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(54) Title: GPR17 MODULATORS, METHOD OF SCREENING AND USES THEREOF

(57) Abstract: The invention provides GPR17 modulators, methods of screening and use thereof for diagnosis and therapy of diseases or dysfunctions involving GPR17 activation, particularly ischemic brain damage.

GPR17 MODULATORS, METHOD OF SCREENING AND USES THEREOF

The present invention relates to GPR17-modulating agents, in particular agents able to modify or block GPR17-receptor activity, and their use in the diagnosis and therapy of diseases or dysfunctions involving the same receptor.

BACKGROUND OF THE INVENTION

Extracellular nucleotides are universal and phylogenetically-ancient signalling molecules acting through specific membrane receptors: the seven ligand-gated P2X channels, and the eight G-protein-coupled P2Y receptors (the $P2Y_{1,2,4,6,11,12,13,14}$ receptor subtypes)[1,2]. Endogenous ligands for P2Y receptors include adenine (ATP, ADP), uracil nucleotides (UTP, UDP) and, as more recently recognized, sugar nucleotides (e.g., UDP-glucose and UDP-galactose). Conversely, cysteinyl-leukotrienes (cysLTs) are peptide-conjugated lipid mediators generated by 5-lipoxygenase metabolism of arachidonic acid with established roles in bronchial asthma, acting through the $CysLT_1$ and $CysLT_2$ receptors[3]. Both P2Y receptors and CysLT receptors belong to the ∂ group of the GPCR rhodopsin family (the purin receptor cluster), which also includes the thrombin receptors and a large number of orphan GPCRs[4]. Among receptors in the purin cluster, GPR17[4] is one of the closest receptors to both P2Y and CysLT receptors, with a mean amino acid sequence identity of 31% with the eight recognized P2Y receptors and of 32 and 35% with $CysLT_1$ and $CysLT_2$[4].

STATE OF THE ART

The identification of the nucleotide and amino acid sequences of cysteinyl-leukotriene GPR17 human receptor is reported in GB2360586. Also proposed therein are screening methods that may be used to identify agonists

or antagonist modulating cysLT-receptor activity. According to GB2360586, the agents modulating cysLT-receptor activity may be used in the treatment and/or prophylaxis of several disorders.

DESCRIPTION OF THE INVENTION

5 The invention is based on the finding that GPR17 represents a dualistic receptor responsive to unrelated families of signaling molecules acting through specific G-protein-coupled receptors, namely nucleotides and cysLTs. It has also been found that inhibition of GPR17 by either antagonist ligands or in vivo antisense technology in an animal ischemia model markedly reduces 10 brain damages, indicating that GPR17 represents a common molecular target mediating the neuroinflammatory effects of nucleotides and cysLTs.

The possibility of modulating brain damage by interfering with a receptor responding to two distinct classes of ligands may significantly improve the therapeutic approach to diseases involving an excessive receptor 15 activation, especially cardiovascular, neurodegenerative disorders and kidney ischemia. New chemical entities able to act on both the cys-LT and nucleotide component of GPR17, in particular, may prove extremely more effective in preventing brain damage and thus open up entirely new therapeutic strategies.

In a first embodiment, the invention provides a method for the 20 identification of GPR17 modulators other than the leukotrienes or analogues thereof, which essentially comprises the following steps:

1) in vitro contacting GPR17 with a candidate compound, which is preferably a nucleotide derivative or analogue unable to interact with cysLT receptors;

25 2) determining the receptor response.

As used herein, "GPR17" indifferently indicates the human or rat receptor. The compounds able to bind the receptor and modulate its activity may be further investigated for their therapeutic potential.

The screening method may be applied to the identification of agonists, antagonists, inverse- or partial-agonists. In a preferred embodiment, the screening method is applied to the identification of compounds having receptor-antagonistic activity. In this case, step 1 above is carried out in the
5 presence of a reference compound able to activate the receptor by binding to its nucleotide- or leukotriene-recognition site. Examples of compounds binding to the nucleotide recognition site of GPR17 include UDP, UDP-glucose and UDP-galactose. The assay can be carried out in a cell-based system or using cell preparations or fractions. Preferably, the pharmacological
10 characterization of the receptor is carried out using the [^{35}GTP]γS binding assay or the functional calcium imaging assay.

In the [^{35}GTP]γS assay, upon agonist-binding the receptor undergo a conformational change which induces activation of the G-proteins responsible for signal-transduction. GPR17 activation can be assessed by testing the
15 ability of exogenously added ligands to increase [^{35}GTP]γS binding to purified membranes. In 1321N1 cells (which do not constitutively express any P2Y or CysLT receptors), heterologous hGPR17 expression induced the appearance of specific concentration-dependent responses to the cysLTs LTD_4 and LTC_4 and to the uracil nucleotides UDP, UDP-glucose and UDP-galactose. The ligand
20 specificity of the human receptor was also confirmed in COS-7 and HEK-293 cells. Both AR-C69931MX (which has been reported as a selective $P2Y_{12}$ and $P2Y_{13}$ antagonist[10,11,15]), and the selective $P2Y_1$-receptor antagonist MRS2179[7] concentration-dependently inhibited the [^{35}S]GTPγS binding stimulated by UDP-glucose in membranes of cells expressing the human
25 receptor. Conversely, the $CysLT_1$ antagonists montelukast and pranlukast[3,13] concentration-dependently inhibited the activation of human and rat receptors induced by LTD_4. The different potencies between the two classes of ligands (nanomolar for cysteinyl leukotrienes and micromolar for nucleotides)

suggests that GPR17 can undergo differential activation under specific physiological and pathological conditions. In particular, receptor activation by cysteinyl leukotrienes is believed to occur in physiological conditions, while in conditions of stress and injury the effect of nucleotides becomes important.
5 In the latter situation, in fact, the concentration of nucleotides significantly increases in consequence of their release by hypoxic cells and their production by hydrolysis of nucleic acids in dead cells.

According to a further embodiment, the invention provides the use of GPR17-receptor antagonists for the preparation of a therapeutic agent for the
10 treatment of diseases involving GPR17 activation, particularly neuroprotective, anti-inflammatory and preferably anti-ischemic agents for the treatment of cerebral, cardiac and renal ischemia. The antagonists may be identified with the method according to the invention, or they can be selected from the compounds having purinergic-receptor modulating activity. A
15 comprehensive review of these latter can be found in Jacobson K. et al., "Molecular recognition at purine and pyrimidine nucleotide (P2) receptors", Current Topics in Medicinal Chemistry 2004, vol. 4, pp. 671-686, herein entirely incorporated by reference. According to the invention, the antagonists MRS2179 (N6-methyl-adenosin-3',5'-bis-phosphate, compound no. 46 in the
20 reference) and AR-C69931-MX (N6-methylthio-ethyl-2-trifluoromethyl-ethylthio-adenosin-5'beta-methylene, γ-dichloromethylene trisphosphate, compound no. 57) are particularly preferred.

The invention further provides the use of combinations or associations of compounds acting on the GPR17-receptor sites respectively involved in the
25 recognition of nucleotides and leukotrienes. The compounds acting on the GPR17-receptor site involved in the recognition of leukotrienes are preferably selected from:

- MK-571, 3-(3(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)-(3-

dimethylamino-3-oxo-propyl)thio)methyl)thio)propanoic acid, described in Jones TR et al., (1989) Pharmacology of L-660, 711 (MK-571): “a novel potent and selective leukotriene D4 receptor antagonist”. Can J Physiol Pharmacolo 67:17-28;

- Pranlukast ONO-1078, described in Obata T et al., (1985) New antagonists of leukotrienes: ONO-RS-411 and ONO-RS-347. Adv Prostaglandin Thromboxane Leukot Res 15:229-231;
- the inverse agonists MK-571 and Montelukast, described in Dupre DJ et al., (2004) Inverse agonist activity of selected ligands of the cysteinyl-leukotriene receptor 1. J Pharmacol Exp Ther 309: 102-108.

The above bibliographic citations are herein entirely incorporated by reference.

For use in therapy, the GPR17 antagonists can be simultaneously administered, for example in a single pharmaceutical form or preparation, or separately, using different administration forms and routes. Besides the synthetic compounds (or “small molecules”) indicated above, the therapeutic approach to diseases involving GPR17 activation can be based on:

- expression vectors comprising the nucleotide sequence encoding the receptor protein, deletion or mutation variants thereof, for example plasmids, viruses or phages containing the regulatory sequences necessary for the correct expression of vector polynucleotide sequences (promoters, enhancers, initiation and termination sequences, polyadenylation sequences and, optionally, translation initiation and termination sequences);
- polypeptides having binding affinity to the receptor, able to modify the purinergic or leukotriene activity thereof, including synthetic oligopeptides, monoclonal or polyclonal antibodies recognizing and

binding the GPR17 receptor;

- expression vectors comprising polynucleotides derived from the receptor-encoding sequence and governing the synthesis of antisense RNA;
- synthetic antisense polynucleotides as therapeutic agents. These polynucleotides may include molecules (aptamers) able to interact with the receptor or decoy molecules able to link nuclear proteins or regulatory sequences modulating the receptor expression on genomic DNA.

In an established animal model of permanent ischemic damage (the monolateral middle cerebral artery occlusion in the rat, MCAo), either montelukast or AR-C69931MX (GPR17 antagonists) markedly prevented increase of brain damage determined by Magnetic Resonance Imaging. The same result was observed when the expression of GPR17 was knocked down by utilizing antisense oligonucleotides. Of several antisense oligonucleotides designed on the sequence of rGPR17 mRNA, SEQ ID NO: 1 and SEQ ID NO: 2 (herein also referred to as oligo616 and oligo241, respectively) were able to reduce the in vitro expression of rGPR17 and, when intracerebroventricularly injected in rats, to significantly attenuate infarct size evolution in the lesioned cerebral site.

Therefore, in a particularly preferred embodiment, the invention provides antisense oligonucleotides according to SEQ ID NO: 1 and 2, and the use thereof for the preparation of a therapeutic agent for the treatment of ischemic brain damage.

The oligonucleotides sequences may be chemically modified or conjugated to improve their stability, in vivo delivery and pharmacokinetic profile. For example, the oligonucleotide backbone may be modified to contain 2'-O- (Cl-C3) alkylribonucleotides, 2'- deoxyribonucleotides,

phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkyl phosphonates, phosphinates, phosphoroamidates, thionophosphoroamidates,thionoalkylphosphonates or phosphotriesters groups. Other modifications may involve the sugar moiety,
5 the internucleosidic bond or the purine or pyrimidine bases, e. g. by introducing purines and pyrimidines variously substituted on the heterocyclic rings, for example by alkyl, hydroxy-or halo-alkyl, halogen, hydroxyl, sulfur, amino or aza groups. Moreover, the oligonucleotides of the invention can be conjugated with different groups or functionalities able to increase their
10 activity, distribution or cellular uptake. Such groups or functionalities include lipids, aliphatic chains, poliethilenglycol chains, polyamines and phospholipids.

For use in therapy, the pharmacological agents according to the invention, in particular small molecules, peptides and antisense
15 oligonucleotides, may be suitably formulated together with physiologically acceptable excipients or carriers. Suitable pharmaceutical forms may vary depending on the specific compound or substance and on the administration route. The dosage of active ingredient will be determined on a case by case basis, depending on the severity of the disease to be treated and on the general
20 conditions of the patients.

Suitable pharmaceutical compositions may be prepared following the indications provided in Remington's Pharmaceutical Sciences, XVIII Ed. Mack Publishing Co.

In a further embodiment, the invention relates to a diagnostic
25 composition containing a compound, ligand, peptide, antibody or oligonucleotide able to interact with the purinergic site of the GPR17 receptor, particularly useful for the study of receptor functionality under physiological or pathological conditions.

The invention will be further illustrated by the following examples and by the annexed Figures.

DESCRIPTION OF THE FIGURES

Figure 1 **(a)** Multialignment of human, mouse and rat aminoacid
5 GPR17 sequences highlighting the seven TM domains and the conserved H-X-X-R motif in TM6. **(b)** RT-PCR amplification of the human (1087 bp) or rat (1079 bp) cDNA sequences in brain, kidney, heart, and in human umbilical vein endothelial cells (HUVEC) and breast adenocarcinoma MCF7 cells. No signal was found in peripheral blood mononuclear cells (PBMC), myeloid
10 U937, hepatocellular carcinoma Hep-G2, cervix carcinoma (HeLa) and neuroblastoma SK-N-BE cells. Parallel expression of the house-keeping gene beta-actin is shown. **(c)** Phylogenetic tree generated with the program Mega 2.1, showing the evolutionary relationships between GPR17 and P2Y and CysLT receptors.

15 **Fig. 1.1** Nucleotide and deduced amino acid sequence of hGPR17. The cloned sequence was 99% identical to that reported in the public database (GeneBank accession No: U33447), with the only exception of a T–to-C nucleotide substitution in position 721, which had no effect on the encoded Aminoacid (Leu). Black triangles and circles indicate potential sites for
20 N-linked glycosylation and phosphorylation, respectively. These sites were identified by utilising CBS Prediction Servers (NetPhos 2.0 and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services). Structural analysis for indicated putative TM domain determination of the protein sequences were performed with TMpred software (http://www.searchlauncher.bcm.tmc.edu/seq-
25 search/struc-predict.html).

Figure 2 Determination of GPR17 agonist specificity by [^{35}S]GTPγS binding in cells expressing the human and rat receptors. **(a)** Agonist-response curve to cys-LTs and nucleotides in 1321N1 cells expressing hGPR17, as

shown by presence of a specific 1087 bp amplification product **(b).** No products were detected in samples that did not undergo retrotranscription (RT) (indicated as –RT), nor in cells transfected with the empty vector. **(c)** and **(d)**, same as **(a)** and **(b)** for rGPR17. **(e)** Responses to cys-LTs in COS-7
5 cells expressing hGPR17. **(f)** Responses to nucleotides in HEK-293 cells expressing hGPR17. For each agonist, EC_{50} values are reported. Each point in graphs represents the mean±SD of triplicate determinations from 4-10 independent experiments.

Figure 2.1 **(a)** Sequence multialignment of hGPR17 with
10 $hP2Y_{1,2,3,4,6,11,12,13,14}$ and CysLT1 and CysLT2 receptors. Sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Entrez/). Determination of putative open reading frame was performed with DNA Strider 1.2..BLAST searches were performed through the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/blast/). Amino acid
15 sequences were aligned with ClustalX1.8. Dark and light gray shading indicates presence of, respectively, at least 50% identical or homologous residues. Boxes indicate putative TM1-7. **(b)** Amino acid sequence identity and, (c), similarity (i.e., presence of homologous amino acids) of hGPR17 with known P2Y and CysLT receptors.

20 **Figure 3** Effect of P2Y and $CysLT_1$ receptor antagonists on activation of recombinant GPR17 in the $[^{35}S]GTP\gamma S$ binding assay. **(a)** Antagonism of UDP-glucose stimulation of $[^{35}S]GTP\gamma S$ binding by the indicated P2Y antagonists in 1321N1 cells expressing the human receptor. **(b)** same as in **(a)**, in 1321N1 cells expressing the rat receptor. **(c)** Antagonism of LTD_4
25 stimulation of $[^{35}S]GTP\gamma S$ binding by indicated $CysLT_1$ antagonists in 1321N1 cells expressing the human receptor. **(d)** same as in **(c)** in 1321N1 cells expressing the rat receptor. Each point in graphs represent the mean±SD of triplicate determinations from 4-7 independent experiments. For each

antagonist, IC_{50} values are reported.

Figure 3.1 Abolition of responses to nucleotides and cys-LTs in membranes of cells pretreated with the Gi protein inhibitor PTX.

1321N1 cells expressing hGPR17 were exposed in culture to either
5 vehicle (empty columns) or 100ng/ml PTX (black columns) for 18 hours before membrane preparation. $[^{35}S]GTP\gamma S$ binding was then measured in the absence (basal) or presence of either nucleotides or cys-LTs, as indicated. Data are the mean of 3 experiments run in triplicate. In membranes preincubated with PTX, responses induced by agonists (black column) were in
10 all cases significantly lower ($P<0.001$) with respect to responses detected with the same compounds in membranes from untreated cells (white columns).

Figure 4 Single cell calcium imaging in 1321N1 or COS-7 cells expressing hGPR17. Each trace shows response recorded from one single cell. **(a-c)** Approximately 30% of 1321N1 cells expressing hGPR17 showed
15 responses to uracil nucleotides (mean calcium response to UDP-glucose: $\Delta F340/380=0.34\pm0.11$, n=7), or **(d)** to LTD_4. **(e-h)** The same agonists induced no responses in cells transfected with the empty plasmid. **(i)** Approximately 45% of COS-7 cells transfected with $hCysLT_1$ receptor showed calcium transients to LTD_4 (mean calcium response; $\Delta F340/380= 0.3\pm0.08$, n=17).
20 **(j)** Similar responses were recorded from approximately 50% of COS-7 cells expressing hGPR17 (mean calcium response: $\Delta F340/380= 0.18 \pm 0.03$, n=22). **(k)** No responses to LTD_4 were recorded in cells transfected with the empty plasmid.

Figure 4.1 Target sequences of synthesized anti-sense oligonucleotides
25 on rGPR17 mRNA. **(a)** The various anti-sense sequences were named based on the rat coding sequence (GenBank accession No.: AC112062); black arrows indicate beginning and ending of the coding sequences. All oligonucleotides, including the "scrambled" sequence, were designed as

described in Methods. All these sequences were tested in vitro on HEK-293 cells expressing rGPR17 in order to select the most appropriate oligonucleotides to be utilized for the in vivo studies. Two typical experiments are shown in **(b)**, one aimed at assaying oligo616, oligo241 and oligo99, and
5 the other one aimed at assaying oligoSTART and oligoEND, all in comparison with scrambled oligo sequences run in parallel. On day 2 after plating, cells were transfected with pcDNA3.1 containing the construct encoding for rGPR17 together with the neomycin resistance gene, here utilized as a reported gene. The various oligonucleotides described in **(a)** (all utilized at a
10 0.3μM final concentration) were added to cells twice in a small Fugene volume (125 μl): 16 and 40 h after transfection of rGPR17. Twenty-four h after the last Fugene addition, RNA was extracted from cells as described in Methods, and the transcripts for both rGPR17 and the neomycin resistance gene determined as specific RT-PCR amplification products of 1079 and
15 357bp, respectively, as indicated. Only oligo616 and, to a lesser extent, oligo241 were able to attenuate rGPR17 mRNA in HEK-293 cells. Based on this, only these 2 antisense oligonucleotides were tested in vivo (see Fig. 5). Similar data have been obtained in 11 different experiments.

Figure 5 Effect of montelukast (Mtk), AR-C69931MX (AR-C) and
20 oligo616 on evolution of brain infarct size as determined by MRI at 2, 24, 48 h after MCAo. Quantitative analysis of infarct size volume at 24 and 48 h after MCAo from rats receiving vehicle (C1, n=5) or Mtk (2 mg/kg intravenously, i.v., 10 min after MCAo; n=6); vehicle (C2, n=5) or AR-C (4.5 μg/animal intracerebroventricularly, i.c.v., 10 min after MCAo, n=5), vehicle (C3, n=5)
25 or either oligo616 or scrambled-oligo (400 ng/animal, i.c.v., 48, 24 before and 10 min after MCAo, n=5). Data are expressed as percentage variation of infarct volume at 24 and 48 hours after MCAo compared to 2 h considered as 100% (§ $p < 0.05$, # $p < 0.01$ *vs* volume at 2 hr; * $p < 0.05$, ** $p < 0.01$ *vs*

corresponding control evaluated at the same time point).

METHODS

Cell culture and treatments. Human astrocytoma cells (ADF cells), 1321N1, COS-7 and HEK-293 cells were cultured as previously described[11,24].
5 For [^{35}S]GTPγS, 10^6 1321N1, COS-7 or HEK-293 cells were seeded on 75 cm^2 flasks and transfected by the calcium phosphate precipitation method as previously described[11]. For experiments with anti-sense oligonucleotides (see also below), after plating HEK-293 cells were trasfected with the various oligos in Fugene™. For calcium imaging studies, 1321N1 and COS-7 cells
10 were seeded on 2.4-cm diameter glass coverslips (100 x 10^3 cells). In selected experiments, cells were exposed to 100 ng/ml PTX (Sigma) for 18 h before membrane preparation, to inhibit PTX-sensitive Gi proteins. For treatment of cultured cells with oligonucleotides, 13 x 10^4 HEK-293 cells were seeded on 9 cm^2 dishes and treated as described in Supplementary Fig. 4.

15 **Reagents.** All culture media and sera were from Celbio. All reagents for RT-PCR, cloning and transfection were from Invitrogen, with the exception of Fugene™, which was from Roche Diagnostics. LTD_4 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Anti-sense oligonucleotides were selected according to the general criteria for oligo
20 designing and synthetized by MWG-Biotech AG. In particular, thermodynamic criteria were set according to previous indications[25] and care was taken to avoid internal loop, palindrome of 6 or more base pairs, nucleotides repetition (more then 3 base pairs), and where possible, a AGGG consensus sequence shown to target RNAse degradation was included in the
25 designed oligos (see oligo 616 and 241 in Supplementary Figure 4)[26]. Oligo antisense sequence was mapped on GPR17 RNA secondary structure predicted using GeneBee service (http://www.genebee.msu.su/services/rna2_reduced.html) to choose oligo

mapping in the loop part of the hairpin; the scrambled oligonucleotide was randomly generated on the basis of the 616oligo. We chose to use unmodified oligonucleotides to avoid possible toxicity, while the stability issue was faced by a multiple delivery experimental design (see Fig. 5 legend and text). Each
5 oligo antisense sequence was challenged with rat Genebank using BLASTA programme (http://www.ncbi.nlm.nih.gov/BLAST/) to exclude the presence of multiple target sequences in the rat genome. All other reagents were from Sigma-Aldrich.

Total RNA isolation and PCR Analysis. Total RNA was extracted
10 using the TRIZOL® Reagent (Invitrogen) according to manufacturer's instructions. Retrotranscription to cDNA and PCR reactions were carried out as previously described[11].

Cloning and heterologous expression of human and rat GPR17. The following specific oligonucleotide PCR-primers external to the open reading
15 frame (ORF) of the previously reported human receptor sequence (GenBank accession no U33447) were utilized to amplify a 1087 bp product from human astrocytoma ADF cell:

Fw: 5'-GAC-TCC-AGC-CAA-AGC-ATG-AA-3'

Rw: 5'-GGG-TCT-GCT-GAG-TCC-TAA-ACA-3'

20 The amplification product was cloned into a pcDNA3.1 expression vector using the pcDNA3.1/V5-His©TOPO® TA Expression Kit (Invitrogen, Milan, Italy). Interrogation of the rat HTGS database with the nucleotidic sequence of hGPR17 revealed the presence of an highly similar (89% identical) sequence in chromosome 18 supercontig (Genbank accession No.:
25 AC112062). By utilizing specific oligonucleotide primers external to the putative 1020 bp ORF of the rat sequence we also cloned rGPR17 from rat brain. Constructs were verified by sequencing using the Applied Biosystems Terminator cycle sequencing kit. A partial sequence of the mouse ortholog of

GPR17 is reported in Genbank (AY255543) and the complete sequence 98% identical to the rat receptor was found in a BAC clone (AC131761) using the rat sequence as a probe in the mouse HTGS database.

[^{35}S]GTPγS Binding Assay. 1321N1 cells, COS-7 and HEK-293 cells (control and transfected cells) were homogenized in 5 mM TRIS-HCl, 2 mM EDTA, pH 7.4 and centrifuged at 48000 g for 15 min at 4°C. The resulting pellets (plasma membranes) were washed in 50 mM TRIS-HCl, 10 mM $MgCl_2$, pH 7.4 and stored at -80°C until used. Measurement of nucleotide-stimulated [^{35}S]GTPγS binding to membranes of cells expressing the human or rat GPR17 receptor was performed as previously described[9-11].

Functional calcium imaging assay. Measurements of intracellular calcium concentrations ($[Ca^{2+}]_i$) were carried out as previously described[27]. Forty-eight h after transfection, 1321N1 and COS-7 cells were loaded with 2 μM Fura-2 pentacetoxy methylester in Krebs-Ringer solution, washed and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, N.Y.) equipped with a calcium imaging unit. Polychrome IV (TILL Photonics, Germany) was used as light source. Fura-2 and EGFP fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, Forest City, CA) and analyzed with the Axon Imaging Workbench 2.2 software (Axon Instruments). Images were acquired at 1–4 340/380 ratios/s.

Induction of focal brain ischemia in the rat. Male Sprague-Dawley rats (Charles River) underwent permanent middle cerebral artery occlusion (MCAo) as previously described[28,29]. Drug treatments were as follows: montelukast (2 mg/kg, intravenously, i.v., single bolus of 200 μl in physiological solution) and AR-C69931MX (4.5 μg/animal, intracerebroventricularly, i.c.v., 5 μl in physiological solution) were administered 10 minutes after MCAo. AR-C was utilized here to simply test the involvement of GPR17 in brain ischemia, and, being a very polar molecule which is likely to very poorly permeate the blood

brain barrier, it was administered i.c.v. Oligo-616 and oligo-scramble (400 ng in 5 μl of physiological solution) were administered i.c.v three times to each rat 48, 24 h before and 10 minutes after MCAo. Control groups received corresponding vehicle i.c.v.

5 **Magnetic Resonance Imaging analysis**. MRI measurements were performed 2, 24 and 48 h after MCAo using a 4.7T, vertical superwidebore magnet of a Bruker AMX3 spectrometer with micro imaging accessory. Animal preparation, image acquisition, trace of the diffusion tensor map computation, ischemic volume determination and progression of the ischemic 10 damage over time was as previously described[30].

Statistical analysis. For [^{35}S]GTPγS binding data, analysis and graphic presentation was performed by the non-linear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad). For calcium imaging, data were normalized to the mean F340/380 increase recorded in control cells. All 15 data are presented as mean±SEM of 4-18 experiments run in triplicate. Statistical analysis was performed by either Student's *t* test or one-way ANOVA (Scheffe' test). Significance refers to results where $P<0.05$ was obtained.

RESULTS

GPR17: a close relative of both P2Y and CysLT receptors

20 In search for the natural ligand of GPR17, we first cloned and analyzed the coding sequences from the human and rat receptors. The previously unidentified rat hortholog displayed a 89% aminoacid identity with the human sequence (**Fig. 1a**; **Fig. 1.1**). The hydropathic profile of deduced putative proteins was consistent with the typical seven transmembrane (7TM) structure 25 of a GPCR (ibidem). Multialignment of rat, mouse and human proteins showed almost complete overlapping of TM3, TM6 and TM7 and conservation of a typical amino acid motif in TM6 (**H-X-X-R**) that is also present in all known P2Y and CysLT receptors and has been proposed (at least

for nucleotide receptors) to be essential for binding to endogenous ligands[5-7] (**Fig. 1a**). In line with previous tissue distribution data[8], both human and rat GPR17 showed highest expression levels in brain, followed by kidney and heart, with no significant expression in liver and lung (**Fig. 1b**); hGPR17
5 mRNA was also found in some of the cell lines tested (see **Fig. 1** legend). Identity and similarity of hGPR17 to the known P2Y and CysLT receptors is shown in **Fig. 2.1**. Similarity was highest with $P2Y_1$ (56%), followed by $CysLT_2$ (54%), $CysLT_1$ (53%), $P2Y_4$ (53%), $P2Y_2$ (52%), $P2Y_{11}$ (50%), $P2Y_{13}$ (49%), $P2Y_{14}$ (48%), $P2Y_{12}$ (47%), $P2Y_6$ (45%). The phylogenetic
10 relationships among these receptors are shown in **Fig. 1c**. As expected, $CysLT_1$ and $CysLT_2$ receptors cluster together, whereas P2Y receptors cluster in two phylogenetically-distinct subgroups, one encompassing $P2Y_{1,2,4,6,11}$ and the other one encompassing $P2Y_{12,13,14}$[2]. GPR17 is located at an intermediate position between the $P2Y_{12,13,14}$ subgroup and the $CysLT_1$ and $CysLT_2$ group.
15 Thus, its ligand specificity cannot be predicted simply based on its phylogenetic position and remains unknown (**Fig. 1c**).

Functional characterization unveils the dual pharmacology of GPR17

To identify the endogenous ligand of GPR17, the cDNAs from human
20 and rat GPR17 were cloned into the mammalian expression vector pcDNA3.1 and transfected in 1321N1, COS-7 and HEK-293 cells for functional characterization. GPR17 activation was assessed by testing the ability of exogenously-added ligands to increase [^{35}S]GTPγS binding to purified membranes obtained from transfected cells[9-11]. In 1321N1 cells (which do not
25 constitutively express any functional P2Y or CysLT receptors[12] (Rovati GE & Abbracchio MP, unpublished observations), heterologous hGPR17 expression (**Fig. 2b**) induced the appearance of specific concentration-dependent responses to the cysLTs LTD_4 and LTC_4 (with a rank order of potency of

$LTC_4 >> LTD_4$), and to the uracil nucleotides UDP, UDP-glucose and UDP-galactose (with a rank order of potency of UDP-galactose=UDP>UDP-glucose) (**Fig. 2a**). No other nucleotides or nucleosides (i.e., ATP, ADP, 2-methyl-thio-ADP, UTP, α,βmethylenATP and guanosine, all tested at 10
5 and 50 μM concentrations) had any effect. Thus, the agonist response profile of GPR17 to cysLTs is different from that of both $CysLT_1$ and $CysLT_2$[3,13], and, for nucleotides, is intermediate between $P2Y_6$ and the $P2Y_{14}$ receptor[1,2]. Interestingly, the concentrations giving half-maximal response (EC_{50}) for agonist-stimulation of GPR17 were in agreement with the characteristics of
10 already known CysLT and P2Y receptors[1,3,13], i.e., in the nanoMolar (nM) range for cys-LTs and in μMolar (μM) range for uracil nucleotides (**Fig. 2**; see also **Table 1**). This suggests that GPR17 may respond only to cys-LTs, or to both cys-LTs and nucleotides, depending upon different ligand concentrations reflecting specific pathophysiological conditions (see also below). In a similar
15 way, transfection of the newly-cloned rat receptor in 1321N1 cells resulted in rGPR17 expression (**Fig. 2d**) and appearance of specific responses to nM LTD_4 and LTC_4 and to μM UDP and UDP-glucose (**Fig. 2c**). However, interesting differences could be detected by comparing the pharmacological response profiles of the human and rat receptors. At variance from the human
20 receptor, at the rat receptor UDP-glucose was more potent than UDP, and UDP-galactose induced no effect; moreover, the relative potency of cys-LTs was inverted, with LTD_4 approximately 10-fold more potent than LTC_4 (**Fig. 2** and **Table 1**). The ligand specificity of the human receptor was also confirmed in COS-7 and HEK-293 cells. Transfection in COS-7 cells (which
25 constitutively do not respond to cys-LTs[13]) induced responses to LTD_4 and LTC_4 (Fig. 2e). COS-7 cells do express several P2Y receptors[14] (Fumagalli M, Verderio C and Abbracchio MP, unpublished observations), so the "purinergic" component of GPR17 could not be studied in these cells.

Transfection of hGPR17 in HEK-293 cells also induced responses to UDP, UDP-glucose and UDP-galactose, with a rank order of potency and EC_{50} values similar to those observed in 1321N1 cells (**Fig. 2f**; **Table 1**). In either cell system, no responses were ever observed in cells transfected with corresponding empty vectors (data not shown). The specificity of GPR17 responses were also challenged by assessing the ability of some purinergic and leukotriene receptor antagonists to counteract the increase of $[^{35}S]GTP\gamma S$ binding evoked by nucleotides and cys-LTs in 1321N1 cells expressing either the human or rat receptor. Both AR-C69931MX (which has been reported as a selective $P2Y_{12}$ and $P2Y_{13}$ antagonist[10,11,15]), and the selective $P2Y_1$-receptor antagonist MRS2179[7] concentration-dependently inhibited the $[^{35}S]GTP\gamma S$ binding stimulated by 50 μM UDP-glucose in membranes of cells expressing the human receptor, with concentrations giving half-maximal inhibition (IC_{50}) in the nM range (**Fig. 3a**; **Table 1**). These same antagonists also inhibited the effects induced by 50 μM UDP-glucose on the rat receptor (**Fig. 3b**; **Table 1**). However, both antagonists were significantly more potent in inhibiting UDP-glucose-induced activation of the rat receptor (**Fig. 3b**; **Table 1**), as shown by IC_{50} values in the pMolar (pM) range (**Fig. 3b**). Moreover, at variance from the human receptor, on rGPR17, MRS2179 was more potent than AR-C69931MX (ibidem). These species differences, together with the differences in agonist-response profile reported above for human and rat GPR17 (**Fig. 2**) have to be taken into account when using rodent animals as models for preclinical studies aimed at identifying selective modulators of the pathophysiological functions of GPR17. Conversely, the $CysLT_1$ antagonists montelukast and pranlukast[3,13] concentration-dependently inhibited the activation of human (**Fig. 3c**) and rat receptors induced by 100 nM LTD_4, with IC_{50} values in the nM range and similar relative potencies (**Fig. 3d**; see also **Table 1**). The demonstration that GPR17 can bind to ligands known to act as

selective antagonists only at some P2Y or CysLT receptor subtypes is consistent with its phylogenetic position with respect to more recent members of the "purin cluster". The ability to bind to these ligands may have been progressively lost in parallel with the evolution towards phylogenetically more recent receptors. Because both P2Y[2,11,12] and CysLT receptors[3,13] may couple to G proteins of the Gi subfamily, to evaluate the involvement of this class of G-proteins, we preincubated 1321N1 cells expressing hGPR17 with pertussis toxin (PTX) which inactivates Gi proteins, prior to membrane preparation and $[^{35}S]GTP\gamma S$ binding. PTX strongly inhibited $[^{35}S]GTP\gamma S$ binding stimulated by either UDP, UDP-galactose, UDP-glucose or LTD_4, thus establishing an essential role for this type of G protein in GPR17 responses (**Fig. 3.1**). Finally, based on data demonstrating that both P2Y and CysLT receptors can also couple to phospholipase C and increase intracellular calcium $[Ca^{2+}]_I$ [2,3,13], agonist response specificity of GPR17 was also investigated by single cell calcium imaging. Expression of hGPR17 in 1321N1 cells induced rises of $[Ca^{2+}]_i$ upon application of either UDP-glucose (**Fig. 4a**), UDP (**Fig. 4b**), UDP-galactose (**Fig. 4c**) or LTD_4 (**Fig. 4d**), although only in approximately 30% of cells. No response was observed in cells transfected with the empty vector (**Fig. 4e-h**). Expression of hGPR17 in COS-7 cells induced a $[Ca^{2+}]_i$ transient to LTD_4 in approximately 50% of cells (**Fig. 4j**); these responses were similar to those observed in approximately 45% of cells transfected with $hCysLT_1$ receptor, here utilized as a positive control (**Fig. 4i**). No responses were detected in COS-7 cells transfected with the empty plasmid (**Fig. 4k**).

Table 1

Agonist or antagonist	**1321N1 cells expressing human GPR17 EC_{50} or IC_{50} ± SEM**	**1321N1 cells expressing rat GPR17 EC_{50} or IC_{50} ± SEM**
UDP (agonist)	1.14 ± 0.2 µM	4.6 ± 0.6 µM
UDP-glucose (agonist)	15 ± 1.1 µM	530 ± 24 nM
UDP-galactose (agonist)	1.1 ± 0.09 µM	No effect
LTD (agonist)	7.2 ± 0.3 nM	5.9 ± 0.4 nM
LTC (agonist)	0.33 ± 0.011 nM	65 ± 4.2 nM
AR-C69931MX (antagonist)	0.7 ± 0.02 nM	22 ± 1.5 pM
MRS 2179 (antagonist)	508 ± 29 nM	0.18 ± 0.02 pM
Montelukast (antagonist)	60 ± 4.3 nM	196 ± 13 nM
Pranlukast (antagonist)	10.5 ± 1.2 nM	31 ± 2.4 nM

Table 1. Potency of various ligands on $[^{35}S]GTP\gamma S$ binding to membranes obtaind from 1321N1 cells-transfected with human or rat GPR17. This table summarized the EC_{50} and IC_{50}values for agonists and antagonists respectively, of the various ligand tested in vitro on the recombinant human or rat GPR17, upon expression in 1321N1 cells. All data represent the mean of triplicate determinations from 4-9 independent experiments.

Inhibition of GPR17 prevents evolution of ischemic brain damage

In order to characterize the pathophysiological roles of GPR17, based on data suggesting massive accumulation of both cys-LTs and nucleotides in traumatic and ischemic tissues (Burnstock & Knight, 2004; Ciceri et al., 2001, Ohtsuki et al., 1995) and also based on our previous results demonstrating restricted receptor expression in organs that typically undergo ischemic

damage (**Fig. 1b**), we tested the involvement of GPR17 in brain ischemia by utilizing an established animal model of permanent ischemic damage (the monolateral middle cerebral artery occlusion in the rat, MCAo). As expected, Magnetic Resonance Imaging (MRI) of developing damage in the same living
5 animals at 2, 24 and 48 h after MCAo showed that, in control (vehicle-treated) rats, brain infarct volume increased dramatically between 2 and 48 h in the lesioned hemisphere with respect to the controlateral unlesioned side (see Control animals indicated as C1, C2 and C3 in **Fig. 5**). *In vivo* treatment of ischemic animals with either montelukast or AR-C69931MX (proved to be
10 effective antagonists of GPR17 in heterologous expression systems *in vitro*, see **Fig. 3**), administered 10 min after MCAo (see legend for details) markedly prevented increase of damage with respect to 2h (**Fig. 5**), suggesting a contribution of GPR17 to development of ischemic injury. However, since montelukast and AR-C69931MX are also potent antagonists at $CysLT_1$ and
15 $P2Y_{12,13}$ receptors, respectively[3,10,11,15] and some of these receptors are expressed in rat brain (ibidem), to prove the specific involvement of GPR17 in prevention of brain damage, we selectively knocked-down the expression of GPR17 *in vivo* by utilizing an anti-sense oligonucleotide strategy[18] which has been proven to very efficiently and specifically down-regulate the expression
20 of several other GPCRs in the brain[19,20]. To do so, several anti-sense oligonucleotides have been designed on the sequence of rGPR17 mRNA (indicated as oligoSTART, oligo99, oligo241, oligo-616 and oligoEND in **Fig. 4.1**) and tested for their ability to down-regulate the mRNA for rGPR17 heterologously expressed in vitro in HEK-293 cells. A randomly generated
25 "scrambled" oligonucleotide sequence was utilized in parallel as an internal control. Of all these anti-sense oligonucleotides, only oligo616 and, to a lesser extent, oligo241 were able to reduce the *in vitro* expression of rGPR17 in the HEK-293 cells (see **Fig. 4.1**), and were thus selected for the *in vivo* MCAo

study. These oligonucleotides were administered *in vivo* by employing a multiple delivery experimental protocol. In a similar way to montelukast and AR-C69931MX, repeated intracerebroventricular (i.c.v.) injections (400 ng/animal) of oligo616 to ischemic rats 48 and 24 h before and 10 min
5 after MCAo markedly and significantly attenuated infarct size evolution in the lesioned hemisphere (**Fig. 5**). A smaller protective effect was observed with oligo241 (data not shown). No effect on the extent of ischemic damage was observed in animals injected with a non-specific "scrambled" oligonucleotide (**Fig. 5**). In subsequent experiments, a single cumulative (1200 ng/ml) i.c.v.
10 administration of oligo616 given only 10 min after MCAo also resulted in similar protection against brain damage (data not shown). These data suggest that inhibition of GPR17 by either antagonist ligands or receptor knock-down results in protection against ischemic brain damage, thus confirming a crucial role for this receptor in injury development. This protection can be attained
15 even if receptor inhibition or knock-down is accomplished after (and not before) ischemia is induced, making this receptor a highly relevant biological target for the development of new therapeutic approaches to stroke treatment.

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CLAIMS

1. A method for the identification of a GPR17 modulator, which essentially comprises the steps of:

5 a) contacting GPR17 with a candidate compound, the latter being a nucleotide derivative or analog unable to interact with cysteinyl leukotriene receptors;

b) determining the receptor response.

2. A method according to claim 1, which is carried out in a cell-based
10 system or using cell preparations or fractions.

3. A method according to claim 1, wherein the receptor response is determined by means of the [^{35}S]GTPγS binding assay.

4. A method according to claim 1, for the identification of receptor antagonists.

15 5. A method according to claim 1, wherein step (a) is carried out in the presence of a compound able to bind to GPR17 nucleotide-recognition site.

6. A method according to claim 5, wherein said compound is selected from UDP, UDP-glucose and UDP-galactose.

7. An antisense oligonucleotide selected from SEQ ID NO: 1 and SED ID
20 NO: 2, or a functional analog thereof.

8. The use of a GPR17 antagonist interacting with the receptor nucleotide binding site, for the preparation of a therapeutic agent for the treatment of disorders involving GPR17 activation.

9. The use according to claim 7, of a compound selected from N6-methyl-
25 adenosin-3',5'-bis-phosphate and N6-methylthio-ethyl-2-trifluoromethyl-ethylthio-adenosin-5'beta-methylen γ-dichloromethylene trisphosphate.

10. The use of a combination of GPR17 antagonists respectively acting on the nucleotide and on the leukotriene receptor-binding site, for the preparation

of a therapeutic agent for the treatment of disorders involving GPR17 activation.

11. The use according to claim 9, of a compound selected from N6-methyl-adenosin-3',5'-bis-phosphate and N6-methylthio-ethyl-2-trifluoromethyl-
5 ethylthio-adenosin-5'beta-methylen γ-dichloromethylene trisphosphate in combination with a compound selected from Montelukast, Pranlukast and 3-(3(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)-(3-dimethylamino-3-oxo-propyl)thio)methyl)thio)propanoic acid.

12. The use of a compound interacting with the GPR17 nucleotide binding
10 site, for the preparation of a diagnostic composition.

13. The use according to claims 7-10, for the preparation of a neuroprotective, anti-inflammatory and anti-ischemic agent.

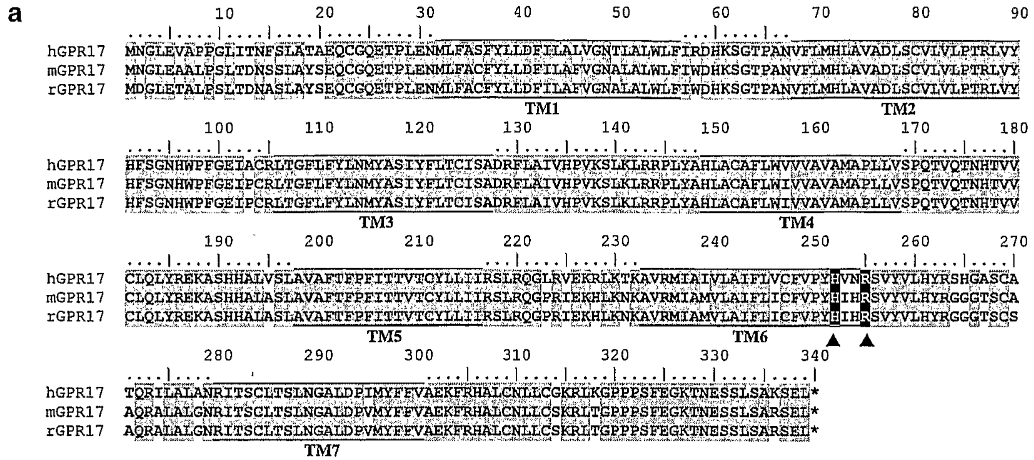
14. The use according to claim 12, for the preparation of a therapeutic agent useful for the treatment of cerebral, cardiac and renal ischemia.

15 15. The use according to claim 13 of an antisense oligonucleotide selected from SEQ ID NO: 1 and SEQ ID NO: 2.

16. The use of an antisense oligonucleotide selected from SEQ ID NO: 1 and SEQ ID NO: 2 for the treatment of ischemic brain damage.

17. The use of small interference RNAs directed against SEQ ID NO: 1 and
20 SEQ ID NO: 2 for the preparation of a therapeutic agent for the treatment of ischemic brain, heart and renal damage.

a



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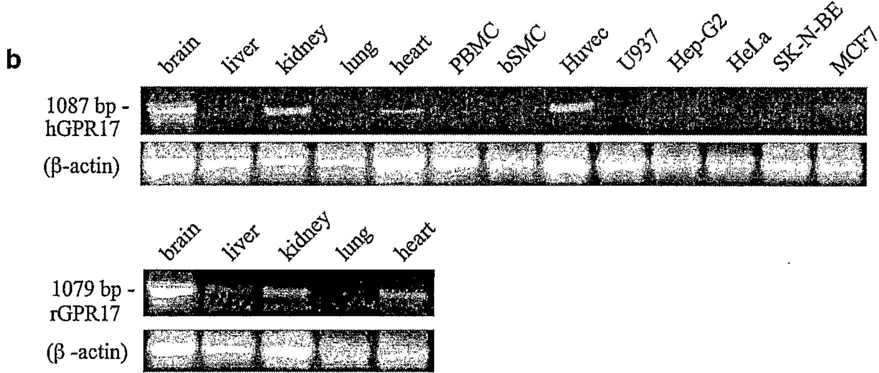

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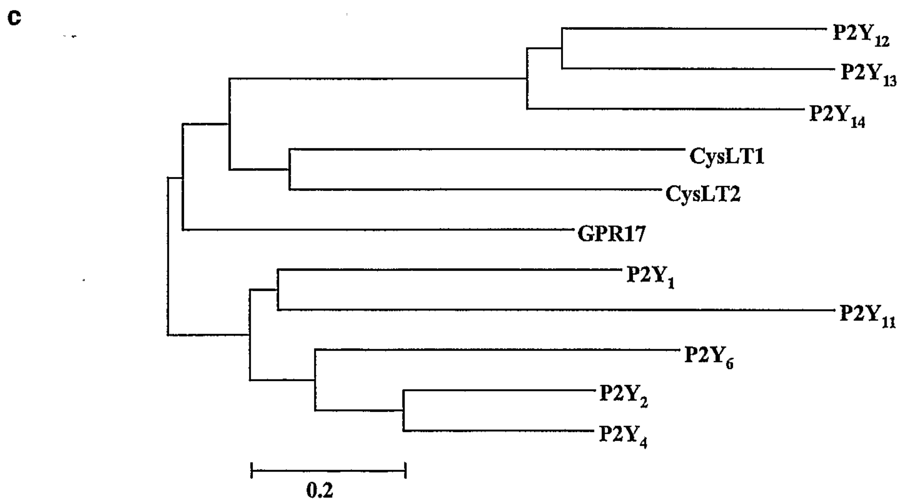

Figure 1

Figure 1.1

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1    ATGAATGGCCTTGAAGTGGCTCCCCCAGGTCTGATCACCAACTTCTCCCTGGCCACGGCAGAGCAATGTGGCCAGGAGACGCCACTGGAG 90
      M  N  G  L  E  V  A  P  P  G  L  I  T  N  F  S  L  A  T  A  E  Q  C  G  Q  E  T  P  L  E
                                               s                                   ●
91   AACATGCTGTTCGCCTCCTTCTACCTTCTGGATTTTATCCTGGCTTTAGTTGGCAATACCCTGGCTCTGTGGCTTTTCATCCGAGACCAC 180
      N  M  L  F  A  S  F  Y  L  L  D  F  I  L  A  L  V  G  N  T  L  A  L  W  L  F  I  R  D  H
                                             TM1
181  AAGTCCGGGACCCCGGCCAACGTGTTCCTGATGCATCTGGCCGTGGCCGACTTGTCGTGCGTGCTGGTCCTGCCCACCCGCCTGGTCTAC 270
      K  S  G  T  P  A  N  V  F  L  M  H  L  A  V  A  D  L  S  C  V  L  V  L  P  T  R  L  V  Y
                                                                TM2
271  CACTTCTCTGGGAACCACTGGCCATTTGGGGAAATCGCATGCCGTCTCACCGGCTTCCTCTTCTACCTCAACATGTACGCCAGCATCTAC 360
      H  F  S  G  N  H  W  P  F  G  E  I  A  C  R  L  T  G  F  L  F  Y  L  N  M  Y  A  S  I  Y
                                                                                          TM3
361  TTCCTCACCTGCATCAGCGCCGACCGTTTCCTGGCCATTGTGCACCCGGTCAAGTCCCTCAAGCTCCGCAGGCCCCTCTACGCACACCTG 450
      F  L  T  C  I  S  A  D  R  F  L  A  I  V  H  P  V  K  S  L  K  L  R  R  P  L  Y  A  H  L
                                                                                     ●
451  GCCTGTGCCTTCCTGTGGGTGGTGGTGGCTGTGGCCATGGCCCCGCTGCTGGTGAGCCCACAGACCGTGCAGACCAACCACACGGTGGTC 540
      A  C  A  F  L  W  V  V  V  A  V  A  M  A  P  L  L  V  S  P  Q  T  V  Q  T  N  H  T  V  V
                 TM4                                                              s
541  TGCCTGCAGCTGTACCGGGAGAAGGCCTCCCACCATGCCCTGGTGTCCCTGGCAGTGGCCTTCACCTTCCCGTTCATCACCACGGTCACC 630
      C  L  Q  L  Y  R  E  K  A  S  H  H  A  L  V  S  L  A  V  A  F  T  F  P  F  I  T  T  V  T
                                 ●                                               TM5
631  TGCTACCTGCTGATCATCCGCAGCCTGCGGCAGGGCCTGCGTGTGGAGAAGCGCCTCAAGACCAAGGCAGTGCGCATGATCGCCATAGTG 720
      C  Y  L  L  I  I  R  S  L  R  Q  G  L  R  V  E  K  R  L  K  T  K  A  V  R  M  I  A  I  V
                                                                  ●
721  TTGGCCATCTTCCTGGTCTGCTTCGTGCCCTACCACGTCAACCGCTCCGTCTACGTGCTGCACTACCGCAGCCATGGGGCCTCCTGCGCC 810
      L  A  I  F  L  V  C  F  V  P  Y  H  V  N  R  S  V  Y  V  L  H  Y  R  S  H  G  A  S  C  A
              TM6                              s
811  ACCCAGCGCATCCTGGCCCTGGCAAACCGCATCACCTCCTGCCTCACCAGCCTCAACGGGGCACTCGACCCCATCATGTATTTCTTCGTG 900
      T  Q  R  I  L  A  L  A  N  R  I  T  S  C  L  T  S  L  N  G  A  L  D  P  I  M  Y  F  F  V
                                          ●                         TM7
901  GCTGAGAAGTTCCGCCACGCCCTGTGCAACTTGCTCTGTGGCAAAAGGCTCAAGGGCCCGCCCCCCAGCTTCGAAGGGAAAACCAACGAG 990
      A  E  K  F  R  H  A  L  C  N  L  L  C  G  K  R  L  K  G  P  P  P  S  F  E  G  K  T  N  E
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      ●        ●
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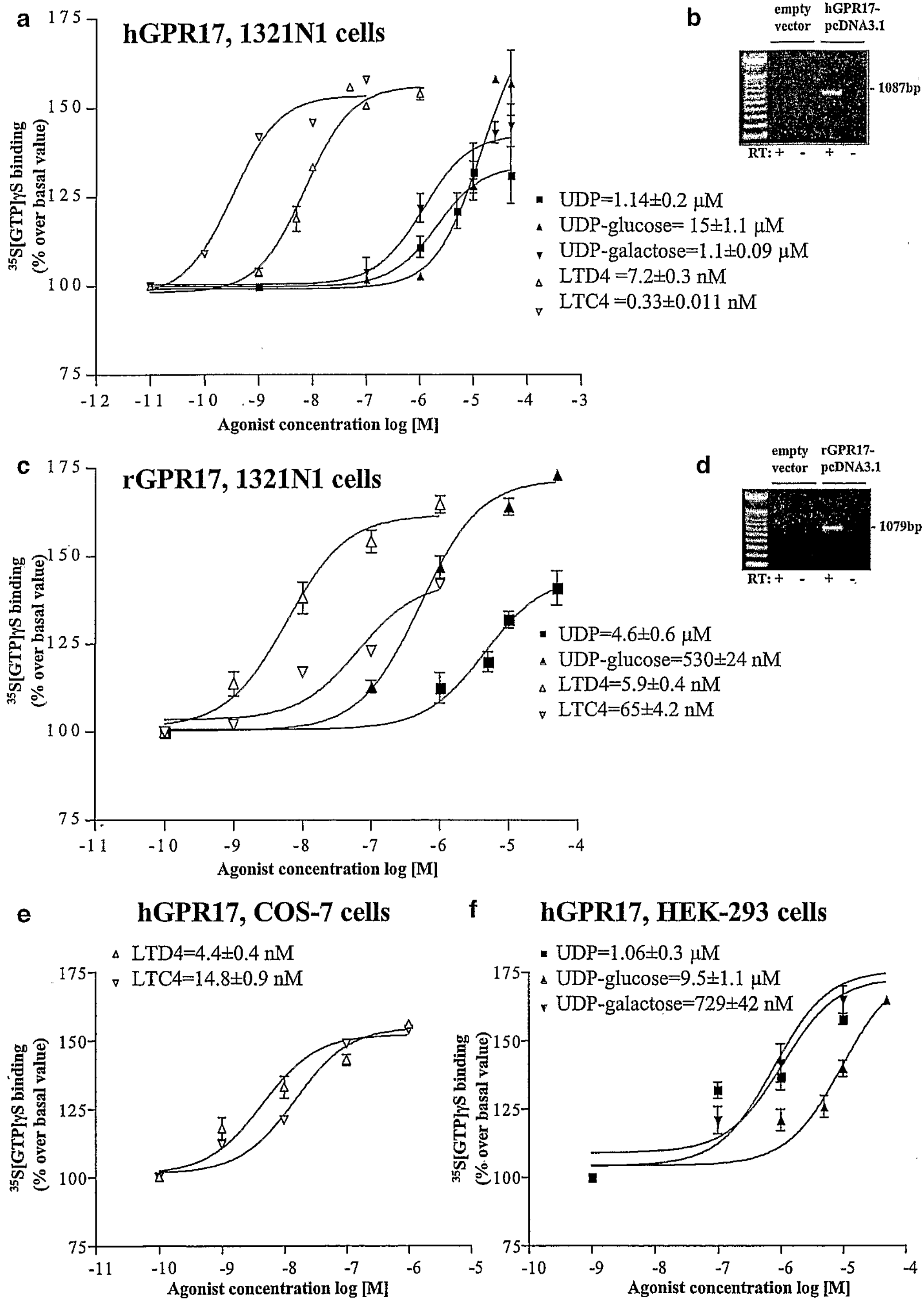
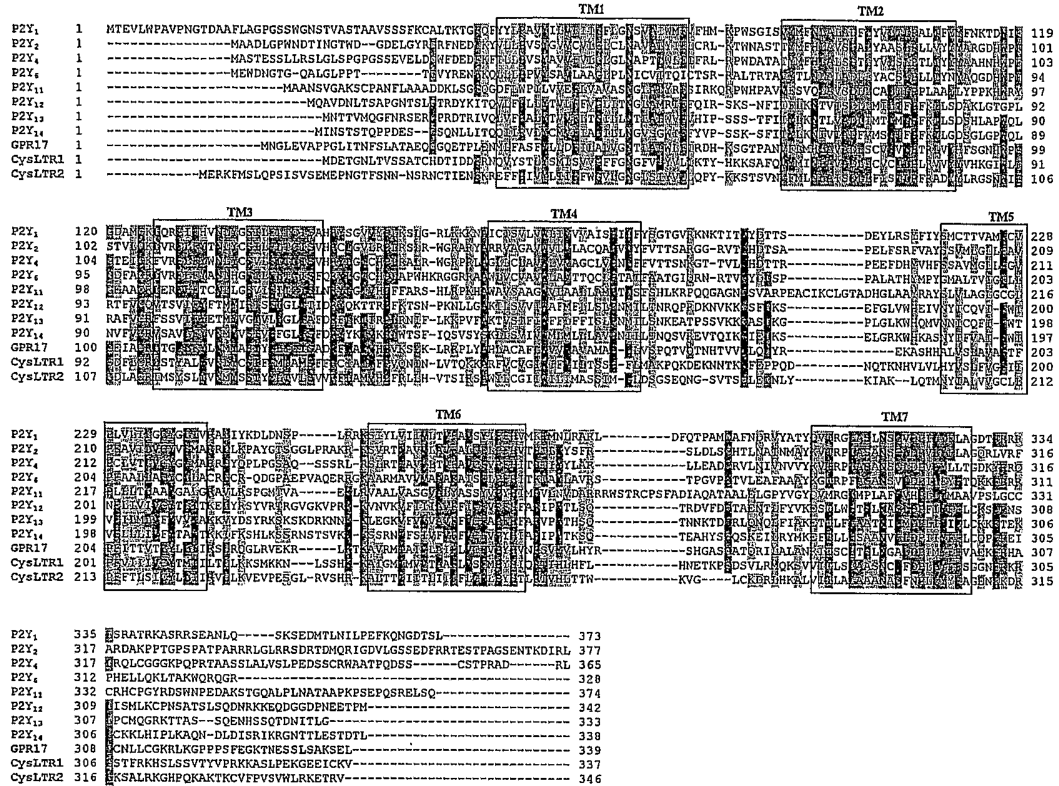


Figure 2

Figure 2.1

a



b

	P2Y$_1$	P2Y$_2$	P2Y$_4$	P2Y$_6$	P2Y$_{11}$	P2Y$_{12}$	P2Y$_{13}$	P2Y$_{14}$	CysLT1	CysLT2
GPR17	33	34	37	32	32	28	27	28	32	35

c

	P2Y$_1$	P2Y$_2$	P2Y$_4$	P2Y$_6$	P2Y$_{11}$	P2Y$_{12}$	P2Y$_{13}$	P2Y$_{14}$	CysLT1	CysLT2
GPR17	56	52	53	45	50	47	49	48	53	54

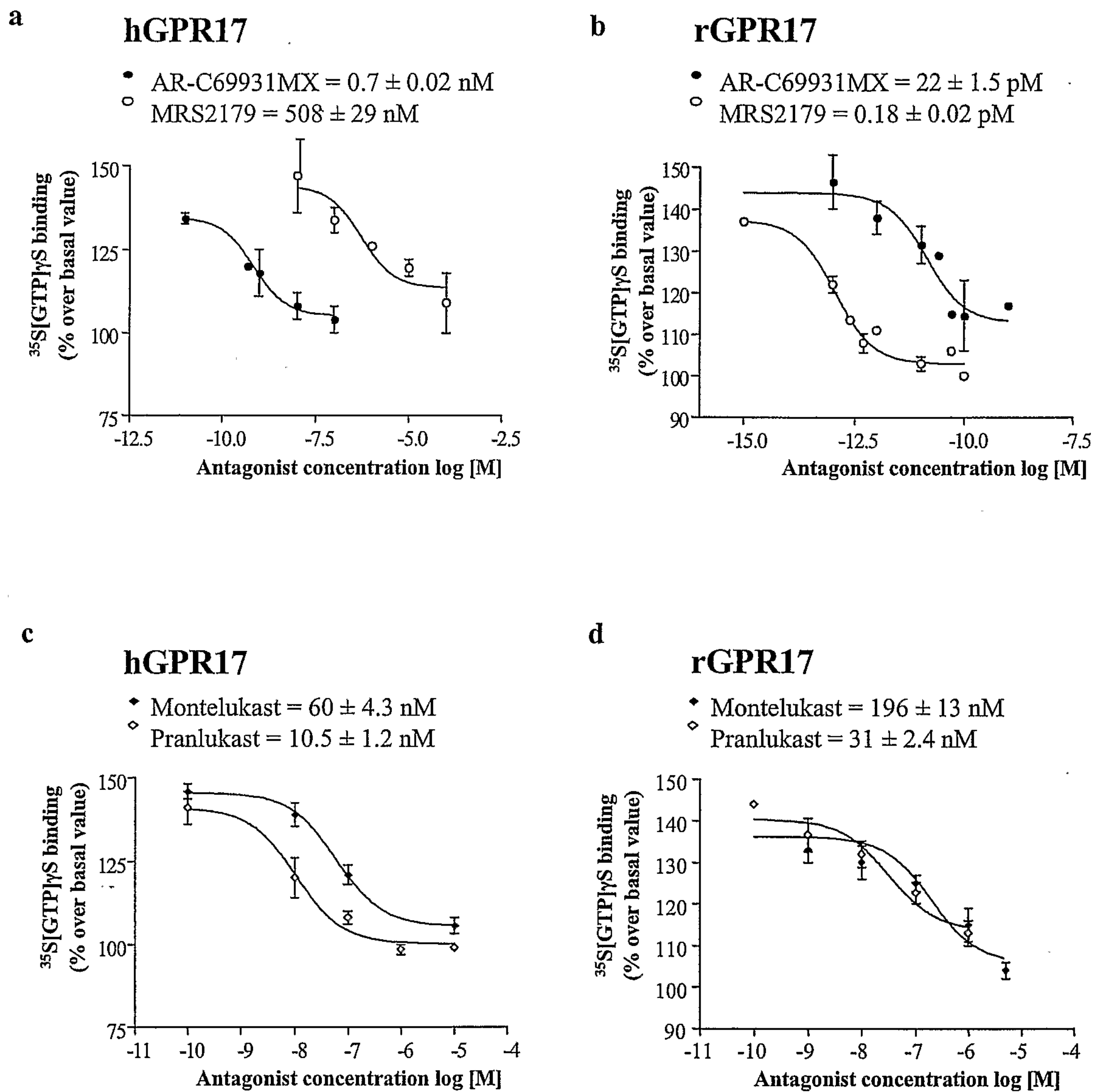
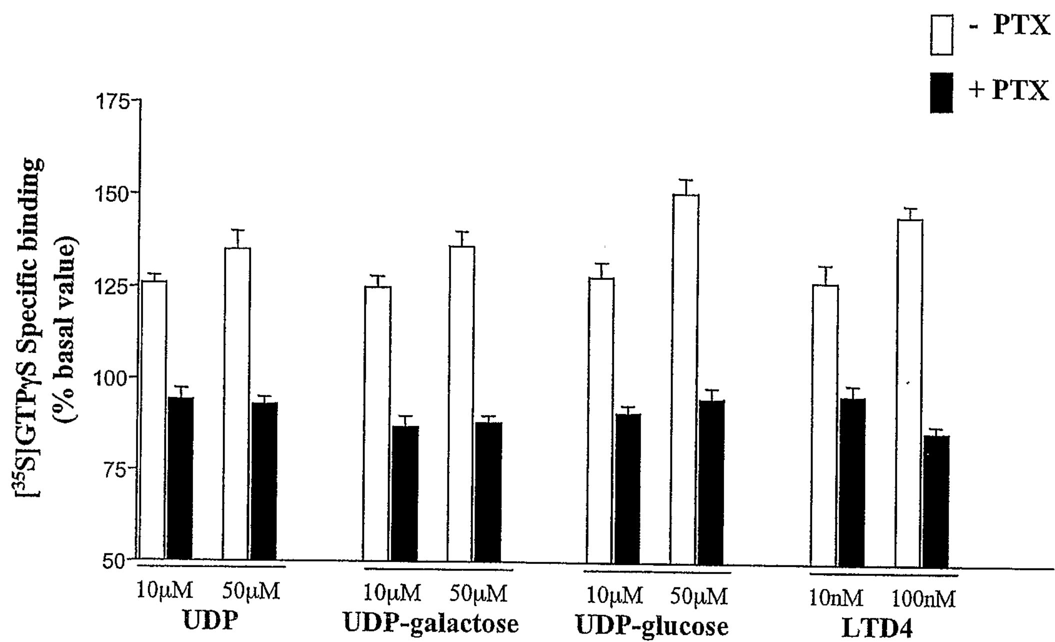


Figure 3

Figure 3.1



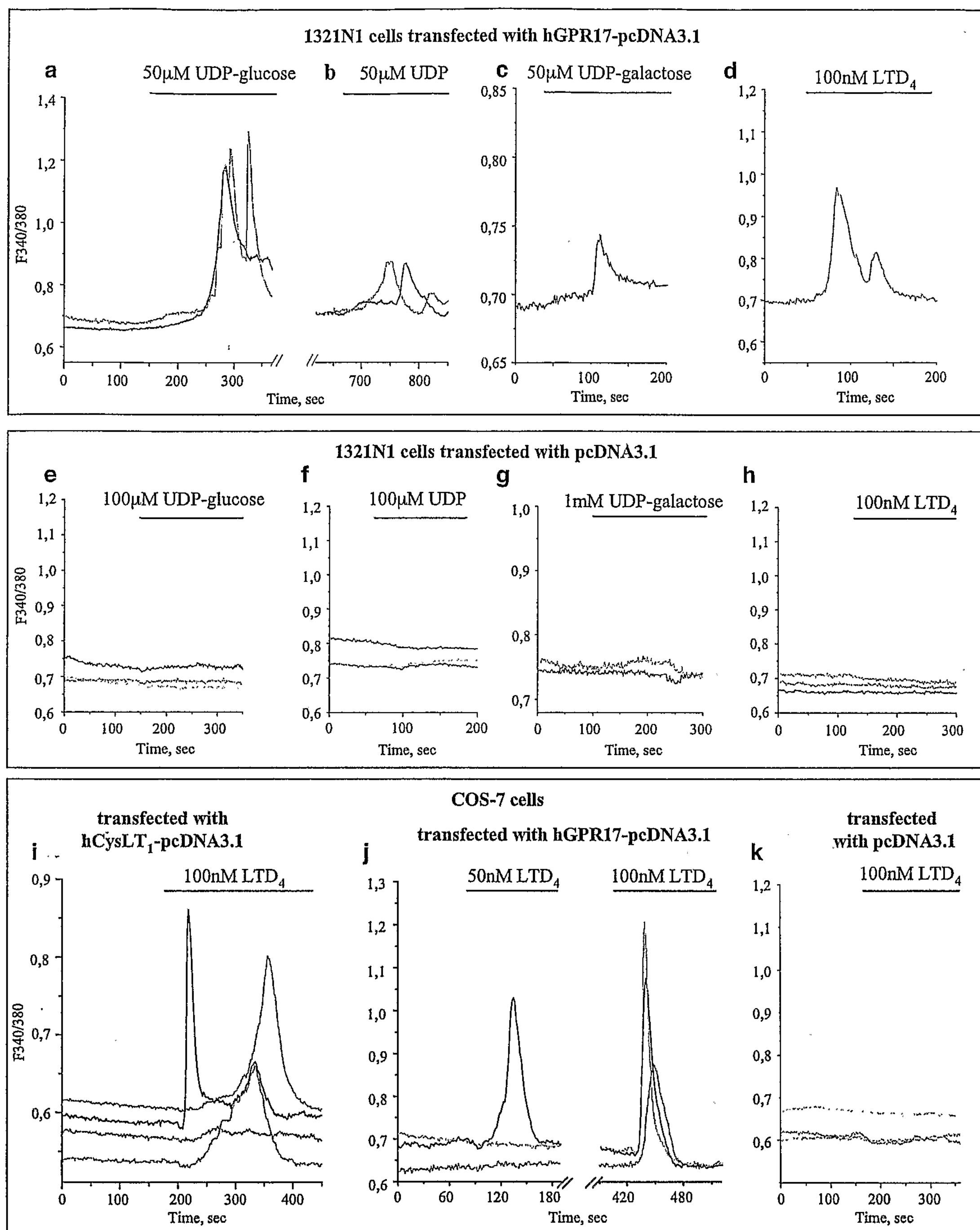
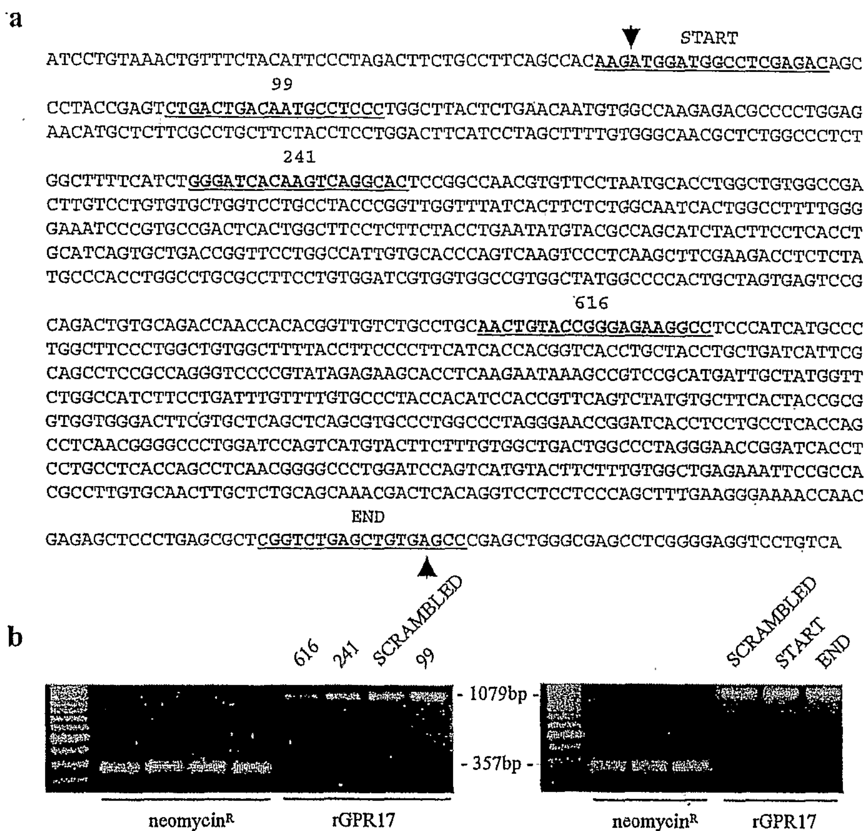


Figure 4

Figure 4.1



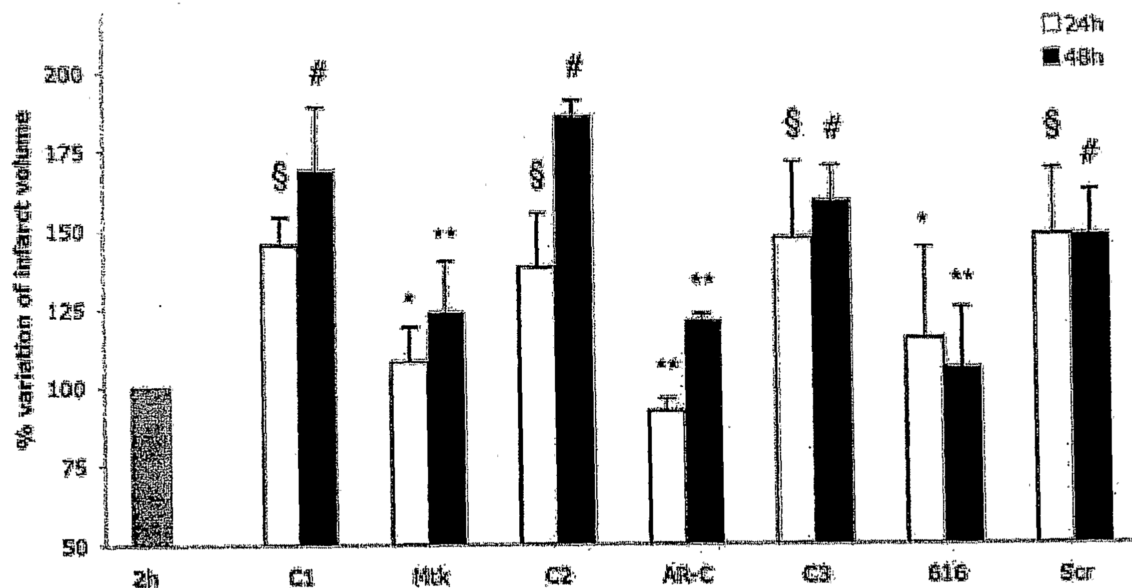


Figure 5

SEQUENCE LISTING

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