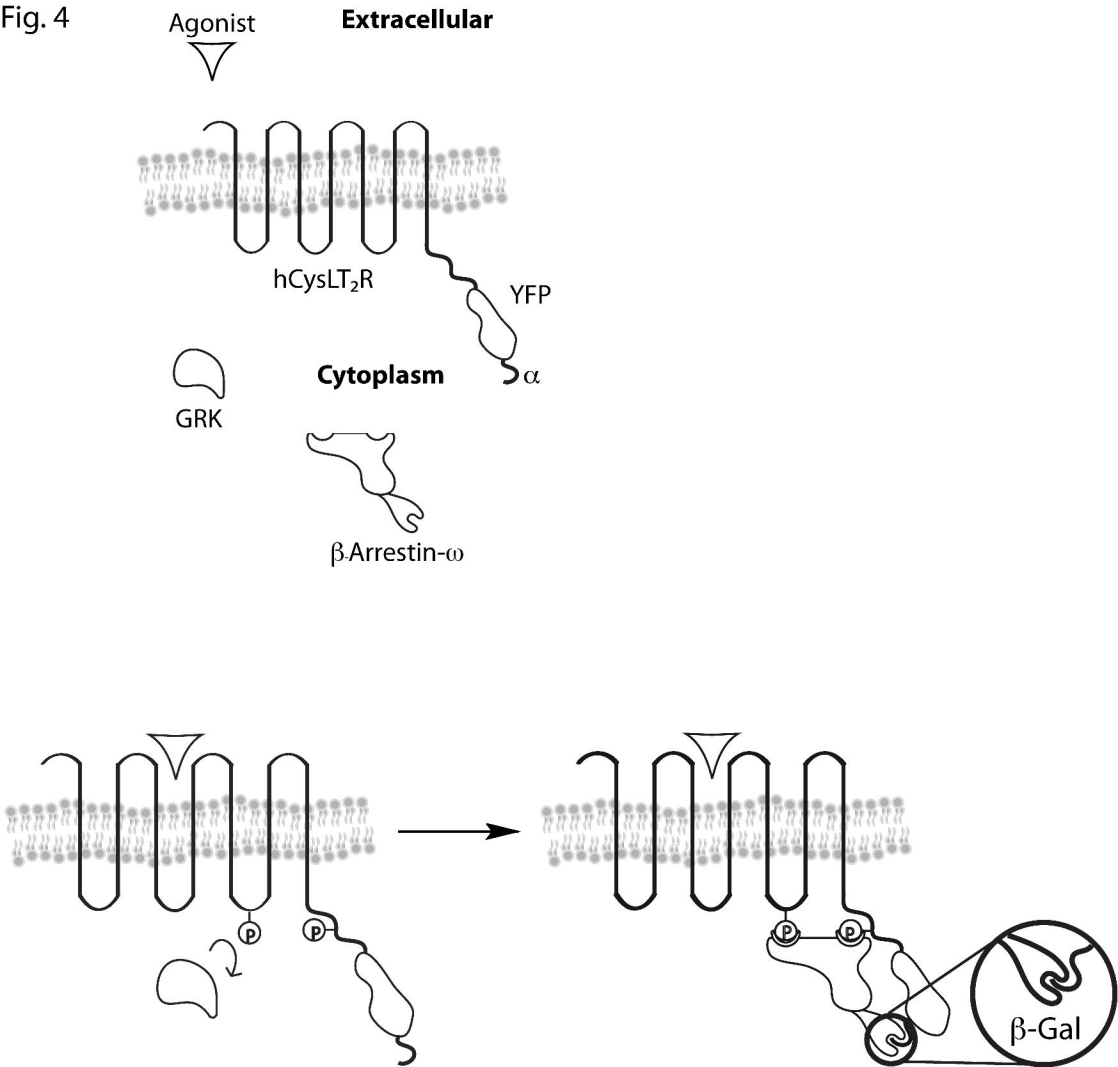

Agonist
Extracellular
hCysLT2R
YFP
Cytoplasm
α
GRK
β-Arrestin-ω
P
P
P
P
β-Gal

Fig. 5

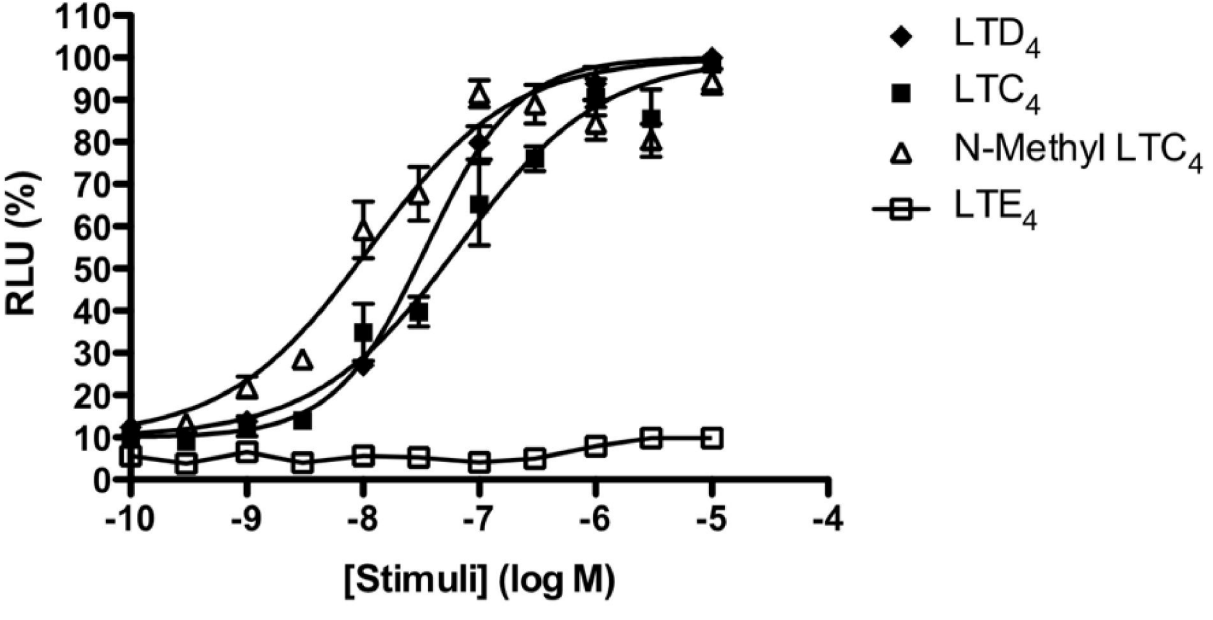

110
100
90
80
70
60
50
40
30
20
10
0
RLU (%)
-10
-9
-8
-7
-6
-5
-4
[Stimuli] (log M)
LTD4
LTC4
N-Methyl LTC4
LTE4

Fig. 6A

$NMLTC_4$

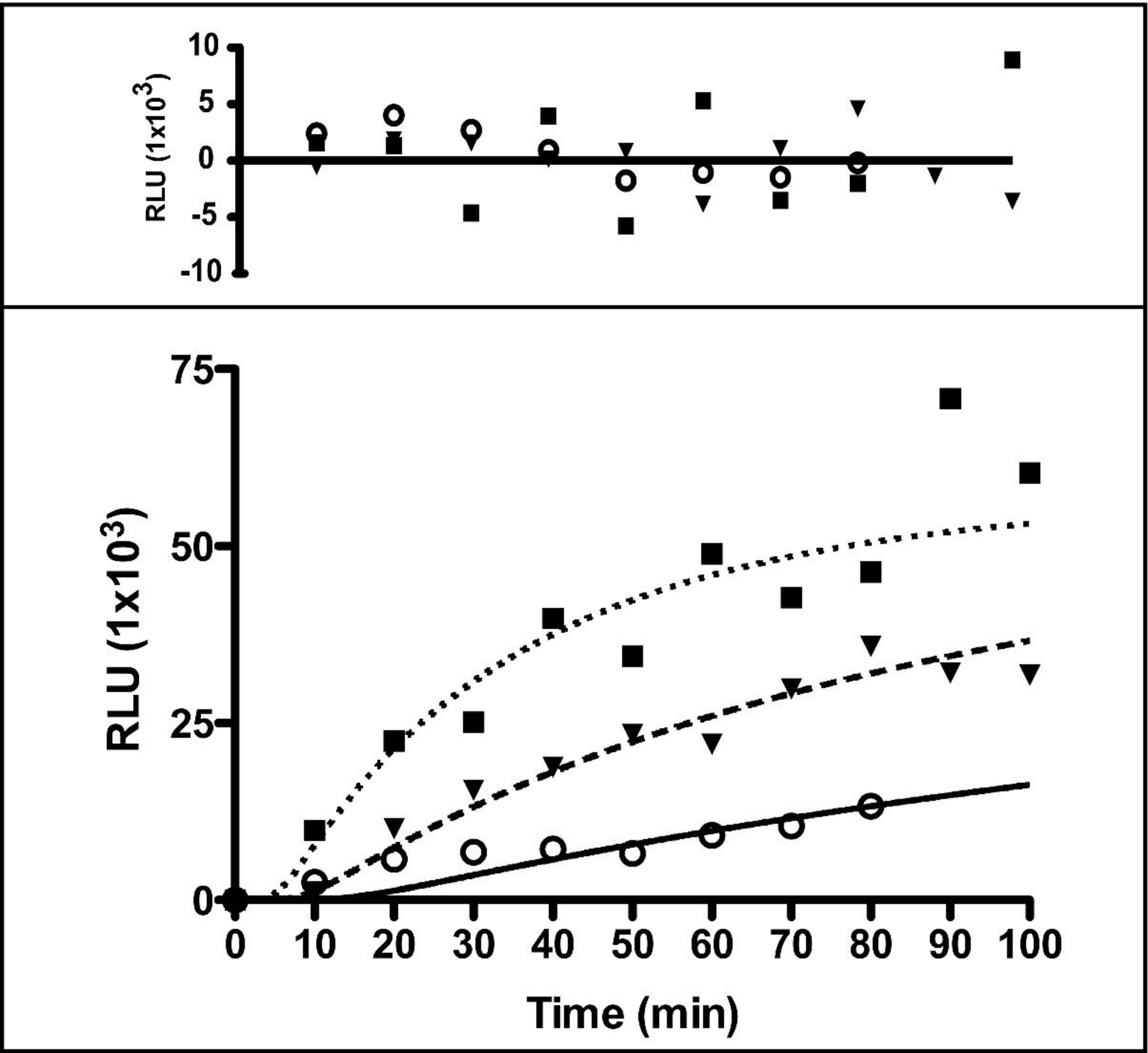


Fig. 6B

LTD_4

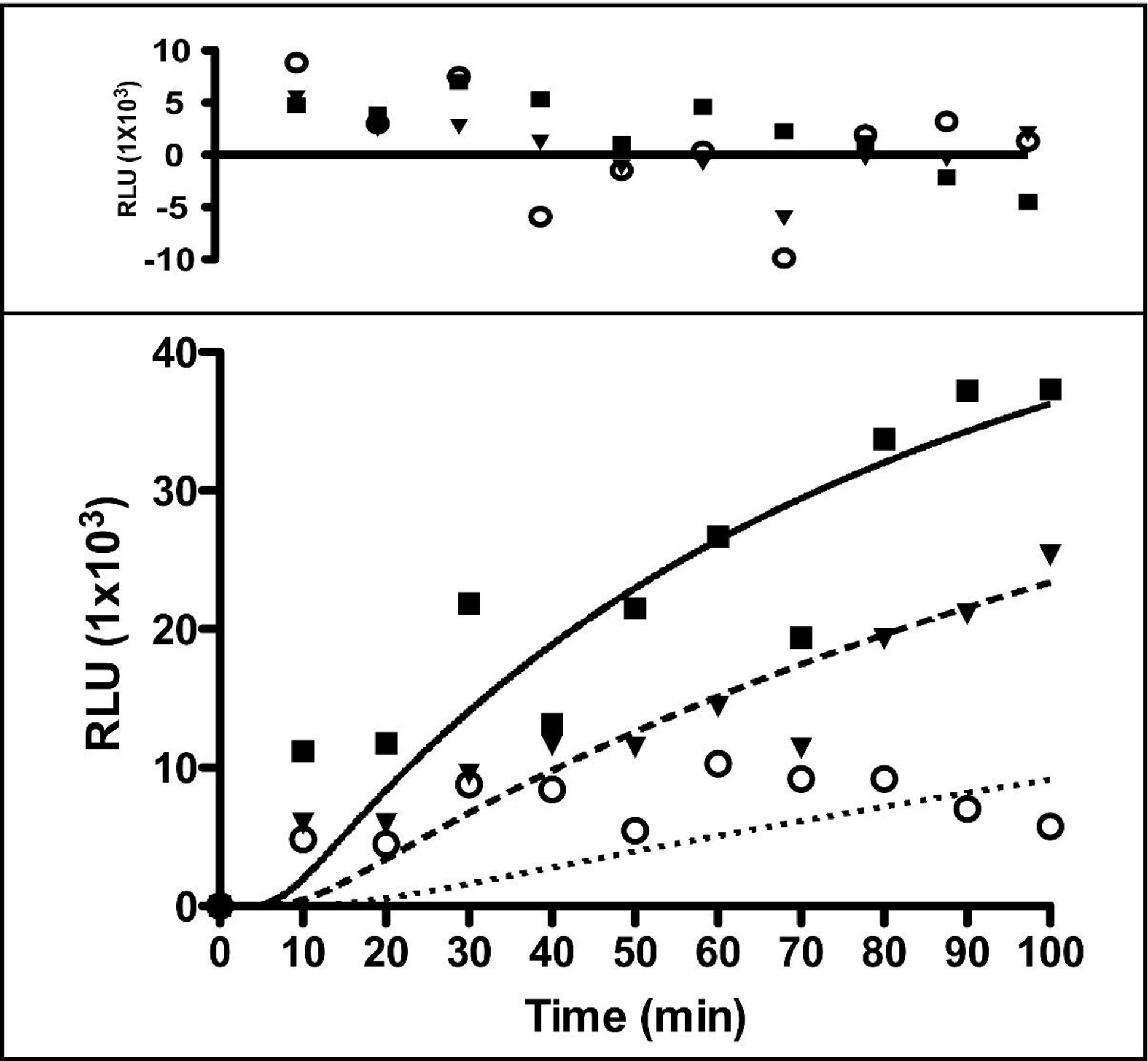

RLU (1X10³)
10
5
0
-5
-10
RLU (1x10³)
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
Time (min)

Fig. 6C

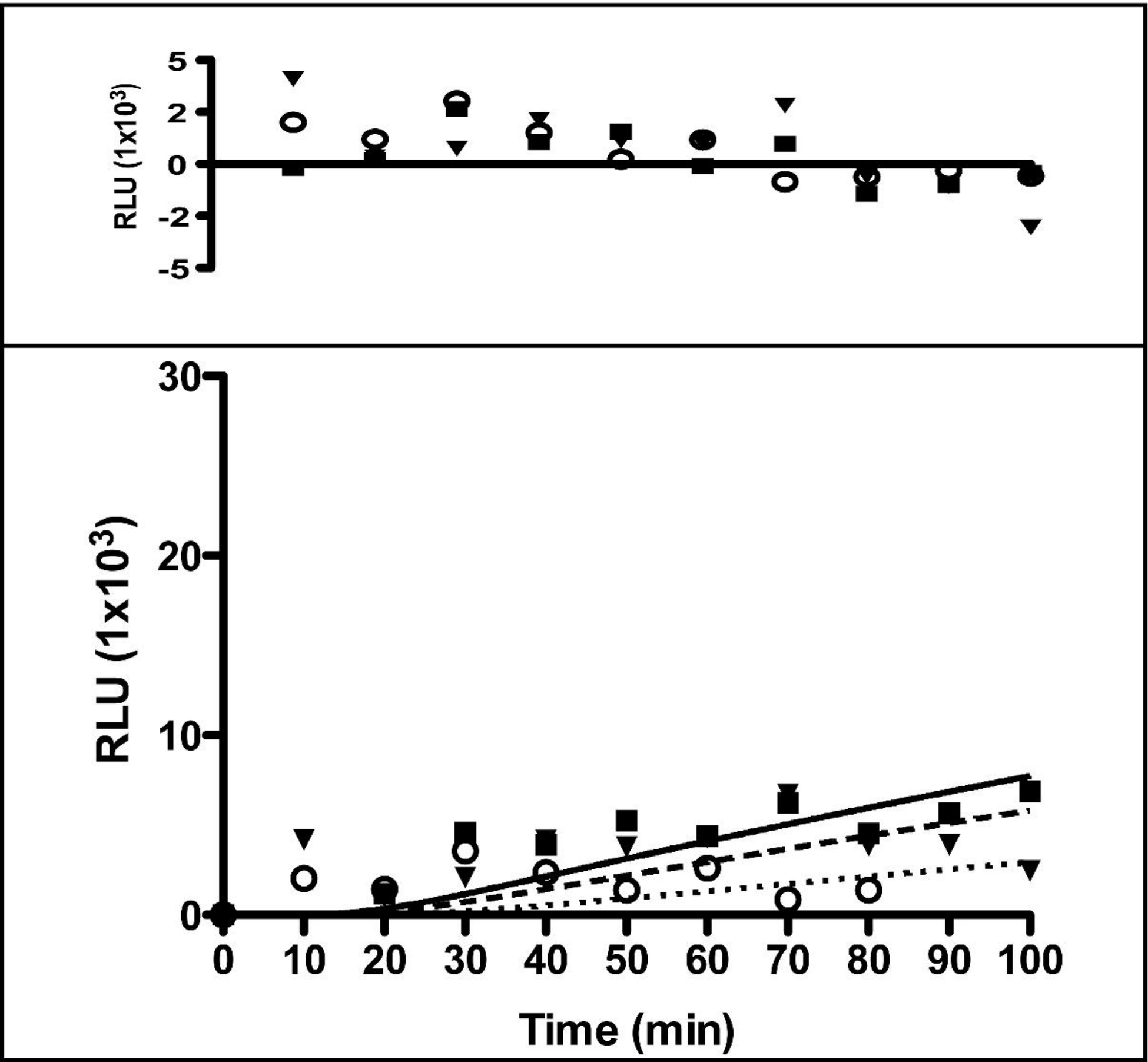

LTE4
RLU (1x10^3)
5
2
0
-2
-5
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
Time (min)

Fig. 7