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(54) Title: SCREENING METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASES

(57) Abstract: The present invention relates to screening methods and pharmaceutical compositions for the treatment of inflammatory bowel diseases. More particularly, the present invention relates to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease comprising the steps consisting of (a) testing each of the test substances for its ability to restore the integrated stress response and (b) and positively selecting the test substances capable of restoring said integrated stress response. A further aspect of the invention relates to an agent capable of restoring the integrated stress response (ISR) for use in the treatment of an inflammatory bowel disease.

SCREENING METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASES

FIELD OF THE INVENTION:

The present invention relates to screening methods and pharmaceutical compositions for the treatment of inflammatory bowel diseases.

BACKGROUND OF THE INVENTION:

Ulcerative colitis (UC) is a chronic intermittent and relapsing inflammatory bowel disease (IBD) of the colon characterized by superficial mucosal lesions that extend through the rectum and progress upstream. The natural history of UC is characterized by the progression of colonic lesions in up to 50% of subjects. This suggests that the colonic mucosa has a "global" susceptibility to environmental factors.

Although genome-wide association studies have already identified more than 40 gene loci associated with UC, the primary defects initiating the cascade of events in colonic epithelial cells and leading to inflammation remain largely unknown. Recent data in animal models have shown that gut inflammation may be linked to an inability to manage the Unfolded Protein Response (UPR) in the epithelial barrier (Heazlewood, C.K. et al. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 5, e54 (2008). Kaser, A. et al. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 134, 743-56 (2008).; Brandl, K. et al. Enhanced sensitivity to DSS colitis caused by a hypomorphic Mbtps1 mutation disrupting the ATF6-driven unfolded protein response. Proc Natl Acad Sci U S A 106, 3300-5 (2009).; Van der Sluis, M. et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131, 117-29 (2006).; Zhao, F. et al. Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice. Dev Biol 338, 270-9 (2010).; Hammer, R.E., Maika, S.D., Richardson, J.A., Tang, J.P. & Taurog, J.D. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. Cell 63, 1099-112 (1990)). It has been suggested that the UPR might help intestinal epithelial cells cope with endoplasmic reticulum (ER) stress and thus deter pro-inflammatory pathways allowing the cell to adapt and respond to environmental changes. The UPR is mediated by i)- IRE1 that signals through

a transcription factor X-box protein 1 (XBP-1) to activate UPR target genes, ii)- ATF6α that induces *XBP-1* mRNA and enhances the ability of the cell to cope with the load of unfolded proteins, and iii)- PERK that phosphorylates the alpha subunit of translation initiation factor 2 (eIF2α) which abolishes the eIF2α-GTP-Met-$tRNA_i^{Met}$ ternary complex formation and inhibits translation of most mRNAs. This adaptation is believed to protect cells against toxic malfolded proteins that can accumulate under stress and to conserve ATP and amino acids in ER stressed cells. Paradoxically, eIF2α phosphorylation stimulates the translation of a subset of genes including ATF4, an inducer of the integrated stress response (ISR) and its downstream transcriptional target, *DDIT3* (*CHOP*).

Although deregulation of some ER stress markers has been partially explored in subjects with IBD (Heazlewood, C.K. et al. "Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis". PLoS Med 5, e54 (2008).; Kaser, A. et al. "XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease." Cell 134, 743-56 (2008). Shkoda, A. et al. "Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation." Gastroenterology 132, 190-207 (2007).), comprehensive biochemical and ultrastructural evidence of ER stress and the consequence of abnormal ER stress responses on mucosal barrier dysfunction are necessary to clarify the role of ER stress in the pathogenesis of UC. Another important question is whether alterations in ER stress could be a predisposing factor that precedes histological colitis in patients with UC. So far, the ER stress abnormalities as predisposing factor predating histological colitis in UC subjects have not yet been investigated.

SUMMARY OF THE INVENTION:

The present invention relates to screening methods and pharmaceutical compositions for the treatment of inflammatory bowel diseases.

More particularly, the present invention relates to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease comprising the steps consisting of (a) testing each of the test substances for its ability to restore the integrated stress response and (b) and positively selecting the test substances capable of restoring said integrated stress response.

A further aspect of the invention relates to an agent capable of restoring the integrated stress response (ISR) for use in the treatment of an inflammatory bowel disease.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors show that unaffected UC mucosa exhibit inappropriate ER stress compared to control subjects. The results show unexpected impairment of the ISR pathway due to a net decrease in eIF2α phosphorylation and thus suggest that the defect in this central stress response associated with the absence of stress granule assembly and increased expression of key components of the translational initiation machinery cause the reprogramming of protein translation in UC. This is supported by a genome-wide microarray analysis of polysome-bound mRNAs isolated from the unaffected mucosa of UC subjects *vs.* controls. These stress-modulated processes affect several key functions of the epithelial barrier which might explain the susceptibility of colonic UC mucosa to environmental stresses. Accordingly ISR represents an attractive target for new therapeutic options to maintain UC subjects in long-term remission.

Definitions:

As used herein the term "integrated stress response" or "ISR" has its general meaning in the art and refers to the pathway described in Schroder & Kaufman ER stress and unfolded protein response. Mut Res. 2005, 569: 29; Ron & Walter, "Signal integration in the endoplasmic reticulum unfolded protein responses", Nat Rev Mol Cell Biol 2007, 8: 519 and in Moenner et al., "Integrated stress response in cancer", Cancer Res. 2007, 67: 10631, The term 'integrated stress response" is also named as the "eIF2α pathway".

The phosphorylation event of eIF2α integrates various types of environmental and endogenous stress signals beyond ER stress, such as amino acid deprivation, exposure to double-stranded viral RNA, osmotic stress, UV light exposure, heme deficiency, hypoxia, and oxidative stress (Harding et al., (2003) ".An integrated stress response regulates amino acid metabolism and resistance to oxidative stress" Mol Cell, 11 (3), 619-33) These divergent signals activate four different eIF2α kinases including PERK (which is activated by ER stress, radiation, or hypoxia), general control nonderepressible-2 (GCN2, which is activated by uncharged tRNAs in amino acid-starved cells), heme-regulated inhibitor (HRI, which is activated by heme deficiency in erythroid precursor cells), and PKR (which is activated by double-stranded RNA and in some contexts, ER stress (Nakamura et al., (2010) "Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis",Cell, 143 (3), 338-48.

In response to various assaults, mammalian cells activate a protective mechanism to prevent damage of vital cellular processes required for homeostasis, once the stress is relieved (Nover et al., (1989), "Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs" Mol Cell Biol, 9 (3): 1298-308). The rapid formation of stress granules (SGs) in the cytoplasm is one of the main mechanisms by which the cell inhibits translation of mRNAs encoding for "housekeeping" functions to prioritize the synthesis of chaperones and enzymes needed for the stress response (Anderson and Kedersha, (2002) "Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation" Cell Stress Chaperones, 7 (2), 213-21). The process that inhibits translation during the ER stress, and which also acts as a stimulus for SG assembly, targets specifically the initiation phase of translation (Anderson and Kedersha, (2006) "RNA granules" J Cell Biol., 172 (6), 803-8). Indeed, it has been shown that arsenite (AS) - and heat shock-mediated SG formation induce the phosphorylation of eIF2, leading to a reduction in the cellular levels of eIF2•GTP•Met-tRNAMet ternary complexes, and a concomitant decrease in translation initiation rates. As a consequence, 40S ribosomes and some translation initiation factors are recruited to SGs. SG formation by mitochondrial poisons has been documented to occur in the absence of eIF2 phosphorylation (Kedersha et al., (2002) "Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules", Mol Biol Cell, 13 (1), 195-210). This suggests that inhibition of translation initiation by stimuli that do not induce eIF2α phosphorylation may also be capable of inducing SG formation.

As used herein the term "eIF2α" has its general meaning in the art and refers to the eukaryotic translation initiation factor 2A that is a 65-kD protein that catalyzes the formation of puromycin-sensitive 80S preinitiation complexes (Zoll WL et al. (2002). "Characterization of mammalian eIF2A and identification of the yeast homolog". J Biol Chem 277 (40): 37079–87..; Merrick WC (1992). "Mechanism and regulation of eukaryotic protein synthesis". Microbiol Rev 56 (2): 291–315).

As used herein the term "PERK" has its general meaning in the art and refers to the eukaryotic translation initiation factor 2-alpha kinase 3 (Shi Y, et al. (1998) "Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control". Mol Cell Biol. 18(12):7499-509).

As used herein the term 'GCN2" has its general meaning in the art and refers to the eukaryotic translation initiation factor 2 alpha kinase 4 (Berlanga JJet al. (1999) "Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2alpha kinase". Eur J Biochem.;265(2):754-62).

A used herein the term "ATF4" has its general meaning in the art and refers to the Activating transcription factor 4 (tax-responsive enhancer element B67) (Tsujimoto Aet al. (1991). "Isolation of cDNAs for DNA-binding proteins which specifically bind to a tax-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I". Journal of Virology 65 (3): 1420–6.. Karpinski BAet al. (1992). "Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element". Proceedings of the National Academy of Sciences of the United States of America 89 (11): 4820–4.)

As used herein, the term "GADD34" for "DNA damage-inducible protein 34" or MyD116 or PPP1R15A has its general meaning in the art and refers to a protein inhibitor 1 (I-1) interacting protein that associates with the C terminus of human I-1. GADD34, whose expression in mammalian cells is elevated by growth arrest, DNA damage, and other forms of cell stress has structural homology to a region of the herpes simplex virus (HSV-1) neurovirulence factor ICP-345, previously shown to bind PP1. GADD34 is an effector of a negative feedback loop that terminates UPR signaling and recruits a catalytic subunit of protein phosphatase 1 (PP1c) to dephosphorylate eIF2α.

As used herein, the term "protein phosphatase 1 (PP1)" denotes a major eukaryotic protein serine/threonine phosphatase that regulates an enormous variety of cellular functions through the interaction of its catalytic subunit (PP1c) with over fifty different established or putative regulatory subunits.

As used herein, the term "an inhibitor of the formation of the PP1/GADD34 complex" denotes an inhibitor able to compete in the µM range with GADD34 to form a complex with PP1 and thereby render said complex non functional, or to block GADD34 expression or to render GADD34 structurally inactive. In another term, "an inhibitor of the formation of the PP1/GADD34 complex" will have an EC50 not greater than 50µM and preferably not greater than 25µM.

As used herein the term "inflammatory bowel disease" has its general meaning in the art and refers to any inflammatory disease that affects the bowel. The term includes but is not limited to ulcerative colitis, Crohn's disease in a state that affect specifically the colon with or without ileitis, microscopic colitis (lymphocytic colitis and collagenous colitis), infectious colitis caused by bacteria or by virus, radiation colitis, ischemic colitis, pediatric colitis, undetermined colitis, and functional bowel disorders (described symptoms without evident anatomical abnormalities).

As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably, a subject according to the invention is a human.

Screening methods of the invention:

An aspect of the invention relates to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease comprising the steps consisting of (a) testing each of the test substances for its ability to restore the integrated stress response and (b) and positively selecting the test substances capable of restoring said integrated stress response.

In one embodiment step (a) of the screening method may consist in determining whether the test substances i) increase the phosphorylation of eIF2α, ii) activate the expression of ATF4 gene, ii) activate the expression of some genes targeted by ATF4 as depicted in Table 1, iii) activate the kinases that promote eIF2α phosphorylation, iv) inhibit the dephosphorylation of phosphorylated eIF2α.

RANKL (receptor activator of NF-kB)	Yang & Karsenty, 2004
E selectin	Liang & Hai, 1997;
VEGF	Roybal et al. 2005
Gadd153	Fawcett et al 1999
Asparagine synthase	Chen, & Kilberg, 2004

TRB3	Ohoka et al 2005
Nrf2 and HO1	Cullinan and Diehl 2006.

Table 1: ATF4 target genes (non exhaustive list)

Accordingly, the present invention is directed to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease, which comprises the steps of i) testing each of the test substances for its ability to activate the expression of ATF4 gene, and ii) identifying the test substance which activates the expression of ATF4, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the invention is directed to a method, which comprises the steps of i) contacting the test substance or each of the test substances with a cell transfected with a reporter gene operatively linked to all or part of the promoter of the ATF4 gene, ii) assessing the level of expression of said reporter gene, and iii) identifying the test substance which activates the expression of said reporter gene, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the reporter gene encodes one of the groups consisting of GFP, CAT, GAL, LUC, and GUS. In another embodiment, the cell is one of the groups consisting of a CHO, BHK, 3T3, and HEK293 cell line. In a particular embodiment, the invention is directed to a method, which comprises the steps of i) contacting the test substance or each of the test substances with a cell capable of expressing the ATF4 gene, ii) assessing the level of expression of said gene, and iii) identifying the test substance which activates the expression of said gene, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the level of expression is assessed by determining the level of transcription of said gene. In a further embodiment, the determination of the level of translation of said gene is effected by means of an immunoassay.

The invention is also directed to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease, which comprises the steps of i) testing each of the test substances for its ability to activate the expression of a gene depicted in Table 1, and ii) identifying the test substance which activates the expression of said gene, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the method comprises the steps of i) contacting the test substance or each of the test substances with a cell transfected with a

reporter gene operatively linked to all or part of the promoter of said gene depicted in Table 1, ii) assessing the level of expression of said reporter gene, and iii) identifying the test substance which activates the expression of said reporter gene, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the reporter gene encodes one of the groups consisting of GFP, CAT, GAL, LUC, and GUS. In another embodiment, the cell is one of the groups consisting of a CHO, BHK, 3T3, and HEK293 cell line.

Activation of expression of any gene can be assessed by determining either the level of transcription or the level of translation in the presence of the test substance in comparison with control assays performed in the absence of the test substance. Such assays are well known in the art and are depicted herein after. For example such assays may be performed on cells capable of expressing the gene (host cells).

The invention is also directed to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease, which comprises the steps of i) testing each of the test substances for its ability to increase phosphorylation of eIF2α, and ii) identifying the test substance which increases phosphorylation of eIF2α, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the invention is also directed to the method, which comprises the steps of i) contacting the test substance or each of the test substances with a cell capable of expressing eIF2α, ii) assessing the level of phosphorylation of eIF2α, and iii) identifying the test substance which increases the phosphorylation of eIF2α, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the assessment of the level of phosphorylation of eIF2α is effected by an immunoassay using an antibody that specifically recognizes the phosphorylated form of eIF2α. In another embodiment, the assessment of the level of phosphorylation of eIF2α, is effected by tracking the covalent binding of a radiolabeled phosphate group to eIF2 α,

The invention is also directed to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease, which comprises the steps of i) testing each of the test substances for its ability to inhibit the dephosphorylation of

eIF2α, and ii) identifying the test substance which inhibits the dephosphorylation of eIF2α, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the invention is directed to a method, which comprises the steps of i) contacting the test substance or each of the test substances with a cell-free composition containing GADD34 and PP1c proteins in the form of a purified complex and eIF2α, in a phosphorylated form, ii) assessing the level of phosphorylation of eIF2α, in comparison with the level of phosphorylation determined in the absence of test substances, in a cell-free composition containing GADD34 and PP1c proteins in the form of a purified complex and eIF2α, in a phosphorylated form, and iii) identifying the test substance which provides a higher level of phosphorylation of eIF2α, in comparison with the level of phosphorylation determined in the absence of test substance, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the assessment of the level of phosphorylation of eIF2α is effected by an immunoassay using an antibody that specifically recognizes the phosphorylated form of eIF2 α. In another embodiment, the assessment of the level of phosphorylation of eIF2α, is effected by tracking the covalent binding of a radiolabeled phosphate group to eIF2α,

The invention is also directed to a method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease, which comprises the steps of i) testing each of the test substances for its ability to activate an eIF2α,a kinase and ii) identifying the test substance which activates an eIF2α, kinase, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease. In one embodiment, the kinase is PERK. In one embodiment, the kinase is GCN2. In another embodiment, the kinase is HRI. In a further embodiment, the kinase is PKR.

Host Cells:

Abroad variety of host-expression vector systems may be utilized to express the genes used in the assays of this invention. These include, but are not limited to, mammalian cell systems such as human cell lines derived from colon adenocarcinoma including HT-29, Caco-2, SW480, HTC116 cell lines. The mammalian cell systems may harbour recombinant expression constructs containing promoters derived from the genome of mammalian cells or from mammalian viruses (e.g., the adenovirus late promoter or the vaccine virus 7.5K promoter).

Additional host-expression vector systems include, but are not limited to, microorganisms such as bacteria (e.g., *E. 5 coli* or *B. subtilis)* transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing PTK or adaptor protein coding sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the protein or peptide oding sequences; insect cell systems, such as Sf9 or Sf21 infected with recombinant virus expression vectors (e.g., baculovirus) containing the protein or peptide coding sequences; amphibian cells, such as Xenopus oocytes; or plant cell systems infected with recombinant virus express- 15 sion vectors (e.g., cauliflower mosaic virus, CMV; tobacco mosaic virus, TMV) or transformed with recombinant plamid expression vectors (e.g., Ti plasmid) containing the protein or peptide coding sequence. Culture conditions for each of these cell types is specific and is known to those familiar with the art.

DNA encoding proteins to be assayed can be transiently or stably expressed in the cell lines by several methods known in the art, such as, calcium phosphate-mediated, DEAE-dextran mediated, liposomal-mediated, viral-mediated, electroporation-mediated and microinjection delivery. Each of these methods may require optimization of assorted experimental parameters depending on the DNA, cell line, and the type of assay to be subsequently employed.

In addition native cell lines that naturally carry and express the nucleic acid sequences for the target protein may be used.

Methods for determining the expression of a gene:

Determination of the expression level of a gene can be performed by a variety of techniques. Generally, the expression level as determined is a relative expression level.

More preferably, the determination comprises contacting the sample with selective reagents such as probes, primers or ligands, and thereby detecting the presence, or measuring the amount, of polypeptide or nucleic acids of interest originally in the sample. Contacting may be performed in any suitable device, such as a plate, microtiter dish, test tube, well, glass, column, and so forth In specific embodiments, the contacting is performed on a substrate coated with the reagent, such as a nucleic acid array or a specific ligand array. The substrate may be a solid or semi-solid substrate such as any suitable support comprising glass, plastic, nylon, paper, metal, polymers and the like. The substrate may be of various forms and sizes, such as a slide, a membrane, a bead, a column, a gel, etc. The contacting may be made under any condition suitable for a detectable complex, such as a nucleic acid hybrid or an antibody-

antigen complex, to be formed between the reagent and the nucleic acids or polypeptides of the sample.

In a preferred embodiment, the expression level may be determined by determining the quantity of mRNA.

Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the subject) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis) and/or amplification (e.g., RT-PCR). Preferably quantitative or semi-quantitative RT-PCR is preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous.

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (e. g. avidin/biotin).

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are “specific” to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular

probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210)

Other methods for determining the expression level of said genes include the determination of the quantity of proteins encoded by said genes.

Such methods comprise contacting a biological sample with a binding partner capable of selectively interacting with a marker protein present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with an antibody against the protein to be tested. A biological sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate (s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Measure of Phosphorylation of eIF2α and the Activation of Kinases:

The levels of phosphorylation of proteins can be assessed by various methods, including immunoassays or radiolabeling. The levels of phosphorylation of target proteins can be assessed by various methods, including immunoassays or radiolabelling. Specifically, the increase of phosphorylation of eIF2α may be measured, activation of the kinases that promote eIF2α phosphorylation may be assayed, and inhibition of dephosphorylation of phosphorylated eIF2α may also be determined by these techniques.

In a particular embodiment, the level of phosphorylation of a protein is assessed by utilizing a binding partner, which should be highly specific for the target protein. It is preferred that the binding partner be an antibody. It is preferably generated against a unique epitope of the substrate. In an alternative, the binding partner should be specific for the phosphorylated form of the target protein. The detection procedure used to assess the phosphorylation state of eIF2α may for instance employ an anti-phosphoserine antibody or a peptide that recognizes and binds to phosphorylated serines. The detection antibody is preferably a polyclonal antibody to maximize the signal, but may also be specific monoclonal antibodies which have been optimized for signal generation.

In one example, levels of eIF2α phosphorylated on serine 51 (in yeast eIF2α, corresponding to residue 52 in rodents or humans) can be measured by immunoblot or immunocytochemistry utilizing a commercially available antibodies, for example, product #9721 from Cell Signalling Technology. In one embodiment, the commercially available antisera to phosphorylated eIF2α may be used to develop high throughput screening assays for test substances that promote the accumulation of phosphorylated eIF2α.

In another example, inhibition of dephosphorylation of eIF2α on serine 51 (in yeast eIF2α, corresponding to residue 52 in rodents or humans) may be assayed by screening a test substance's ability to inhibit the activity of the PPlc and GADD34 complex (Novoa, I., et al. (2001). "Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α". J. Cell. Biol. 28;153(5):1011-22). The PPlc and GADD34 complex is active in vitro, and its activity may be reconstituted using recombinant proteins. A cell-free assay may be used with the PPlc/GADD34 complex in combination with phosphorylated eIF2α and test substances. By utilizing an ELISA assay, dephosphorylation of eIF2α by the PPlc/GADD34 complex and inhibition of this dephosphorylation by a test substance, may be monitored by measuring the decrease in phosphorylated eIF2α signal.

In a further example, activation of the eIF2α kinases, PERK, GCN2, HRI, and PKR, may be measured. Activation of the kinases is associated with an autophosphorylation event on known residues in the kinase (e.g., threonine 898 of mouse GCN2 and threonine 980 of mouse PERK). By using antisera, which recognize the phosphorylated and activated forms of the kinases, activation of the kinases may be detected using immunoblot or immunochemistry, such as with an ELISA. Antisera for the phosphorylated forms of the kinases PERK and GCN2 have been developed. (Harding, H., et al. (2000). "Regulated translation initiation controls stress induced gene expression in mammalian cells". Mol. Cell 6, 1099-1108).

Alternatively, immunoassays may be replaced by the detection of radiolabeled phosphate according to a standard technique. This involves incubating cells with the test substances and radiolabeled phosphate, lysing the cells, separating cellular protein components of the lysate using as SDS-polyacrylamide gel (SDS-PAGE) technique, in either one or two dimensions, and detecting the presence of phosphorylated proteins by exposing X-ray film.

The phosphorylation of a protein may also be conveniently detected by migration on an electrophoresis gel and Western blot, to thereby observe whether a shift of the molecular

weight of the protein occurs, a phosphorylated protein being heavier than the corresponding non-phosphorylated form.

Test substances:

According to a one embodiment of the invention, the test substance of may be selected from the group consisting of peptides, peptidomimetics, small organic molecules, antibodies, aptamers or nucleic acids. For example the test substance according to the invention may be selected from a library of compounds previously synthesized, or a library of compounds for which the structure is determined in a database, or from a library of compounds that have been synthesized de novo.

In a particular embodiment, the test substance may be selected form small organic molecules.

As used herein, the term "small organic molecule" refers to a molecule of size comparable to those organic molecules generally sued in pharmaceuticals. The term excludes biological macromolecules (e.g.; proteins, nucleic acids, etc.); preferred small organic molecules range in size up to 2000 Da, and most preferably up to about 1000 Da.

High throughput screening methods

The above assays may be performed using high throughput screening techniques for identifying test substances for developing drugs that may be useful to the treatment or prevention of an inflammatory bowel disease. High throughput screening techniques may be carried out using multi-well plates (e.g., 96-, 389-, or 1536-well plates), in order to carry out multiple assays using an automated robotic system. Thus, large libraries of test substances may be assayed in a highly efficient manner.

A preferred strategy for identifying test substances starts with cultured cells transfected with a reporter gene fused to the promoter of any gene that is activated by the stress response pathway. More particularly, stably-transfected HT-29 cells growing in wells of micro-titer plates (96 well or 384 well) can be adapted to high through-put screening of libraries of compounds. Compounds in the library will be applied one at a time in an automated fashion to the wells of the microtitre dishes containing the transgenic cells described above.

Once the test substances which activate one of the target genes are identified, it is preferable to then determine their site of action in the Integrated Stress Response pathway. It is particularly useful to define the site of action for the development of more refined assays

for in order to optimize the target substance. Assays to determine the site of action of the target substance in the ISR may be carried out using high throughput techniques.

In one example, the ELISA-based assay for measuring ATF4 translation is particularly adapted to rapid high throughput screening. Similarly, an ELISA assay for measuring phosphorylated eIF2α, by means of commercially available antiserum, may be developed for high throughput screening. Alternatively, antisera to phorphorylated eIF2α kinases may be advantageously used in ELISA-based high throughput screens to focus on upstream components of the pathway.

ELISA-type assays may be performed in microtitre plates. See, for example, Peraldi et al., (1992), J. Biochem. 285: 71-78; Schraag, et al., (1993), Analytical Biochemistry 211: 233-239; Cleavland, (1990), Analytical Biochemistry 190: 249-253; Farley, (1992), Analytical Biochemistry 203: 151-157; and Lczaro, (1991), Analytical Biochemistry 192: 257-261. For evaluating the effects of a test substance on phosphorylation within the normal cellular context, one can also used the rapid and quantitative assays systems described in U.S. Pat. No. 5,763,198. For example, two embodiments may be contemplated as follows.

The extent of phosphorylation of a target protein may be measured by exposing cells that express the target protein to a test substance and, thereafter, lysing the cell to release the cellular contents. The target protein is isolated by incubating the cell lysate with a binding partner to a solid support and thereafter washing away non-bound cellular components. A detection procedure is performed to assess the presence or absence of phosphorylated residues on the protein as compared to lysates of control cells, which were not exposed to the test substance. Alternatively, the binding partner may be directed against the phosphorylated forms of the target protein, so that the steps of isolation and of detection of phosphorylation are performed simultaneously.

These assays offer several advantages. The exposure of the test substance to a whole cell allows for the evaluation of its activity in the natural context in which the test substance may act. In addition, radioactive labeling of the target cell proteins is not required in the assay. Because this assay can readily be performed in a microtitre plate format, the assays described can be performed by an automated robotic system, allowing for testing of large numbers of test samples within a reasonably short time frame.

An alternative embodiment of the invention relates to methods for determining the effect of a test substance on the ability of kinases to phosphorylate eIF2α in a cell-free system. To assess modulation of enzyme activity, the test substance is added to a reaction

mixture containing the kinase and eIF2α bound to a solid support by an antibody. The kinase reaction may be initiated by the addition of ATP. A detection procedure as described herein is performed on the substance to assess the presence or absence of the phosphorylated residues, and results are compared to those obtained for controls, i.e., reaction mixtures to which the test substance was not added.

The assays of the invention can be used as a screen to assess the activity of a previously untested compound or extract, in which case a single concentration is tested and compared to controls. These assays can also be used to assess the relative potency of a compound by testing a range of concentrations, in a range of 100 μM to 1 μM, for example, and computing the concentration at which the amount of phosphorylation is increased by one-half (IC50) compared to controls.

The whole cell assay of the invention described herein can be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay, such as a solid phase coated with a binding partner to a protein of interest, or a detection molecule. The cell-free assays of the invention may be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay.

Therapeutic methods of the invention:

A further aspect of the invention relates to an agent capable of restoring the integrated stress response (ISR) for use in the treatment of an inflammatory bowel disease.

In one embodiment, said agent is able to i) increase the phosphorylation of eIF2α, ii) activate the expression of ATF4 gene, ii) activate the expression of some genes targeted by ATF4 as depicted in Table 1, iii) activate the kinases that promote eIF2α phosphorylation, iv) inhibit the dephosphorylation of phosphorylated eIF2α and v) promote stress granule formation.

A further aspect of the invention relates to an agent capable of restoring the integrated stress response (ISR) for use in maintaining subjects affected by an inflammatory bowel disease in a long term remission after standard treatment or surgery. Currently standard treatment of the inflammatory bowel diseases, especially ulcerative colitis include administration of corticosteroids, immunosuppressive drugs, aminosalicylates sulfasalazine,

such as Mesalazine (also known as 5-aminosalicylic acid, mesalamine, or 5-ASA. Brand name formulations include Apriso, Asacol, Pentasa, Mezavant, Lialda, Fivasa, Rovasa and Salofalk.), Sulfasalazine (also known as Azulfidine), Balsalazide (also known as Colazal or Colazide (UK)), Olsalazine (also known as Dipentum), immunosuppressors (azathioprine, 6-mercaptopurine, methotrexate, rapamycine, cyclosporine and tacrolimus) or biological treatments such as Infliximab, Visilizumab, Adalimumab, or Vedolizumab.

In a particular embodiment, the agent that inhibits the dephosphorylation of phosphorylated eIF2α is salubrinal (3-phenyl-N-[2,2,2-trichloro-1-[[(8-quinolinylamino)thioxomethyl]amino]ethyl]-2-propenamide) (Boyce et al (2005) "A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress". Science 307 935. Long et al (2005) "Structure-activity relationship studies of salubrinal lead to its active biotinylated derivative". Bioorg.Med.Chem.Lett. 15 3849). Salubrinal is a cell-permeable, selective inhibitor of cellular phosphatase complexes that dephosphorylate eIF2α. Salubrinal is available from Alexis Biochemicals or Tocris Bioscience (Cat No. 2347), or other source as known to one of skill in the art.

In another particular embodiment, the agent that inhibits the dephosphorylation of phosphorylated eIF2α is guanabenz (2-(2,6-dichlorobenzylidene) hydrazinecarboximidamide). Guanabenz is a small-molecule which bound to a regulatory subunit of protein phosphatase 1, PPP1R15A/GADD34, selectively disrupting the stressinducedstress induced dephosphorylation of the α subunit of translation initiation factor 2 (eIF2α) (Tsaytler P, Harding HP, Ron D, Bertolotti "A.Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis.". Science. 2011 Apr 1;332(6025):91-4)

In another embodiment, the agent that inhibits the dephosphorylation of phosphorylated eIF2 is an inhibitor of GADD34 or PP1 gene expression.

Inhibitors of expression for use in the present invention may be based on anti-sense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of GADD34 or PP1 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of GADD34 or PP1, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding GADD34 or PP1 can be synthesized, e.g.,

by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of expression for use in the present invention. GADD34 or PP1 gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that GADD34 or PP1 gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836). All or part of the phosphodiester bonds of the siRNAs of the invention are advantageously protected. This protection is generally implemented via the chemical route using methods that are known by art. The phosphodiester bonds can be protected, for example, by a thiol or amine functional group or by a phenyl group. The 5'- and/or 3'- ends of the siRNAs of the invention are also advantageously protected, for example, using the technique described above for protecting the phosphodiester bonds. The siRNA sequences advantageously comprise at least twelve contiguous dinucleotides or their derivatives.

As used herein, the term “siRNA derivatives” with respect to the present nucleic acid sequences refers to a nucleic acid having a percentage of identity of at least 90% with erythropoietin or fragment thereof, preferably of at least 95%, as an example of at least 98%, and more preferably of at least 98%.

As used herein, “percentage of identity” between two nucleic acid sequences, means the percentage of identical nucleic acid, between the two sequences to be compared, obtained with the best alignment of said sequences, this percentage being purely statistical and the differences between these two sequences being randomly spread over the nucleic acid acids sequences. As used herein, “best alignment” or “optimal alignment”, means the alignment for which the determined percentage of identity (see below) is the highest. Sequences comparison between two nucleic acids sequences are usually realized by comparing these sequences that have been previously align according to the best alignment; this comparison is realized on segments of comparison in order to identify and compared the local regions of similarity. The best sequences alignment to perform comparison can be realized, beside by a manual way, by

using the global homology algorithm developed by Smith and Waterman (Ad. App. Math., vol.2, p:482, 1981), by using the local homology algorithm developped by Neddleman and Wunsch (J. Mol. Biol., vol.48, p:443, 1970), by using the method of similarities developed by Pearson and Lipman (Proc. Natl. Acd. Sci. USA, vol.85, p:2444, 1988), by using computer softwares using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple alignment algorithms (Edgar, Robert C., Nucleic Acids Research, vol. 32, p:1792, 2004). To get the best local alignment, one can preferably used BLAST software. The identity percentage between two sequences of nucleic acids is determined by comparing these two sequences optimally aligned, the nucleic acids sequences being able to comprise additions or deletions in respect to the reference sequence in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical position between these two sequences, and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

shRNAs (short hairpin RNAs) can also function as inhibitors of expression for use in the present invention.

Ribozymes can also function as inhibitors of expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of GADD34 or PP1 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable.

Both antisense oligonucleotides and ribozymes useful as inhibitors of expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA

molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides, siRNAs, shRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid to the cells and preferably cells expressing GADD34 or PP1. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging

cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

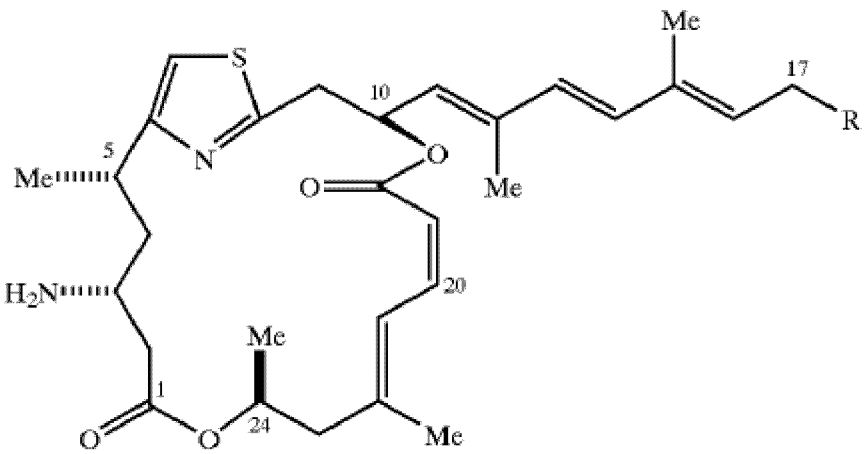
Preferred viruses for certain applications are the adenoviruses and adeno-associated (AAV) viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. Actually 12 different AAV serotypes (AAV1 to 12) are known, each with different tissue tropisms (Wu, Z Mol Ther 2006; 14:316-27). Recombinant AAV are derived from the dependent parvovirus AAV2 (Choi, VW J Virol 2005; 79:6801-07). The adeno-associated virus type 1 to 12 can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species (Wu, Z Mol Ther 2006; 14:316-27). It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA

delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

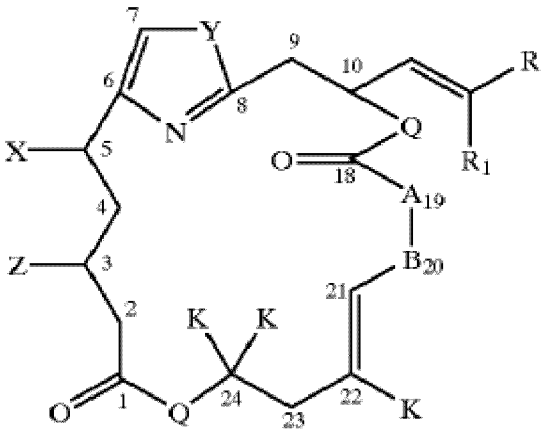
In a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter.. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

In a particular embodiment, the agent that promotes stress granule formation is pateamine A. Pateamine was first isolated from the marine sponge Mycale found off the shores of New Zealand. Northcote, P. T. et al, Tetrahedron Lett., 32:6411-6414 (1991). The natural form bears a thiazole and an E,Z-dienoate within a 19-membered macrocycle and a trienylamine side chain. Two additional pateamines, pateamines B and C, were also isolated. Their structures differ from pateamine A only in the nature of the terminal group of the trienylamine side chain. The structure for all three isolated natural forms is shown below:



Pateamine A: R = NMe_2
B: R = NHMe
C: R = $N(O)Me_2$

In another embodiment, the agent that promotes stress granule formation is a derivative of pateamine A which as the general formula:



=wherein

A-B is ethane, (E) and (Z)-ethene, (E) and (Z)-substituted ethene, ethyne,

K is hydrogen or C1-C3 alkyl,

Q is NH or O,

X is hydrogen, hydroxy, alkoxy, alkyl, aminocarbonyl, amino, alkylamino, dialkylamino, alkoxycarbonylamino,

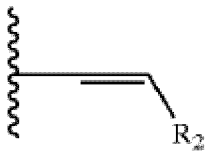
Y is S, NH, or O,

Z is hydrogen, hydroxy, aminocarbonyl, alkylamino, dialkylamino, alkoxycarbonylamino, but not t-butoxycarbonylamino when R4 is dimethylamino,

R1 is hydrogen or C1-C3 alkyl, and

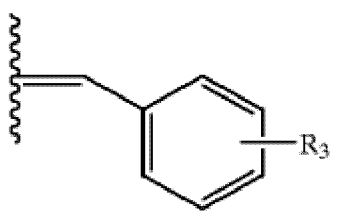
R is selected from the following:

(a) Alkene of the formula:



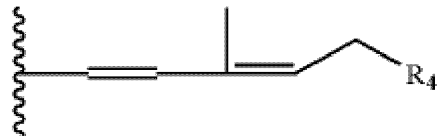
wherein R2 is optionally substituted with one or more substituents selected from alkyl, alkylhydroxy, alkylalkoxy, alkylamino, alkylaminoalkyl, or alkylaminodialkyl;

(b) Alkenylaryl of the formula:



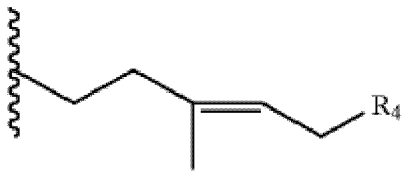
wherein R3 is optionally substituted with one or more substituents selected from hydrogen, alkyl, alkyenyl, ankynyl, hydroxy, alkoxy, amino, alkylamino, dialkylamino, trifluromethane, or fluoro; and

(c) Methyldienylpentyl of the formula:



wherein R4 is optionally substituted with one or more substituents selected from hydrogen, alkyl, alkyenyl, alkynyl, hydroxy, alkoxy, amino, alkylamino, or dialkylamino; and

(d) Methylalkenylpentyl of the formula:



wherein R4 is optionally substituted with one or more substituents selected from hydrogen, alkyl, alkyenyl, alkynyl, hydroxy, alkoxy, amino, alkylamino, or dialkylamino.

and its pharmaceutically accepted salts.

Another object of the invention relates to a method for treating and/or preventing inflammatory bowel disease comprising administering a subject in need thereof with an agent capable of restoring the integrated stress response (ISR), as above described.

The agent capable of restoring the integrated stress response (ISR) may be administered in the form of a pharmaceutical composition, as defined below.

Preferably, said inhibitor is administered in a therapeutically effective amount.

By a "therapeutically effective amount" is meant a sufficient amount of the agent capable of restoring the integrated stress response (ISR) to treat and/or to prevent an inflammatory bowel disease at a reasonable benefit/risk ratio applicable to any medical treatment.

It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed,

the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Another object of the invention relates to a method for treating an inflammatory bowel disease comprising:

i) a first step consisting of administering a subject in need thereof with a standard treatment selected from the group consisting of corticosteroids, immunosuppressive drugs, aminosalicylates sulfasalazine, such as Mesalazine (also known as 5-aminosalicylic acid, mesalamine, or 5-ASA. Brand name formulations include Apriso, Asacol, Pentasa, Mezavant, Lialda, Fivasa, Rovasa and Salofalk.), Sulfasalazine (also known as Azulfidine), Balsalazide (also known as Colazal or Colazide (UK)), Olsalazine (also known as Dipentum), immunosuppressors (azathioprine, 6-mercaptopurine, methotrexate, rapamycine, cyclosporine and tacrolimus) or biological treatments such as Infliximab, Visilizumab, Adalimumab, or Vedolizumab or a combination thereof

ii) second step consisting of administering said subject in need thereof with an agent capable of restoring the integrated stress response (ISR) for maintaining said subject in a long term remission after the standard treatment of step i).

In one embodiment, step i) and ii) are performed concomitantly or preferably step ii) is performed sequentially after step i).

The agent capable of restoring the integrated stress response (ISR) may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms (suppository and enemas).

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The agent capable of restoring the integrated stress response (ISR) of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or

such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl

solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The agent capable of restoring the integrated stress response (ISR) of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules ; and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE 1: UNBALANCED ENDOPLASMIC RETICULUM STRESS REPROGRAMS TRANSLATION IN THE INACTIVE COLON OF PATIENTS WITH ULCERATIVE COLITIS

Material & Methods:

Patients and Biopsy Specimens.

All patients included in this study were followed in the Department of Gastroenterology (Beaujon's hospital). The protocol was approved by the local Ethics Committee and written informed consent for this study was obtained from all patients before enrollment. Colonic pinch biopsies were obtained during endoscopic investigations in 11 patients with UC. Surgical colon samples were collected from 15 patients with UC who underwent a colectomy. The diagnosis of UC was based on Lennard-Jones criteria and clinical disease activity was assessed according to the Colitis Activity Index as previously described[12]. Non-inflamed areas of colonic tissue were punctured endoscopically in each UC patient (5 to 10 biopsies/patients). Surgical samples were processed following the same protocol. Two biopsies were set apart from each patient for histopathological grading. The

control group included 20 subjects who underwent total colonoscopy (n=9) for colon polyp screening, or colectomy for colorectal cancer (n=11). Biopsies (5 to 10) were taken from the right and left colon of each healthy control subject and processed as above. Endoscopic findings were normal in all subjects and biopsy specimens were systematically diagnosed as nonsignificant abnormalities with HE staining. Surgical samples were taken from healthy colon mucosa at least 10 cm from the cancer site, and processed as above.

Histopathological Analyses

Biopsies were routinely stained with HE. Histological assessment of mucosal damage and inflammatory cell infiltrate were graded by the same expert pathologist using a previously validated score to characterize colonic involvement of IBD[12].

Immunohistochemistry and Immunofluorescence Staining.

Immunohistological methods were performed on serial 4 µm deparaffinised sections from control and unaffected UC mucosa. After endogenous peroxidase removal, sections were incubated with specific antibodies. For immunofluorescence studies, cryoslide sections from control and unaffected UC mucosa were incubated with anti-TIA-1 and anti-eIF3 antibodies, and then labeled with secondary antibodies. Nuclei were stained using DAPI or TO-PRO-3 iodide. Fluorescence was detected by confocal laser scanning microscopy (CLSM-510-META, Zeiss). All images were acquired by using the Zeiss LSM Image Browser software. Quantification of stress granules was determined by counting the number of epithelial cells per millimeter of colonic epithelium that contained 5 or more stress granules using Image Pro software (Stress granule Index). At least three microscopic fields were counted on every control (n=10) and UC patient (n=15).

Real-Time PCR.

Total RNA was extracted with RNAble® and quantified using an ND-1000 NanoDrop spectrophotometer. Purity/integrity was assessed with disposable Agilent RNA chips and an Agilent 2100 Bioanalyzer. Primer sequences are reported in Supplemental Informations. mRNA levels were determined by qPCR using a LightCycler 480 instrument.

Western Blot.

Colon biopsies were homogenized as previously described[13]. Equal amounts of total protein (50-75 µg) were then subjected to SDS-PAGE, transferred using iBlotGel Transfer

Device (Invitrogen), and probed with primary antibodies (see Supplemental Informations). Labeled protein bands were scanned with an HP Scanjet 5500, and the relative protein content was determined by densitometric analysis.

Electron Microscopy.

Normal colon biopsies from control (n=8) and UC patients (n=8) were extemporaneously fixed in 3% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated with ethanol and embedded in Epon. Ultrathin sections stained with lead citrate were examined on a Jeol 1010 EM. ER area was calculated by determining the relative surface density as previously reported[14].

Isolation of Polysomes and RNA.

Three independent pools of normal colon biopsies from 6 controls (n=18) and 6 UC patients (n=18) were separately processed for polysome extraction. Samples were lysed and nuclei were removed by centrifugation (3,000g, 5 min, at 4°C). The supernatant was layered onto 10 ml linear sucrose gradients (10 to 50% sucrose) and centrifuged in a SW41Ti rotor for 120 min at 35,000g, at 4°C. The absorbance at 254 nm was measured. RNA was recovered from the individual fraction by phenol/chloroform extraction and isopropanol precipitation. The quality of RNA was determined with an Agilent 2100 Bioanalyzer, as well as in a control gradient in which pooled colonic tissue from 2 UC patients and 2 healthy subjects were lysed in the presence of 25 mM EDTA (pH 8.0). The resulting polysome profile demonstrated a complete shift in mRNAs from the polysome-bound fraction towards lighter sucrose fractions (data not shown) verifying proper identification of polysomal peaks.

Whole Genome Microarray Analysis.

After polysome fractionation and RNA isolation, RNAs from polysomes (fractions 11-22) were pooled and purified. RNA samples were amplified and Cy3 labeled following the manufacturer's protocol. The hybridization procedure was performed by Tebu-Bio according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit and Agilent Whole Human Genome Oligo Microarrays 4x44K which covers more than 41,000 genes and transcripts. Normalized data were serially filtered in the following order: eliminate genes flagged as absent in all groups, select genes up- or downregulated by at least two fold with $P<0.05$ (unaffected UC mucosa vs. control, Student t-test with Benjamini and Hochberg false discovery rate as multiple testing correction).

Statistical analysis

Statistical significance of differences was determined using the non-parametric Mann-Whitney test. All statistical analyses were performed using SPSS software (v15.0; SPSS Inc, Chicago). A P value <.0.05 was considered statistically significant.

Results

Unaffected colonic UC mucosa exhibit extended IRE1β and ATF6α branch signaling

UPR activation was assessed by determining IRE1β-mediated splicing of *XPB-1* in unaffected colonic mucosa from UC and healthy individuals. Spliced *XBP-1* (XBP-1s) mRNA levels were significantly increased in UC mucosa and the ratio of spliced to unspliced *XBP-1* (XBP-1s/XBP-1u) was 1.8 and 3.8 in controls and UC, respectively. Consistent with hyperactivation of the IRE1β/XBP-1 arm, increased expression of the UPR-related target genes *GRP94*, *GRP78*, and *EDEM1* was observed in UC patients compared to controls. Immunoblotting showed increased GRP78 and GRP94 protein expression in unaffected UC mucosa (Figure 1B). In addition to splicing *XBP-1* mRNA, IRE1 can also activate the c-Jun-N-terminal kinases (JNK) which regulate apoptosis and/or proinflammatory gene expression through the TRAF2/MAP3K cascade[15]. Interestingly, TRAF2 protein expression and phosphorylation of both JNK and its downstream target c-jun were increased in unaffected UC mucosa compared to controls. Although it has been suggested that the activation of JNK plays a role in apoptosis, unaffected UC mucosa did not display increased apoptosis as demonstrated by the active caspase 3 and Bax and Bcl-2 expression levels. On the contrary, unaffected UC mucosa exhibited a regenerative response with a 27% increase in Ki67 positive cells detected in 81% of UC patients (9 out of 11) and increased transcription of pro-inflammatory immune mediators.

ATF6α functions as a proximal inducer of the UPR as p50ATF6α, the active bZIP transcription factor converted from the latent p90ATF6α, binds ER stress-responsive elements of genes including *GRP78*, *GRP94* and *XBP-1u*[9]. The generation of p50ATF6α was reproducible in UC patients and was associated with enhanced *XBP-1u* mRNA levels compared to controls.

To determine whether ER stress mainly affected the colonic epithelium of UC patients, immunohistological detection of GRP78 and transmission electron microscopy experiments were conducted on unaffected UC and control mucosa. The GRP78 expression level was increased in the epithelial cells of UC patients compared to controls demonstrating that exacerbated ER stress essentially resides in the colonic epithelial cells of UC mucosa. Electron microscopy analyses identified distinctive ultrastructural changes in both apparently uninvolved (endoscopically and histologically) areas of the colon and in inactive UC with gross distortion of ER morphology in goblet cells. Semi-quantitative measurements of the ER surface area revealed a significant 3-fold increase in ER membrane surface areas in unaffected UC mucosa compared to control mucosa.

Aberrant attenuation of the eIF2α-dependent ISR in unaffected colonic UC mucosa

We then monitored phosphorylation of eIF2α (Ser51) in unaffected colonic UC and control mucosa. Lysates from controls showed marked phosphorylation of eIF2α which reflected physiological ER stress in the colon of healthy subjects. In contrast, no significant modulation of phospho-eIF2α was found associated with enhanced expression of total eIF2α protein levels in UC patients (5-8 fold, $P<0.0001$ *vs.* control). Densitometric quantification showed an even more significant decrease in the ratio of phospho-eIF2α/eIF2α in UC *vs.* controls. Interestingly, this defective phosphorylation of eIF2α was observed in histologically unaffected colonic mucosa of both active and inactive UC patients. Consistent with detectable eIF2α phosphorylation in controls, we observed an associated induction of *ATF4* and *CHOP* mRNA levels correlated with a measurable expression level of ATF4 and CHOP proteins. In contrast, neither *ATF4* and *CHOP* transcripts nor ATF4 and CHOP protein levels were induced in UC mucosa.

The PERK/eIF2α pathway is subject to negative regulation at numerous levels: *PPP1R15a* (GADD34), a stress-induced gene encoding a regulatory subunit of the protein phosphatase 1, and the CReP (PPP1R15b/PP1c/Nck complex) promote eIF2α dephosphorylation and restore translation[16], whereas $p58^{IPK}$ is thought to inhibit multiple eIF2α kinases including PERK[17]. No significant difference in $p58^{IPK}$ mRNA expression level was detected in unaffected UC (n=10) or normal (n=10) mucosa suggesting that down-regulation of eIF2α phosphorylation was not directly associated with changes in PERK activity. Decreased expression and/or phosphorylation of PERK might contribute to the effect

on eIF2α phosphorylation in UC mucosa. However we have been beset to technical difficulties to detect the phosphorylated and total forms of PERK in UC and control biopsies. Nevertheless, the significant increase in GADD34 protein levels found in UC mucosa might explain in part, the low-level of phospho-eIF2α expression.

The number of stress granules is reduced in unaffected UC mucosa

Stress-induced translational arrest is an important protective cell mechanism for reprogramming gene expression during stress. Formation of stress granules (SGs) is central to this response. SGs are specialized cytoplasmic foci which regulate mRNA translation and form by both eIF2α phosphorylation-dependent[18] and -independent mechanisms[19]. SGs contain stalled mRNA bound to small 40S ribosomal subunits, aggregation-prone ribonucleoproteins (*e.g.* TIA-1 and TIAR), and different translational initiation factors[18]. Cryoslide sections of unaffected UC and control colonic mucosa were immunostained for TIA-1 and eIF3, two components of SGs. Colocalization of TIA-1 and eIF3 was observed in abundant punctuate foci in control colonic epithelial cells. In contrast, a marked loss of SGs was observed in unaffected UC mucosa. Only 10% of epithelial cells from each UC tissue section contained at least 5 SGs compared to over 45% of control mucosal cells. The reduction in the number of SGs was not due to a decrease in TIA-1 or eIF3 protein expression levels since Western blot analysis showed increased expression of these two proteins in unaffected UC mucosa. To the best of our knowledge, this is one of the first studies to show that SGs are present throughout the colonic epithelium of healthy subjects during physiological ER stress and that SG loss is a general feature in unaffected UC colonic mucosa. Because SGs play diverse and essential roles in cell homeostasis by regulating cap-dependent translation initiation, apoptosis, metabolic signaling pathways and inflammatory responses, the inability of unaffected colonic UC mucosa to trigger SG formation could be expected to increase the sensitivity of the epithelial barrier to environmental stresses.

Altered ER stress response entails post-transcriptional reprogramming of mRNA translation in unaffected UC mucosa

Translation is tightly regulated under different stress conditions, mainly at the level of initiation. Besides eIF2α, a key player in translation initiation is eIF4E which is the limiting component of the eIF4F initiation complex. This complex contains two other subunits: eIF4A (an ATP-dependent helicase) and eIF4G (a large scaffolding protein), which associate with

eIF4E and play a crucial role in the eIF2α phosphorylation-independent mechanisms of SG formation[19]. eIF4E expression level was ~35% higher in unaffected UC mucosa than in controls. In addition to its level of expression, availability of eIF4E for translation initiation is controlled by the phosphorylation status of 4E-BP1[20]. The level of hyperphosphorylated 4E-BP1 (Ser65) was found to be increased by about threefold while the level of total 4E-BP1 was not affected in unaffected UC mucosa compared to controls. In addition, eIF4E phosphorylation (Ser209) which stimulates translation efficiency[21] and eIF4A and eIF4G expression levels were significantly increased in unaffected UC mucosa suggesting that eIF4F formation and unwinding of RNA secondary structures are optimized in unaffected UC mucosa.

These findings further support that translation initiation may be altered in unaffected UC mucosa. Hence, we hypothesized that deregulated ER stress may induce post-transcriptional reprogramming of mRNA translation to reconfigure the proteome in unaffected UC mucosa.

Microarray of polysomal RNA and patterns of gene expression in unaffected UC mucosa

The translational status of mRNAs bound to polysomes was assessed in unaffected UC and normal mucosa by velocity sedimentation on sucrose gradients. The results showed significant differences in polysome-bound RNA expression profiles between UC and controls with more than a twofold change (up- or downregulated with $P<0.05$) in 2,582 genes/probe sets (Supplementary Table 2). To identify purely translationally regulated gene candidates, we excluded genes whose concomitant increased/decreased transcriptional expression had already been identified in transcriptomic analyses of total mRNA of non-inflamed UC biopsies[22, 23]. Gene products were grouped into functional categories and molecular functions according to the Gene Ontology (http://www.geneontology.org), GeneCards (http://www.genecards.org/) and GeneNote (http://bioinfo2.weizmann.ac.il/cgibin/genenote) data bases. A subset of translationally regulated genes known to be involved in the pathogenesis of experimental colitis and UC, as well as selected genes that may play a crucial role in cell proliferation, ER stress, immune response and colorectal cancer are represented in Table 1. These include genes coding for ER stress response (ATF4, ATF6, ERO1), translation (eIF2alpha, eIF5A), mucins (MUC2, MUC4, MUC12, MUC20), cell-cell adhesion (ZO1, keratins, claudins), immune response and antibacterial defenses (TLR4, TLR6, IFR5, interleukins), detoxification and antioxidative stress (TST/CES2, SOD1, SOD2), and wound

repair and cell cycle (cdc42, $p21^{WAF/cip}$, HMGB1) indicating that our microarray analysis provided an accurate representation of gene expression in unaffected UC mucosa and revealed new altered biological functions in UC.

To demonstrate that the differences in the recruitment of mRNAs to polysomes between UC and controls result in similar modifications in protein expression levels, six candidate genes identified by microarray analysis with a potential direct or indirect relationship to the pathogenesis of UC were analyzed in an independent cohort of subjects. These included CDKN1A ($p21^{WAF/Cip}$) which is involved in the pathogenesis of UC and UC-dependent carcinogenesis[24], eIF5A which promotes translation elongation, polysome disassembly, SG assembly, and inflammation[25], RanBP2 which functions as the small ubiquitin-related E3 ligase of TCF-4 enhancing the Wnt signaling pathway[26], superoxide dismutase 1 (SOD1) which plays a major role in antioxidant stress defenses [27], the polymeric immunoglobulin receptor (PIgR) which is responsible for transepithelial transport of IgA and IgM in the gut[28], and the thiosulfate sulfurtransferase (TST/CES2), a sulfide-detoxifying enzyme whose deregulation could be essential in the cell loss and inflammation that accompanies UC and colorectal cancer[29]. In accordance with their respective polysome-bound mRNA expression, $p21^{WAF/Cip}$, eIF5A, SOD1, PIgR, and TST/CES2 protein expression levels were significantly decreased while RanBP2 protein levels were increased in unaffected UC *vs.* control mucosa. Protein level changes in PIgR (membrane-associated protein) and TST/CES2 (mitochondrion-associated protein) were confirmed by immunohistochemistry (Figure 5B).

Taken together, these findings point out that unbalanced ER stress and associated reprogramming of mRNA translation in unaffected colonic UC mucosa may induce alterations in epithelial barrier's homeostasis facilitating pathological mucosal responses to environmental signals.

***In silico* characterization of translationally downregulated RNA recognized by a restricted subset of microRNAs up-regulated in unaffected UC mucosa**

It is interesting to note that 45.5% (1173 genes) of the 2,582 genes whose expression was altered in microarray analysis, were translationally repressed in unaffected UC mucosa. We hypothesized that these repressed genes might be partially post-transcriptionally silenced by microRNAs (miRNAs). This hypothesis was based on our recent study[12] identifying 8 miRNAs (miR-15a, miR-26a, miR-29a, miR-29b, miR-30c, miR-185, miR-196a, miR-324-3p) whose expression is strongly up-regulated in unaffected UC mucosa *vs.* control mucosa. We therefore searched for miRNA target sequences among the translationally repressed genes

in unaffected UC mucosa using the PITA catalog (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). This computational analysis showed that 25.4% of repressed genes (298/1173 genes) could be recognized by one or more selected miRNAs in unaffected UC mucosa. This suggests that compensatory miRNA-mediated translational repression might explain some of the translationally repressed genes.

Discussion:

There are three original findings in this study. First the coordinated expression of all three branches of the UPR identified in controls is impaired in unaffected UC mucosa; second unaffected UC mucosa are prone to ER stress due to impairment of the ISR and third the coordinated changes in eIF2α phosphorylation, SG formation, and translation initiation selectivity identified in unaffected UC mucosa represent a novel level of regulation in the pathogenesis of UC.

This study shows that the three known UPR transducers are coordinately activated in the colonic mucosa of controls in association with SG formation. This suggests that an integrated UPR strategy exists in normal mucosa which provides a dynamic adaptive response to bacterial burden and environmental stresses to maintain colonic homeostasis and immune vigilance/tolerance.

Our study identified a defective ISR pathway in unaffected UC mucosa leading to reduced ATF4 and CHOP expression and increased chaperone expression. The ISR is a pivotal system of translational regulation coupling diverse stressful conditions with a common downstream event (eIF2α phosphorylation). This suggests that i)- defects in eIF2α phosphorylation impair resistance of UC mucosa to the toxic effects of a large panel of stresses and ii)- inappropriate ER stress renders unaffected UC mucosa highly susceptible to pathological changes in the microenvironment. Thus, ISR repression may be an *in vivo* signature for the susceptibility of unaffected UC mucosa to inflammation. In this context, the association between ER stress and atherosusceptible regions has been revealed by impaired PERK/eIF2α activation in swine demonstrating that deficient eIF2α phosphorylation is linked to spatial susceptibility of the endothelium to atherosclerosis[30]. Moreover, TLR4 which activates IRE1α/XBP-1 signaling cascade for cytokine production in macrophages[31] suppresses ATF4/CHOP expression in response to LPS[32] showing the close link between bacterial signals and ER stress. Of all the environmental risk factors suspected to play a key role in UC, only tobacco plays a protective role. Interestingly, Hengstermann and Müller[33]

showed that cigarette smoke induces an ER stress response in 3T3 cells by activating the PERK/eIF2α axis suggesting that specific activation of the PERK pathway might explain the protective role of tobacco in UC.

Our results suggest that the onset of colonic inflammation in UC depends on the presence of epithelial cells which are compromised in the ability to dynamically remodel eIF2α phosphorylation status and to maintain a more plastic remodeling of mRNA translation. The hypothesis of reprogrammed translation initiation is reinforced by the significant reduction in SGs together with increased proliferation in unaffected colonic UC mucosa. Combined with the increased expression of chaperone proteins this suggests that unaffected UC mucosa has an abnormal ER stress-related pro-survival profile that could explain increased risk of colorectal neoplasia associated with UC. Because there is not enough material for proteomic analysis in human colon biopsies[34], we performed comparative pangenomic microarray analysis from polysome-bound mRNAs. Numerous deregulated genes encode for proteins that have already been associated with the pathogenesis of IBD and others indicate new targets for understanding the pathogenesis of UC. For example the expression of PIgR is reduced in epithelial cells of unaffected UC mucosa implying that impaired anti-bacterial protection may be triggered by reduced transport of secreted IgA into the gut lumen. Anti-oxydative stress protection also appears to be defective in UC as SOD1 and SOD2 and several redox enzymes were strongly downregulated. Decreased TST/CES2 expression in unaffected UC mucosa may increase epithelial cell exposure to the toxic effects of H_2S and lead to or worsen UC, as well as promote colorectal carcinogenesis[29]. The downregulation of critical cytoprotective proteins through the reprogramming of mRNA translation in UC could impair epithelial homeostasis. Thus we identified a large pool of repressed polysomal mRNAs that might be specifically targeted by the subset of 8 miRNAs that share regulated overexpression in unaffected UC mucosa[12]. This could be the first step towards identifying the regulatory networks that control translational repression whose dysregulation could be involved in the pathophysiology of UC.

These results show that patients who are considered to be in clinical, endoscopic and histological remission have persistent cellular and molecular damage to the colon suggesting that existing treatments could be optimized. Thus, our results suggest new perspectives for the treatment of UC to achieve a potential cure and/or more profound remission. Examples include Salubrinal, a phospho-eIF2α dephosphorylation inhibitor[35], and Pateamine A, an immunosuppressive and antiproliferative agent that represses translation and induces SG formation through eIF4A inhibition[19].

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EXAMPLE 2: SALUBRINAL EFFECT IN VIVO

Ulcerative colitis (UC) is characterized by exclusive colonic involvement with superficial mucosal lesions associated with depletion in goblet cells and decreased secretion of mucins in inflammatory colonic mucosa[11]. Although it has been proposed that the epithelium of UC patients is diffusely abnormal irrespective to inflammation[12], early alterations predating inflammation within colonic epithelial cell remain elusive. It is now evident that impairment of proper ERS resolution by altered unfolded protein response (UPR)

in epithelial cells can lead or sensitize to colonic inflammation both in animal[2-8] and human studies[1,2]. However, the consequences of ERS alterations during UC remain misunderstood. The UPR is a carefully orchestrated process involving three proximal sensors PERK, ATF6, and IRE1 that allow cells to cope with a wide variety of stressful conditions. The combined action of these sensors restores cell homeostasis by cessation of protein translation, increase of chaperones production, and degradation of the burden of aberrant proteins. Sustained or abnormal ERS adversely affects normal cell function leading to inflammation and/or apoptosis[13,14].

The relationship between goblet cells, ERS, and inflammation is unclear although goblet cells and mucus barrier have been linked to inflammation. However, knockout of the mucin gene *Muc2* in mice is not sufficient to cause colitis since inflammation appears to arise only on a permissive genetic background[15,16] and patients with UC express MUC2. Furthermore, partial or total depletion in the number of goblet cells[17-20] and therefore in mucus and antibacterial products is insufficient to induce colitis. Finally, accumulation of missense mutated Muc2[ref.7] or HLA-27 protein[9] which is prone to misfolding in the ER, or knockout of the protein disulfide isomerase Agr2[ref.5] induce exaggerated ERS in secretory cells and subsequent inflammation. Thus, the predisposition to colitis might reside in goblet cells themselves and in their inability to manage ERS in the absence of immune dysfunction and in the setting of a normal colonic flora.

To explore the puzzling way by which goblet cells are affected by ERS in UC, we artificially increased the number of goblet cells in conditions of ERS. We crossed Nox1-deficient mice, which exhibit fine deregulation of colonic progenitor cells leading to increased goblet cell expression[10], and IL-10^{KO} mice, which express deregulated ERS in epithelial intestinal cells[8] and develop enterocolitis depending on both genetic background and environmental factors[21]. The relevance of this IL-10/Nox1^{dKO} model relies on the human colonic epithelial cell expression of both IL-10[22] and NOX1[23,24]. Interestingly, NOX1 expression follows the same colonic gradient than goblet cells[25] and UC lesions. Moreover, genome-wide association study demonstrate significant association of the small GTPase *Rac1*[ref.26] (a partner of NOX1[ref.27]) and *IL-10* genes[28] with UC. Here we showed that IL-10 and NOX1 expression levels were markedly altered in uninflamed colonic mucosa from patients with UC (n=12) *versus* healthy controls (n =12).

All SPF-reared C57Bl/6-IL10/Nox1^{dKO} mice developed spontaneously colitis at 6/7 weeks and disease activity index (DAI) scores which became more severe with age, including diarrhea, high incidence of rectal bleeding, change in body and colon weights, and prolapses.

None of WT and single-KO mice developed colitis during the time frame studied. Histopathologic scores revealed that IL10/Nox1^{dKO} mice developed more severe colitis along the proximal-distal axis and exhibited signs of UC without any signs of ileitis including polymorphonuclear infiltrates, crypt abscesses, edema, focal epithelial erosion, and crypt loss. We next measured epithelial permeability of FITC-dextran in segments of distal colon and indigenous bacterial translocation was identified in the spleen of 7 and 12-week-old mice. Consistent with the colitis state, only IL10/Nox1^{dKO} mice demonstrated an increased permeability in the colon and exhibited splenomegaly which was closely correlated with increased Gram-negative commensal bacteria translocation. Interestingly, IL10/Nox1^{dKO} mice showed the main complications of UC such as colitis-associated colorectal cancer and spontaneously primary sclerosing cholangitis at 7/8 months of age. By contrast, IL10^{KO} mice showed mild enterocolitis at low frequency (<20% at 34 weeks), without showing any signs of cholangitis or colorectal cancer (at >8 months of age; this study and[29]). Interestingly, here we report the comprehensive genome-wide screen of 561 microRNAs of colonic epithelial mucosa of Nox1^{KO}, IL10^{KO}, and IL10/Nox1^{dKO} mice (6- and 16-wk-old) *versus* WT mice. Consistent with previous findings in patients with UC[30,31], IL10/Nox1^{dKO} mice expressed almost 50% of microRNAs relevant in defining UC signature.

To analyze cytokine responses at the site of inflammation, colon samples were collected and various cytokines were analyzed at both mRNA and protein levels. IL10/Nox1^{dKO} mice showed increased expression levels of pro-inflammatory cytokines mainly involved in UC. Lymphoid and myeloid cell population analysis in the spleen did not differ between the four genotypes. By contrast, a massive infiltration of FoxP3^{+} T_{reg} was only observed in the colonic tissue in spite of active mucosal inflammation and at lesser extent in the spleen of IL10/Nox1^{dKO} mice consistent with findings in UC[32]. To determine whether the genotype of hematopoietic lineages affected the extent of colitis, we generated bone marrow (BM) chimeric mice in which recipients and donors were WT (CD45.1) and WT, IL10^{KO}, and IL10/Nox1^{dKO} mice (CD45.2), respectively. Interestingly, reconstitution of irradiated WT mice with IL10^{KO} or IL10/Nox1^{dKO} BM was insufficient to cause disease demonstrating that colitis is chiefly inherent to epithelial cells rather than hematopoietic lineages in IL10/Nox1^{dKO} mice.

The colonic epithelium of IL10/Nox1^{dKO} mice showed a paucity of Alcian Blue/PAS positive mucins associated with loss of goblet cells at the ulcerated sites. Accordingly, Muc2 and Muc4 protein expression levels were dramatically low in the inflamed colonic areas of IL10/Nox1^{dKO} mice. Rarefaction and aberrant morphology of goblet cells with few and

immature thecae associated with small amount of mucus and swollen, round mitochondria were similarly observed in the colon of both IL10/Nox1^{dKO} mice and patients with UC.

Colonic section of IL10/Nox1^{dKO} mice displayed an increase in the number of PCNA- and phospho-histone 3-positive cells suggesting increased epithelial proliferation. Scanning electron microscopy (SEM) showed a ~30% increase in crypt length in IL10/Nox1^{dKO} mice. Interestingly, SEM displayed a wide spectrum of identical ultrastructural alterations of the mucosa both in IL10/Nox1^{dKO} mice and in unaffected colonic mucosa of patients with UC including crypt distortion, visible crypt openings disposed in rows, edematous glandular borders, and dilatation of the gland lumen. Notwithstanding increased colonic proliferation, staining and quantitative assessment of apoptotic cells indicated that decreased expression of goblet cells in IL10/Nox1^{dKO} mice was due to increased apoptosis in the colon.

To assess the pathogenic role of goblet cells in UC, WT and Nox1^{KO} mice were subjected to oral administration of DSS or rectal administration of TNBS. No significant differences in DAI and histological damages of colonic mucosa were seen between the two mouse models suggesting that chemically-induced inflammation is likely independent of increased expression of goblet cells. By contrast, tunicamycin treatment, a canonical inducer of ERS, significantly induced a more severe colitis in mice overexpressing goblet cells than in WT mice indicating that goblet cell itself may be a direct participant in the development of colitis as a consequence of ERS. Accordingly, IL10/Nox1^{dKO} mice exhibited ERS disturbances in the colonic mucosa prior inflammatory damages as previously described in patients with UC[1]. IRE1beta and ATF6alpha branch signaling were extended in colonic epithelial cells as evidenced by the increased *XBP-1* mRNA splicing, the induction of GRP78, GRP94, PDI at both mRNA and protein levels, and dilated cisternae and gross distortion of the ER in goblet cells. Interestingly, identical defective phosphorylation of eIF2α correlated with low expression of ATF4 was observed both in unaffected colonic mucosa of IL10/Nox1^{dKO} mice and patients with UC[1]. Consistent with reduced eIF2α phosphorylation, increased expression of PPP1R15A/GADD34, a stress-inducible protein that recruits the catalytic subunit of protein phosphatase 1 and promotes eIF2alpha dephosphorylation, was detected in agreement with our previous data in humans[1]. EIF2alpha phosphorylation is cytoprotective during ERS, because cells are sensitized to cell death when this pathway is genetically ablated[33] and protected when it is ectopically enforced[34]. To test whether a selective pharmacological inhibitor of GADD34-mediated eIF2alpha dephosphorylation may alleviate colitis, IL10/Nox1^{dKO} mice were treated with 1 mg/kg salubrinal[35] for up to three weeks. We showed that salubrinal strongly reduced histological colitis score throughout the

colon, markedly prevented immune cell infiltration, and restored intact mucosal architecture with normal goblet cells. Salubrinal caused robust eIF2alpha phosphorylation and protected colonic mucosa against apoptosis at least in part for its anti-apoptotic activity on CHOP expression. Furthermore, there was a trend toward reduced Grp78/Bip and Grp94 expression in salubrinal-treated mice demonstrating that salubrinal engages the translational control arm of the UPR by inducing eIF2alpha phosphorylation and acts like a proteostasis regulator by lowering protein folding in stressed cells. Interestingly, we demonstrated that salubrinal-induced phosphorylation of eIF2alpha was mainly detected in colonic epithelial cells. Finally, levels of proinflammatory cytokines and amount of colonic and splenic T_{reg} cells were strongly decreased to baseline in salubrinal-treated IL10/Nox1^{dKO} mice.

Our findings strengthen that defective eIF2alpha phosphorylation is a major player in UC and may open new therapeutic avenues. Current treatments of UC cannot change the natural course of the disease. These difficulties to manage UC may be explained by the use of immunomodulators which are mainly designed to modulate the activity of immune cells and are hardly efficient to repair early epithelial abnormalities. Thus, eIF2α modulators could define a new class of drugs specifically based on the intimate mechanisms of UC which might likely shift the paradigm for UC treatment from immunomulators to epitheliomodulators.

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REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS:

1. A method for screening a plurality of test substances useful for the prevention or treatment of an inflammatory bowel disease comprising the steps consisting of (a) testing each of the test substances for its ability to restore the integrated stress response and (b) and positively selecting the test substances capable of restoring said integrated stress response.

2. The method according to claim 1 wherein step (a) of the screening method may consist in determining whether the test substances i) increase the phosphorylation of eIF2α, ii) activate the expression of ATF4 gene, ii) activate the expression of some genes targeted by ATF4 as depicted in Table 1, iii) activate the kinases that promote eIF2α phosphorylation, or iv) inhibit the dephosphorylation of phosphorylated eIF2α.

3. The method according to claim 2 wherein said method comprises the steps of i) testing each of the test substances for its ability to increase phosphorylation of eIF2α, and ii) identifying the test substance which increases phosphorylation of eIF2α, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease.

4. The method according to claim 2 wherein said method comprises the steps of i) testing each of the test substances for its ability to inhibit the dephosphorylation of eIF2α, and ii) identifying the test substance which inhibits the dephosphorylation of eIF2α, thereby to identify a test substance useful as a preventive or therapeutic agent for an inflammatory bowel disease.

5. A method for the treatment of an inflammatory bowel disease in a subject in need thereof or for maintaining a subject affected by an inflammatory bowel disease in a long term remission after standard treatment or surgery comprising administering the subject with an agent capable of restoring the integrated stress response (ISR) for use in the treatment of or for use in

6. The method according to claim 5 wherein the agent is able to i) increase the phosphorylation of eIF2α, ii) activate the expression of ATF4 gene, ii) activate the expression of some genes targeted by ATF4 as depicted in Table 1, iii) activate the

kinases that promote eIF2α phosphorylation, iv) inhibit the dephosphorylation of phosphorylated eIF2α and v) promote stress granule formation.

7. The method according to claim 6 wherein the agent that inhibits the dephosphorylation of phosphorylated eIF2 is salubrinal or guanabenz.

8. The method according to claim 6 wherein the agent is an inhibitor of GADD34 or PP1 gene expression.

9. The method according to claim 6 wherein the agent is pateamine A.

10. A pharmaceutical composition comprising an agent according to any of claims 5 to 9 for use in the treatment of an inflammatory bowel disease or for use in maintaining subjects affected by an inflammatory bowel disease in a long term remission after standard treatment or surgery.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056799

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/101224 A2 (UNIV LELAND STANFORD JUNIOR [US]; KOONG ALBERT C [US]; FELDMAN DOUGLAS) 7 September 2007 (2007-09-07)	1,5,6,10
Y	the whole document, in particular the claims	1-10

Y	KASER A ET AL: "Endoplasmic reticulum stress in the intestinal epithelium and inflammatory bowel disease", SEMINARS IN IMMUNOLOGY, W.B. SAUNDERS COMPANY, PA, US, vol. 21, no. 3, 1 June 2009 (2009-06-01), pages 156-163, XP026140246, ISSN: 1044-5323, DOI: 10.1016/J.SMIM.2009.01.001 [retrieved on 2009-02-23] the whole document	1-10

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2012	02/07/2012

Name and mailing address of the ISA/	Authorized officer
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	KASER ARTHUR ET AL: "Endoplasmic reticulum stress: implications for inflammatory bowel disease pathogenesis.", CURRENT OPINION IN GASTROENTEROLOGY JUL 2010 LNKD- PUBMED:20495455, vol. 26, no. 4, July 2010 (2010-07), pages 318-326, XP009151322, ISSN: 1531-7056 the whole document	1-10
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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