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(71) Applicant (for all designated States except US): **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101, rue de Tolbiac, F-75013 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PLAISANCE, Pascale** [FR/FR]; 12 ter rue Guilloud (allée C), F-69003 Lyon (FR). **BADO, André** [FR/FR]; 29 allée d'Augusta, F-95490 Vaureal (FR). **TSOCAS, Annick** [FR/FR]; 48 rue de Vouillé, F-75015 Paris (FR). **DUCROC, Robert** [FR/FR]; 10 rue Achille Luchaire, Hall 8, F-75014 Paris (FR).

(74) Agents: **LE COUPANEC, Pascale** et al.; Nony & Associés, 3, rue de Penthièvre, F-75008 Paris (FR).

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(54) Title: USE OF LEPTIN FOR THE TREATMENT AND/OR PROPHYLAXIS OF DISEASE ASSOCIATED WITH A MUCUS SECRETION DEFECT

(57) Abstract: A use of leptin, an analog, a prodrug or a derivative thereof for the manufacture of a medicament intended for the treatment and/or the prophylaxis of a disease associated with a mucous secretion defect.



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Use of leptin for the treatment and/or prophylaxis of disease associated with a
mucus secretion defect.

The invention is related to the use of leptin, analogs, prodrugs or derivatives thereof, for the manufacture of a medicament intended for the treatment and/or the
5 prophylaxis of diseases associated with a mucous secretion defect.

The invention is also related to the use of leptin, analogs, prodrugs or derivatives thereof, for the manufacture of a medicament intended for the treatment and/or the prophylaxis of bowel diseases.

The invention is also related to the use of leptin, analogs, prodrugs or
10 derivatives thereof, for the manufacture of a medicament intended for increasing and/or preventing the decrease of mucous secretion within gastro-intestinal tract, and/or various other parts of the body, such as the lungs.

Many of the organs that open to the exterior of the body have their epithelial surface coated with a protective layer of mucous. As for example, the interior of the
15 pulmonary alveolus or the luminal surface of the gastro-intestinal tract are covered by a visco-elastic mucous gel layer that acts as a protective barrier against the harsh luminal environment.

It is well-known that this mucous gel covering these mucosal surfaces is one of the major components of physiological defence mechanisms. Mucous provides protection
20 from noxious substances (acidity, proteolytic enzyme activities, toxins, etc...) and constitutes a local physical barrier against bacteria and pathogens. It also regulates the epithelium hydration, allows lubrication of the cells surface and directly participates in the immune response due to interaction with secreted immunoglobulins.

Mucins, high-molecular weight glycoproteins, are the predominant components
25 of this mucous gel. These glycoproteins consist of a protein core covered almost entirely by O-linked carbohydrate chains which may constitute up to 80% of the total molecular weight.

It has long been acknowledged that the structural characteristics of this barrier are primary indicators of its physiological function and changes to its composition have
30 long been identified with various pathologies.

As for example, it is known that defect in mucous composition and/or structure and/or secretion may be implied in various pathologies such as intestinal metaplasia,

gastric ulcer, *Helicobacter pylori* infection, inflammatory bowel disease, gastric and colorectal cancers, or cystic fibrosis (Corfield *et al.*, Gut, 2000, 47:589-94; Quinton, Physiological Rev., 1999, 79 (1):S3-22).

Inflammatory bowel disease (IBD) is an idiopathic disease, probably involving
5 an immunization of the body to its one intestinal tract. The two major types of IBD are ulcerative colitis and Crohn's disease. The pathophysiology of IBD is not well-established, but it is recognized that the common end pathway is inflammation of the mucosal lining of the intestinal tract, causing ulceration, oedema, bleeding, fluid and electrolyte loss. Although ulcerative colitis and Crohn's disease have significant differences, and are
10 differently located, most of the treatments available for one are also effective for the other.

However, none of these treatments brings full satisfaction and there is still a need for improving the patient conditions.

Cystic fibrosis is a lethal-inherited disease associated with a defect in exocrine gland function due to abnormalities of chloride transport across epithelial cells and
15 mucosal surfaces. As a result, many organs may be subjected to injuries, such as gastro-intestinal tract or lungs. A defect in water secretion at the gastro-intestinal level may result in an increase of mucous viscosity resulting in mechanical problems associated with inflammation, scarring and strictures that may predispose the patient to distal intestinal obstruction syndrome. In the lungs, abnormal, thick and "dehydrated" mucous is found and
20 is associated with chronic infections, although it is not well-established if these infections are cause or consequence of this abnormal mucous (Quinton, Physiological Rev;1999, 79 (1):S3-S22).

Furthermore, the gastro-intestinal tract is a biological barrier regularly being in contact with endogenous (as acid, enzymes) and exogenous compounds or pathogenic
25 organisms (as *Helicobacter pylori*). Therefore, the mucosa integrity may suffer from various injuries resulting from the aggressive attacks of those elements. As an example, chemotherapy and radiotherapy, while intended to be used for the treatment of neoplasia, are also known to induce damages to healthy tissues. Both treatments, and in particular chemotherapy, are known to increase several folds the apoptosis in intestinal crypts,
30 resulting in severe inflammation, associated with lesions and ulcerations (Keefe *et al.*, Gut, 2000, 47:632-7). This disorder, known as mucositis, may end by the invasion of the mucosa by intestinal pathogenic organisms, and sepsis may occur. The overall of this

disorder translates into impairment of mucosal and motor functions. The complexity of mucositis lesions associated with chemotherapy and/or radiotherapy impaired the development of effective palliative and/or preventive treatments that have to simultaneously prevent the injury of gastro-intestinal tract but should allow the treatment of neoplasia. It has been observed that mucin and other salivary constituents could play a protective role in the prevention of chemotherapy-induced mucositis (A. Awidi, *et al. European Journal of Cancer*, 37(16): 2010-2014, 2001).

Hence, there is a need for improving the patient condition associated with defect in mucous secretion, as for example in cystic fibrosis, mucositis or IBD.

There is also a need for improving the protection of mucosal surface subjected to various aggressive conditions, such as pathogen organisms and/or aggressive radiotherapy and/or chemotherapy treatments.

There is also a need for preventing the decrease of mucosecretion into a patient subjected to diseases relative to the gastro-intestinal tract.

Unexpectedly, it has been observed by the inventors that the administration of leptin to an animal resulted in an increase of mucous secretion at the colon level.

It has been also observed by the inventors that the application of leptin on a human colon cell line resulted in the induction of mucin discharge.

More specifically, it has been observed that the intra-colonic perfusion of leptin to an animal was able to increase by four-fold the discharge of mucins. A similar effect was also observed by systemic administration of leptin. This effect has been demonstrated as being specifically mediated by the leptin receptor. It has also been observed that such striking effect of leptin on mucous secretion from colon mucosal could be reproduced by application of leptin on human colon cell line. The effect of leptin is far more effective than the carbachol, the most powerful muco-secretagogue known up to now, and may result in increase of mucin-like glycoproteins secretion up to 2 times the basal level.

Thus, according to one of its first aspect, the instant invention is directed to the use of leptin, an analog, a prodrug or a derivative thereof, for the manufacture of a medicament intended for the treatment and/or the prophylaxis of a disease associated with a mucous secretion defect.

In particular, the invention may allow to prevent and/or reduce the mucosal lesions associated with various gastro-intestinal diseases, as for example, inflammatory bowel disease.

5 The leptin is a 16-kD protein well-known to play a critical role in the regulation of body weight by inhibiting food intake and stimulating energy expenditures. In addition to its well-recognized implication in the development of obesity, it is considered that leptin has a variety of other functions such as the regulation of haematopoiesis, angiogenesis, wound-healing and a role in the immune and inflammatory response.

10 As to its role in inflammation process, it has been suggested that leptin could play an anti-inflammatory effect on colonic inflammatory disease (Cakir *et al.*, Peptide, 2004, 25 (1): 95-104; Fantuzzi & Faggioni, J. Leukoc Biol, 2000, 68(4):447-46). However, contradictory studies have been recently published, wherein intra-rectal administration of leptin into mice resulted in colonic epithelial wall damage and acute intestinal
15 inflammation. This leads to the suggestion that leptin could play an active role in generation of intestinal inflammation disease such as inflammatory bowel disease (Sitaraman *et al.*, Faseb J, 2004, 18(6): 696-8).

This other aspect of the effects of leptin is well in contradiction with the observations of the inventors that an administration of leptin to an animal leads to an
20 increase of mucous secretion, and therefore may have a protective effect against deleterious conditions and/or agents.

Within the scope of the instant invention, the term "analog" is dedicated to refer to any substance that gives rise to a pharmacological and biological effect comparable to those obtained with the leptin. As example of analog suitable for implementing the
25 instant invention, mention may be made of leptin agonists such as described by Rozhavskaya-Arena *et al.* (Endocrinology, 2000, 141(7): 2501-7).

Within the scope of the instant invention, the term "prodrug" is dedicated to refer to any substance that gives rise to a pharmacologically active form of leptin or an analog, although not itself active.

30 Within the scope of the instant invention, the term "derivative" includes any inorganic or organic salts, esters of amides of leptin or of analogs suitable for the purpose of the invention.

Within the following of the specification, the term "leptin" will be used to design also prodrug, analog and derivative thereof, except otherwise indicated.

Within the scope of the instant invention, the expression "mucous secretion defect" is dedicated to refer to a defect related to the quantity of secreted mucous and/or to its quality, *i.e.* to its composition.

Such a defect may translate into an unusually decreased level of secreted mucous in a region of an organ where it should normally be present, as well as to a condition whereby an animal secretes a mucous with an unusually viscosity.

It may also be translated into a mucous displaying a qualitative or a quantitative alteration in its composition. As example, one component of the mucous, *e.g.* mucins, may have alteration in their structure, in their expression or their quantity.

In relation to diseases associated with a defect in mucous secretion and/or composition, the term "treatment" refers to a reduction of the severity of the disease, *e.g.* by reducing the lesion connected with a lack of mucous secretion or an alteration of the structure and/or proportion of biological molecules composing the mucous. As example of lesion associated with a defect in mucus secretion, it may be made mention of lesions or ulcerations within the intestinal epithelium or mucosa.

In relation to diseases associated with a defect of mucous secretion and/or composition, the term "prophylaxis" refers to preventing such disease from occurring, *i.e.* the leptin, or analog, prodrug or derivative thereof, is administered prior to the onset of the disease condition. This means that the compounds of the instant invention can be used as prophylactic agents to impede occurrence of diseases associated with defect in mucus secretion and/or composition.

The invention may also, by increasing the secretion and/or by modifying the composition of the mucous coating the interior surface of various organs open to the exterior of the body, improve the physiological defence mechanisms, for example against intestinal and/or lung pathogens and/or against various aggressive compounds.

The invention may also be used to improve the patient condition affected with a mucosal inflammation, for example by improving its comfort or reducing its pain.

According to another aspect, the instant invention is directed to a use of leptin, an analog, a prodrug or a derivative thereof, for the manufacture of a medicament intended

for the treatment and/or the prophylaxis of a bowel disease associated with a mucous secretion defect.

According to another of its aspects, the instant invention is directed to a use of leptin, an analog, a prodrug or a derivative thereof, for the manufacture of a medicament
5 intended to be used as a colon muco-secretagogue.

According to the instant invention, the term "colon muco-secretagogue" is understood as characterizing a compound stimulating the secretion of mucous by the colonic mucous cell.

According to another embodiment, the instant invention concerns a method of
10 treatment and/or prophylaxis of a disease associated with a mucous secretion defect comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a derivative thereof.

According to another of its aspects, the instant invention is directed to a method of treatment and/or prophylaxis of a bowel disease associated with a mucous
15 secretion defect comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a derivative thereof.

According to another of its embodiments, the instant invention is directed to a method for increasing and/or preventing a decrease of a bowel mucous secretion comprising at least a step of administration to an animal in need thereof an effective
20 amount of leptin, an analog, a prodrug or a derivative thereof.

According to another of its embodiments, the instant invention is directed to a method of treatment and/or prophylaxis of a gastro-intestinal side effect associated with a radiotherapy and/or a chemotherapy treatment comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a
25 derivative thereof.

According to another of its aspects, the instant invention is directed to a pharmaceutical composition comprising as therapeutically active agent at least an effective amount of leptin, an analog, a prodrug or a derivative thereof in a combination with a compound acting on gastro-intestinal tract.

30 Within the scope of the instant invention, the term "compound acting on gastro-intestinal tract" is dedicated to refer to any compound having the properties to induce into this organ a biological effect that may be beneficial or deleterious to it.

A "beneficial effect" has to be understood as meaning that the compound maintain or improve the physiological functions of gastro-intestinal tract such as mucous secretion.

5 A "deleterious effect" has to be understood as meaning that the compound induces an impairment of physiological functions of gastro-intestinal tract.

According to one of its embodiments, the method may be used for the treatment and/or the prophylaxis of a bowel disease.

10 According to another embodiment, the method may be used for increasing and/or preventing a decrease of bowel mucous secretion.

As examples of bowel diseases associated with mucous secretion defect, there are inflammatory bowel diseases, bowel defects connected with cystic fibrosis, gastric ulcer, and gastric or colon cancers, as well as yeasts, parasites, virus and bacteria-related gastrointestinal diseases.

15 As examples of inflammatory bowel diseases, mention may be made of Crohn's disease, ulcerative colitis, mucositis, microscopic colitis (lymphocytic and collagenous colitis), and infection induced colitis, and acute colitis.

The physiopathological state of mucositis may have, for example, as aetiology side effects subsequent to radiotherapy and/or chemotherapy treatment.

20 The radiotherapy treatment may be administered for example by administration of X-rays and/or administration to an animal in need thereof of radioactive elements designed, or not, to target specifically the organ to be treated. For example, such radioactive elements may be associated with an antibody having specificity for a tumour antigen.

25 The chemotherapy treatment may be any known administration of chemotherapy agent, such as methotrexate, taxol or 5-fluoro-uracil.

To prevent or reduce the occurrence of lesions resulting of administration of a radiotherapy and/or chemotherapy treatment, the leptin may be administered before, concomitantly, and/or after the administration of said radiotherapy and/or chemotherapy
30 treatment.

According to one embodiment, the leptin administered may be a recombinant leptin, and more advantageously, a human recombinant leptin, produced through any

known biology molecular tools as described in Sambrook et al. (Molecular Cloning, Cold Spring Harbour Lab, NY 1989) using the DNA sequence numbered P41159, available from Swiss-prot database (www.expasy.org).

5 Instead of leptin or a recombinant leptin, an analog may also be used. Such a compound may be a peptidic or non-peptidic compound having biological effect analogous to the leptin. As example of an analog, there is [D-Le-4]-OB3 compound described by Rozhaskaya-Arena et al. (Endocrinology, 2000, 141 (7): 2501-7).

The leptin or analog, prodrug or derivative thereof is advantageously administered in an effective amount.

10 Within the scope of the instant invention, the term "effective amount" means the minimal amount necessary to observe the expected effect, i.e. a reducing of the size of mucosal lesions and/or an alleviating of the symptoms suffered by the patient.

The leptin or or analog, prodrug or derivative thereof may be administered in a dose ranging from about 0.01 mg/kg/day to 1.6 mg/kg /day of patient body weight, more particularly in a dose ranging from about 0.02 mg/ kg /day to 0.8 mg/kg/day of a patient body weight, and in particular from about 0,02 mg/kg/day to 0,08 mg/kg/day of a patient body weight.

The dose of leptin, analog, prodrug or derivative thereof, to be administered is to be adapted according to the nature of the pathology to be treated and/or prevented, and according to the patient condition. The adapted dose may be routinely determined by any means known by the skilled person in the art.

The leptin may be administered alone or in a combination with any known compound acting on gastro-intestinal tract.

25 Such a compound may have a beneficial effect on said gastro-intestinal tract. Such beneficial effect may be the maintenance and/or the improving of the physiological functions of the gastro-intestinal tract such as, for example, the increase in cytoprotection and in epithelial restitution, the maintenance of mucosal vascular integrity, the intestinal permeability, the absorption of electrolytes, the increase of mucous secretion, the modification of the composition of the secreted mucous, the modification of the motor function.

30 As example of compound that may be administered for a beneficial effect, in combination with leptin, it may be made mention of cholinergic-like compounds such as

acetylcholin, carbacholin, tea-derived extracts, prostaglandins and prostaglandin's analogs, growth factors, polysaccharide antipeptic, opioid agonists, short-chain fatty acids, and combinations thereof.

5 The administration of such a combination may be aimed to obtain an additive or a synergic effect of the leptin and the compounds.

The leptin may also be administered with a compound having a deleterious effect on gastro-intestinal tract.

10 The deleterious effect may result in an impairment of the physiological functions of the gastro-intestinal tract. Such impairment may result as an effect of compound inducing necrosis or apoptosis of cells lining this organ.

As example of such compound, mention may be made of chemotherapy agents, as previously mentioned, salicylic derivatives or non-steroid anti-inflammatory (NSAI) compounds, anti-inflammatory steroid compounds, and combination thereof, which are known to be aggressive for the gastrointestinal mucosa.

15 The administration of such combination is intended to prevent and/or reduce the occurrence of injuries due to the use of compound with deleterious effect.

By "combination", it is understood that the compound with deleterious or beneficial effect may be administered simultaneously or separately as a kit-of-parts with leptin.

20 The skilled person in the art can routinely determine the appropriate dose of each of the components of the combination to be administered to obtain the desired effect, by using any pharmacological tool and animal model known in the field.

The leptin may be administered alone or in a combination with a compound, as previously defined, to any animal in need thereof.

25 The term "animals" includes mammals such as humans and farm (agricultural) animals, especially the animals of economic importance such as gallinaceous birds, bovine, ovine, caprine and porcine mammals, especially those that produce products suitable for the human consumption, such as meat, eggs and milk. Further, the term is intended to include fish and shellfish, such as a salmon, a cod, a tilapia, a clump and oysters. The term
30 also includes domestic animals such as dogs and cats. The term is also used to refer to laboratory animals which include, but are not limited to, rodents such as mouse, rats, guinea pigs or hamsters.

The leptin, alone or in combination with a compound as previously defined, may be administered by any appropriate route suitable to reach the therapeutic and/or prophylactic aimed effect, with the maximum efficiency and/or the minimum side effect.

As example of suitable route, it may be made mention of oral route (*per os*),
5 rectal route, parenteral route or nasal route.

The administration *per os* or by rectal route may be, for example, well-suited for treating and/or preventing pathological conditions localized at the gastro-intestinal level such as inflammatory bowel diseases or gastro-intestinal injuries resulting from side effect following a radiotherapy and/or chemotherapy treatment. For such pathological conditions,
10 the parenteral route, as intravenous route, may also convene.

The leptin, alone or in combination with a compound as previously defined, may be presented in any galenic forms or compositions suitable to the particular route of administration intended to be used. Such a galenic form may be for example tablet, dragee, pill, powder, liquid, suppository or aerosol. Within such a galenic form, the leptin or the
15 combination may be associated with any suitable pharmaceutical excipients. Generally, the formulations are prepared by contacting the components of the present invention, each uniformly and intimately with liquid carriers or finely divided solids carriers or both.

Therefore, according to another embodiment, the instant invention is also concerned with a pharmaceutical composition that comprises as therapeutically active
20 agent at least an effective amount of leptin, alone or in combination with a compound as previously defined.

The pharmaceutical composition of the instant invention may be implemented in any of the methods or uses previously described.

A further aspect of the instant invention is concerned with the use of leptin, or
25 an analog, a prodrug or a derivative thereof for the manufacture of a medicament or a pharmaceutical composition as previously described.

The instant invention is also concerned with the use of such a medicament or pharmaceutical composition to be implemented in the methods or uses as previously described.

30

The invention will be more fully understood by reference to the following examples. Other advantages and characteristics of the invention will fully appear by

reference to the following examples. They should not, however, be construed as limiting the scope of the invention.

Legends to figures

5 Figures 1A and 1B: they show a leptin-induced intracolonic increase mucus secretion. Figure 1A: it shows a time-course effect of leptin on mucous secretion. KRB alone (control; open bar) or 100 nmol/L leptin (black bar) in KRB were perfused intraluminally in colon of anaesthetized rats. Mucous release was collected after 30-180 min and quantified by ELISA. Each time period mucous release was determined
10 separately. As a positive control, the effect of intravenous bolus injection of saline of 1 mmol/L carbachol (grey bar) on mucous secretion was determined over 180 min-period. *P<0.05 vs control. Figure 1B: it shows a dose-response curve for luminal leptin stimulation of mucous secretion in rat colon *in vivo*. Mucous secretion was determined as non-cumulative responses to different concentrations of leptin after a 60-min luminal
15 perfusion. Data are expressed as mucus secretion in μg per mg DNA and are the mean $\pm$ SEM of 4-8 experiments for each dose of leptin.

 Figure 2: it shows histology of control and leptin treated rat colon. The colon loops were luminally perfused with KRB alone (Figure 2A) or with leptin 0.1 nmol/L (Figure 2B) or 1 nmol/L (Figure 2C) in KRB for 60 min. Sections (4 μm) of colon mucosa
20 were stained.

 Figure 3: it shows that the leptin stimulation effect is impaired in leptin-receptor deficient rats (fa/fa). The colon loops of anaesthetized lean (*Fa/fa*) (control) and obese *fa/fa* (leptin-receptor deficient) rats were perfused intraluminally for 180 min with KRB alone (open bar) or with 100 nmol/L leptin in KRB (black bar). Mucous release was
25 collected and quantified by ELISA. The data are expressed as mucus secretion in μg per mg DNA and are the mean $\pm$ SEM of 3-4 rats* P<0.05 vs. controls.

 Figure 4: it shows the luminal vs. systemic effect of leptin on colonic mucous secretion. The colon loops of anaesthetized rats were perfused intraluminally (right bars) with KRB alone (control; open bar) or with 5 nmol/L leptin (black bar) in KRB for 60 min.
30 In another set of experiment (left bar), saline (control; open bar) or 5 nmol/L leptin (black bar) was intravenously injected through the femoral. Mucous release was collected 60 min after the beginning of the injection and quantified by ELISA. These data are expressed as

mucus injection in μg per mg DNA and are the mean $\pm$ SEM of 3-8 rats * $P < 0.05$, significantly different from controls. ** $P < 0.05$ vs i.v.

Figures 5A and 5B: they show that leptin stimulates mucin secretion from HT29-MTX cells. Figure 5A: after 24 hr serum starvation, HT29-MTX cells were treated without (control; open bar) or with 10 nmol/L leptin (black bar) or 1 mmol/L carbachol (grey bar) for 15 to 60 min. The amount of mucin glycoproteins in culture media was measured by ELLA. The data expressed as mucin glycoproteins secretion in percent of control and each bar represents the mean $\pm$ SEM of 3 experiments performed in triplicate. Insert: RT-PCR of Ob-Rb from total RNA extracted from HT29 and HT29-MTX cells. lane 1: DNA markers; lane 2: parental HT29 cells and lanes 3 & 4 HT29 MTX cells. The 246 bp amplicon (arrows) corresponds to human Ob-Rb. Figure 5B: it shows a dose response curve for leptin stimulation of mucin glycoprotein secretion in HT29-MTX cells. The cells were incubated with leptin (●: 0.01-10 nmol/L) or carbachol (○: 1 mmol/L) at 37°C for 60 min. Each point represents the mean $\pm$ SEM of 3-4 experiments performed in triplicate. Samples of medium were also collected after incubation with leptin 10 nmol/L for specific determination of MUC5AC secretion *, $P < 0.05$ vs. controls.

Material and Methods

Male Wistar rats weighing 220-240g, and male 6-8 week-old diabetic Zucker fatty (*fa/fa*) and lean (*Fa/fa*) rats (Charles River Laboratories, L'Arbresle, France) were used throughout the experiments.

For *in vivo* studies of mucous secretion, rats were fasted 16 h in mesh-wire cages to avoid coprophagia with water *ad libitum*. Animals were anaesthetized with ethylurethane (1.2 g/kg, intramuscular) (Prolabo, Paris, France) and equipped with an intraluminal catheter inserted into the right colon after the caecum and another placed 2 cm from rectum.

For the colon perfusion, an inflow cannula was inserted 1 cm below the caecum and the outflow cannula was set up at a distance of 1 cm above the rectum. The colonic segment was flushed with saline solution prewarmed to 37°C to remove residual intestinal contents. Each site was continuously perfused at a rate of 1 ml/15 min (Minipuls

2; Gilson Co., Paris, France) with a Krebs-Ringer buffer (pH 7.5) containing (in nmol/L): 120 NaCl, 4.5 KCl, 0.5 MgCl₂, 0.7 Na₂HPO₄, 1.5 NaH₂PO₄, 15 NaHCO₃, 1.2 CaCl₂ and 10 glucose. After a 30-minute stabilization period, vehicle (control) or recombinant murine leptin (R&D Systems, Europe) (100 nmol/L) in Krebs-Ringer buffer was intraluminally
5 perfused for each time period varying from 30 to 180 minutes and the perfusate was collected. For comparison purpose, carbachol at 1 mmol/L, a well-known stimulant of muco-secretion or leptin, was administered as bolus injection through the femoral vein and mucus secretion over a 180-min duration of the experiment was determined.

For dose-response studies, each dose of leptin was added in KRB solution and
10 the colon segment was luminally perfused for 60 minutes and the fluid content was continuously collected.

At the end of the experimental period, the perfused segments were removed and the mucous adherent to the mucosal surface was collected by manual massage, subsequently flushed with air, and drained. Luminal content (fluid content+ adherent
15 mucus gel) was collected in preweighed tubes and frozen at -20°C for subsequent determination of mucin-like immunoreactivity and luminal DNA content. The empty colonic loops were weighed, measured, immediately frozen in liquid nitrogen and then stored at -80°C. Tissue homogenates were then analysed for DNA content as described below. The amount of mucin secreted from each loop was expressed as micrograms of
20 mucin per milligram of tissue DNA.

For assessing the effect of leptin on a human model, HT29-MTX and HT29 cells were used. HT29-MTX cells, a human colonic carcinoma derived mucin-secreting goblet cell line (Lesuffleur *et al.*, J. Cell. Sci., 1983, 106:771-83), and the parental HT29
25 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen corporation, Cergy Pontoise, France) supplemented with 10% foetal bovine serum (FBS) (Sigma, Saint Louis, MO, USA) and 10 mg/ml penicillin/streptomycin (PS, Invitrogen). Cells were grown in plastic 25 cm² culture flasks and maintained at 37°C in a 5% CO₂ atmosphere within a humidified incubator. Medium was replaced every two days.

30 To study mucin secretion, HT29-MTX cells were seeded in 12-well culture plates (5.10⁵ cells/well) and were given fresh medium every two days. Experiments were performed 21 days after reaching confluency. Twenty four hours before the secretory

studies, the culture medium was replaced by serum-free medium in order to starve the cells from serum and to eliminate any interference from extraneous proteins or hormones on mucin assay. The experimental protocol was then the following: the serum-free medium was removed and the monolayer cultures of HT29-MTX were washed twice with PBS
5 (37°C). Serum-free medium with or without leptin was added to the cells and incubated at 37°C for 15-60 min in a humidified atmosphere. The supernatants were then collected, frozen and stored at -20°C. Cells were then processed with trypsin and the cell number per well was determined. All experiments were performed at least three times in triplicate.

10 The expression of leptin receptor by HT29-MTX cells was controlled by RT-PCR. Total RNA were extracted from HT29 or HT29-MTX cells, using Trizol[®] (Invitrogen corporation) according to the manufacturer's instructions in RNA was reverse-transcribed into cDNA then amplified with Ob-Rb primers. The primers of 5'-GCC AAC AAC TGT GGT CTC TC-3' and 3'-AGA GAA GCA CTT GGT GAC TG -3' for
15 huB219.1 were designed on the basis of the previously published cDNA sequence for human Ob-Rb leptin receptor (Accession number: U52914) which define an amplicon of 246 bp. PCR was performed under the thermocycling conditions as follows: 1-minute denaturation at 94°C, 1-minute annealing at 57°C and 1-minute extension at 72°C for 35 cycles. The last amplification was followed by final ten-minute elongation step at 72°C.
20 PCR products were analysed by electrophoresis in 2% agarose gel in the presence of ethidium bromide.

The amount of mucin secreted by colonic intestinal loops or by human cells was determined with an ELISA assay. Samples of luminal contents from colonic loops
25 were incubated for 24 hours with 100 mmol/L 1,4-dithiothreitol at 4°C for reduction and were then assayed for mucins by ELISA as described in Plaisancie *et al.* (Am. J. Physiol., 1998, 275: G1073-84) and Claustre *et al.*, (Am. J. Physiol. Gastro Intest. Liver Physiol., 2002, 283:G521-8). The polyclonal antiserum 45C was raised in rabbits against rat colonic mucins. Immunoglobine rich fractions were prepared using DEAE-SEPHADEX
30 A50 (pH 7.2), and then used to produce biotinylated labelled antibodies using a succinimide ester of biotin (Guesdon *et al.*, J. Histochem Cytochem, 1979, 27:1131-9). Mucin content of samples was determined from standard curves prepared from purified rat

colonic mucins obtained from CsCl gradient purified material (Plaisancie *et al.* Am. J. Physiol., 1998, 275: G1073-84; Claustre *et al.* Am. J. Physiol. Gastro Intest. Liver Physiol., 2002, 283:G521-8). The amount of mucin secreted from rat colon was expressed as micrograms of mucin per milligram of tissue DNA. The coefficient of variation was 4.5%. For determination of DNA content, rings of tissue were homogenized (Ultra-Turrax, Janke and Kundel, Staufen, Germany) in phosphate buffered saline (PBS), sonicated for 20 s, and then analysed using the fluorometric method of Hinegardner (Hinegardner, Anal Biochem, 1971, 39: 197-201).

The secretion of mucin 5AC from HT29-MTX incubation medium was measured by an ELISA method using the primary monoclonal antibody 45M1 (Santa Cruz, biotechnology, CA, USA). This mouse monoclonal antibody recognizes the peptide core of gastric mucin M1 identified as Mucin 5AC. It reacts with human mucin epitopes (Bara *et al.*, Int J Cancer, 1991, 47: 304-10). ELISA plates (96 wells) were coated directly with samples of cell culture media diluted in carbonate coating buffer (0.5 M, pH 9.6), incubated overnight at 4°C, and then processed as described in Trompette *et al.*, (Eur J Cell Biol, 2001, 11:3499-53). Porcine gastric mucin, previously shown to react strongly with anti-human gastric monoclonal 45M1 antibody (Hutton *et al.*, Glycoconj J, 1998, 15: 283-91), was treated in the same way to obtain a mucin standard curve. The mucin 5AC secreted from HT29-MTX was expressed as nanograms of mucin per 10⁶ cells and the results were given as percent of controls.

The amount of glycoproteins mucins secreted by HT29-MTX cells were assayed according to an enzyme-linked lectin assay (ELLA) as described in Trompette *et al.* (Eur J Cell Biol, 2001, 11:3499-53). Briefly, wells of a microtiter plate was coated with sample diluted in sodium carbonate buffer (0.5 M, pH 9.6) and then incubated overnight at 4°C. The plates were then washed with PBS containing 0.1% Tween (PBS-Tween, pH 7) and blocked with 200 µl of 2% BSA in PBS-Tween for 1 hour at 37°C. After washing five times, 100 µl of biotinylated wheat germ agglutinin (WGA, Vector laboratories, Burlingame, CA, USA) at a concentration of 5 µg/ml in PBS-Tween-BA were added, and the samples were then incubated for 1 h at 37 °C. The plate was washed again. At this stage, 100 µl of avidin-peroxidase conjugate (Vectastain Elite ABC reagent) were added

and allowed to bind for 1 h at room temperature. After washing five times, 100 µl of o-phenyldiamine dihydrochloride solution were then added to each well and the color was allowed to develop in the dark for 5-10 min. The reaction was stopped by adding 25 µl of 3 M sulphuric acid to each well. The absorbance was read at 492 nm on a micro-ELISA plate reader.

Mucin-like glycoproteins content of samples was determined from standard curves prepared from DHE or HT29-MTX mucins isolated from 75-cm² flasks and purified by ultracentrifugation as described in Plaisancie *et al.*, (Am. J. Physiol., 1998, 275: G1073-84). The results were given as percent of controls.

For histological analysis, a segment of colon from control-and leptin-treated rats were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 2 µm, and stained with alcian blue (AB, pH 2.5) followed by the periodic acid-Schiff reaction (PAS) to reveal the goblet cells, and then counterstained with hematoxylin. The AB/PAS method yielded blue colour when mostly acidic mucins were present, purple when neutral mucins were also present and magenta when mainly neutral mucins were present.

The results were expressed as means ± SEM. One way ANOVA with Tukey-Kramer multiple comparison post-test and U-test of Mann-Whitney or Mann-Whitney test alone for single comparisons were performed using GraphPad Prism version 3.0 for Windows (Graphpad software Inc., San Diego, CA). The level of significance was set at P<0.05.

Results

Example I

Effect of leptin on colonic mucous secretion

Mucous secretion was stimulated by intracolonic administration of leptin. The mucous collected at the end of the experiment in control rats, was constituted of the adherent mucous gel plus the basal secretion (or unstimulated mucin release). In control rats, there was a spontaneous increase in mucus measured after 30 min which remained at plateau between 60 and 180 min (Figure 1A). Luminal perfusion of 100 nmol/L leptin induced a time-dependent increase in mucous secretion. Indeed, this increased mucin

discharge was rapid (30 minutes after the beginning of leptin perfusion) and reached a 4-fold increase as compared to control ($815 \pm 150 \mu\text{g}/\text{mg DNA}$ vs. $213 \pm 55 \mu\text{g}/\text{mg DNA}$ in controls; $P < 0.05$). This leptin induced increase in mucous secretion remained high (2.5 and 3.3-fold over basal value) after 180 min of leptin. In these conditions, intravenous
5 infusion of 1 mmol/L carbachol also induced an increase in colon mucin secretion consistent with earlier studies. It could be noted that luminal leptin-induced mucin secretion was higher than induced by intravenous carbachol, although it did not reach statistical significance ($1879 \pm 482 \mu\text{g}/\text{mg DNA}$ vs. $1124 \pm 548 \mu\text{g}/\text{mg DNA}$).

The dose-response effect of luminal leptin on colonic mucous discharge was
10 further determined after 60-min of perfusion. As shown in Fig.1B, leptin-induced a dose-dependent increase in colonic mucus discharge with a significant response observed with nmol/L leptin, and maximal response with 10 nmol/L leptin ($1922 \pm 317 \mu\text{g}/\text{mg DNA}$ vs. $545 \pm 80 \mu\text{g}/\text{mg DNA}$ in controls). No further increase in mucin secretion was observed with 100 nmol/L leptin. The concentration producing a half maximal stimulation of
15 mucous secretion (EC_{50}) calculated from the dose-response curve was 0.8 nmol/L.

Effect of leptin on release of mucus granules from goblet cells.

Histology studies were performed on colon mucosa sections from control- and leptin-treated colon mucosa. Goblet cells in the rat colonic epithelium were stained with
20 alcian blue followed by the periodic acid-Schiff reaction. In the control colon mucosa (Fig. 2A), numerous goblet cells with densely stained granules can be observed along the length of the crypt. Leptin 0.1 nmol/L administered intraluminally did not induce any modification in mucosa morphology consistent with quantitative data (Fig. 2B). By contrast, 1 nmol/L leptin induced a markedly decrease of mucous granules in goblet cells
25 of rat colon (Fig. 2C). A decrease in the number of stained mucous cells was also observed as compared to control and the crypt lumen was expanded according to the increase in mucous secretion.

Example II

Receptor- specificity of leptin effect on colonic mucous secretion

The implication of the activation of leptin receptors in the leptin induced-mucous secretion was assessed by examining the response to leptin in colonic preparations

from Zucker diabetic fatty (ZDF) *fa/fa* rats which have a functional decrease of the leptin receptor (Chua *et al.*, Diabetes, 1996, 45:1141-3). In lean *Fa/fa* perfused rat colon, this increased was impaired and the leptin-induced secretion was not significantly different from control (3732.5 ± 475.3 $\mu\text{g}/\text{mg}$ DNA vs. 2971.5 ± 26.3 $\mu\text{g}/\text{mg}$ DNA $P > 0.05$). It is
5 noteworthy that basal secretion of colonic mucous was extremely high in ZDF (*fa/fa*) rats when compared to lean (*Fa/fa*) or Wistar rats (Figure 3).

Example III

Luminal vs systemic leptin effect on mucus secretion

10 The importance of the route of administration was evaluated by determining the effects of intravenous leptin on colonic mucous secretion. The amount of mucous secreted was not different after intravenous or luminal perfusion of saline. Intravenous as well as luminal perfusion of leptin (5 nmol/L) significantly stimulated mucous secretion (Fig. 4).

15

Example IV

Effect of leptin on human cells

The assessing of the intestinal effect of leptin on human cells was carried out on human colonic HT29-MTX cells, known to synthesize and secrete mucin-like
20 glycoprotein (Lesuffleur *et al.*, J Cell Sci. 106:771-83). Firstly, the expression of the long form of leptin receptor by these cells was controlled. By RT-PCR analysis a 246 bp amplicon was amplified (Fig. 5A insert). After cDNA sequencing, this product was found to be 100% identical to the human Ob-Rb gene transcript.

The consistence of leptin induction of mucin discharge with expression of
25 leptin receptor on HT29-MTX cells was further evaluated. Cells were incubated for 15 to 60 min with medium (basal) or challenged by carbachol (positive control) or leptin (Figure 5A). Addition of carbachol (1 mmol/L) to the incubation medium of HT29-MTX cells elicited an increased exocytotic response (Figure 5) that was detectable 15 min after incubation ($219 \pm 8\%$ of control, $P < 0.05$). Incubation of HT29-MTX cells with leptin (1
30 mmol/L) elicited a rapid (15 min) increased ($216 \pm 13\%$ of control, $P < 0.05$) exocytotic response (Figure 5A). This leptin stimulation of mucin-like glycoprotein secretion was dose-dependent with significant response occurring with 0.1 nmol/L (Figure 5B). Maximal

response was achieved with 1 nmol/L leptin ($85\% \pm 18\%$ of control, $P < 0.05$) and no further increased was observed with 10 nmol/L leptin. Using a specific human MUC5AC ELISA, it has been found that 10 nmol/L leptin induced a specific rise in MUC5AC secretion ($60\% \pm 8\%$ above control, $P < 0.05$).

CLAIMS

1. A use of leptin, an analog, a prodrug or a derivative thereof for the manufacture of a medicament intended for the treatment and/or the prophylaxis of a disease associated with a mucous secretion defect.
5
2. A use of leptin, an analog, a prodrug or a derivative thereof for the manufacture of a medicament intended to be used as a colon muco-secretagogue.
3. The use according to claim 1, wherein said disease is a bowel disease.
4. The use according to anyone of the preceding claims, wherein said
10 medicament is intended for increasing and/or preventing a decrease of bowel mucous secretion.
5. The use according to anyone of the preceding claims, wherein said medicament is for the treatment and/or the prophylaxis of an inflammatory bowel disease.
6. The use, according to claim 5, wherein said inflammatory disease is
15 selected from Crohn's disease, ulcerative colitis, mucositis, microscopic colitis (lymphocytic and collagenous colitis), infection induced colitis, and acute colitis.
7. The use, according to the any of the preceding claims, wherein said leptin is a recombinant leptin.
8. The use, according to anyone of the preceding claims, wherein said
20 medicament is under the form selected from a tablet, a dragee, a pill, a powder, a liquid, a suppository or an aerosol.
9. The use, according to anyone of the preceding claims, wherein said medicament is designed to be administered *per os*.
10. The use according to claims 1 to 9, wherein said medicament is designed to
25 be administered by rectal route.
11. The use according to anyone of claims 1 to 9, wherein said medicament is designed to be administered by parenteral route.
12. The use according to anyone of claims 1 to 9, wherein said medicament is designed to be administered by nasal route.
13. A method of treatment and/or prophylaxis of a disease associated with a
30 mucous secretion defect comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a derivative thereof.

14. The method, according to the preceding claim, wherein said disease is a bowel disease.

15. The method, according to the preceding claim, wherein said bowel disease is an inflammatory bowel disease.

5 16. The method, according to the preceding claim, wherein said inflammatory bowel disease is selected from Crohn's disease, ulcerative colitis, mucositis, microscopic colitis (lymphocytic and collagenous colitis), infection induced colitis, and acute colitis.

10 17. A method for increasing and/or preventing a decrease of bowel mucous secretion comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a derivative thereof.

 18. A method of treatment and/or prophylaxis of a gastro-intestinal side effect associated with a radiotherapy and/or a chemotherapy treatment comprising at least a step of administration to an animal in need thereof an effective amount of leptin, an analog, a prodrug or a derivative thereof.

15 19. The method according to the preceding claim, wherein said leptin, analog, prodrug or derivative thereof is administered before, concomitantly, and/or after the administration of said radiotherapy and/or chemotherapy treatment.

 20. The method, according to anyone of the claims 13 to 19, wherein said leptin is a recombinant leptin.

20 21. The method, according to anyone of the claims 13 to 20, wherein said leptin, analog, prodrug or derivative thereof is under the form selected from a tablet, a dragee, a pill, a powder, a liquid, a suppository or an aerosol.

 22. The method, according to anyone of claims 13 to 21, wherein said leptin, analog, prodrug or derivative thereof is administered *per os*.

25 23. The method according to anyone of claims 13 to 21, wherein said leptin, analog, prodrug or derivative thereof, is administered by rectal route.

 24. The method according to anyone of claims 13 to 21, wherein said leptin, analog, prodrug or derivative thereof, is administered by parenteral route.

30 25. The method according to anyone of claims 13 to 21, wherein said leptin, lepidomimetic compound, prodrug or derivative thereof, is administered by nasal route.

26. A pharmaceutical composition comprising as therapeutically active agent at least an effective amount of leptin, an analog, a prodrug or a derivative thereof, in combination with a compound acting on gastro-intestinal tract.

27. The pharmaceutical composition according to the preceding claim, wherein
5 said compound acting on gastro-intestinal tract has a beneficial effect on said gastro-intestinal tract.

28. The pharmaceutical composition according to the preceding claim, wherein
said compound acting on the gastro-intestinal tract is selected from cholinergic-like
compound, tea-derived extract, prostaglandins and prostaglandin's analogs, growth factors,
10 polysaccharide anti-peptic, opioid agonists, short chain fatty acids, and combinations thereof.

29. The pharmaceutical composition, according to anyone of claims 26 to 28,
for the treatment and/or the prophylaxis of a bowel disease associated with a mucous
secretion defect.

15 30. The pharmaceutical composition, according to anyone of claims 26 to 29,
for the treatment and/or the prophylaxis of an inflammatory bowel disease.

31. The pharmaceutical composition according to claim 26, wherein said
compound acting on the gastro-intestinal tract has a deleterious effect on said gastro-
intestinal tract.

20 32. The pharmaceutical composition according to the preceding claim, wherein
said compound acting on the gastro-intestinal tract is selected from NSAID compound,
chemotherapy agent, anti-inflammatory steroid compound, and combinations thereof.

33. The pharmaceutical composition according to the preceding claim for the
treatment and/or the prophylaxis of a bowel disease resulting from said deleterious effect.

25 34. The pharmaceutical composition, according to anyone of claims 26 to 33,
wherein said bowel disease is selected from Crohn's disease, ulcerative colitis, mucositis,
microscopic colitis (lymphocytic and collagenous colitis), infection induced colitis, and
acute colitis.

35. The pharmaceutical composition, according to anyone of claims 26 to 34,
30 wherein said leptin is a recombinant leptin.

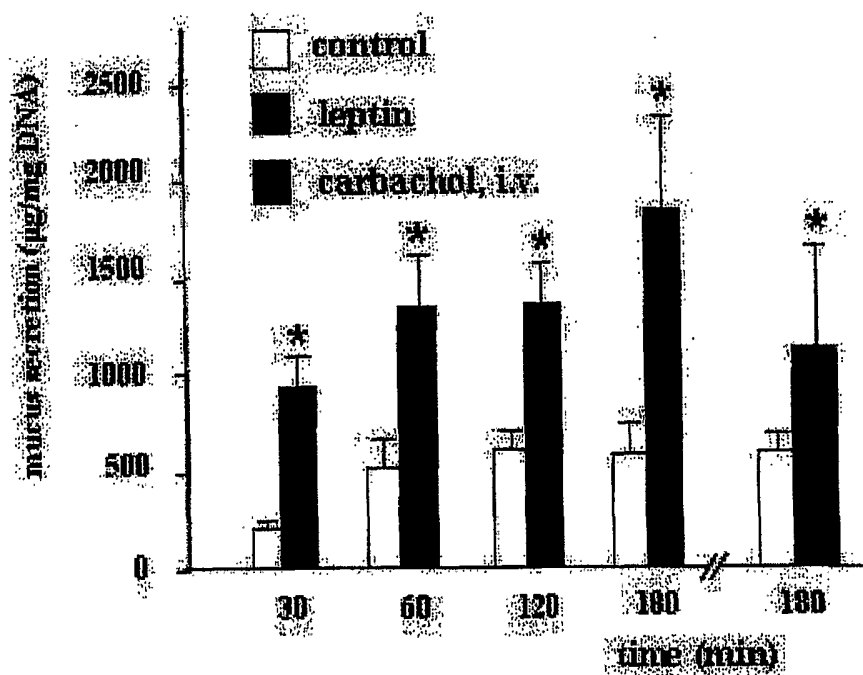
36. The pharmaceutical composition, according to anyone of claims 26 to 35, wherein said composition is administered *per os*, by rectal route, by parenteral route and/or by nasal route.

5 37. The pharmaceutical composition, according to anyone of claims 26 to 36, wherein said composition is under the form of a tablet, a dragee, a pill, a powder, a liquid or a suppository.

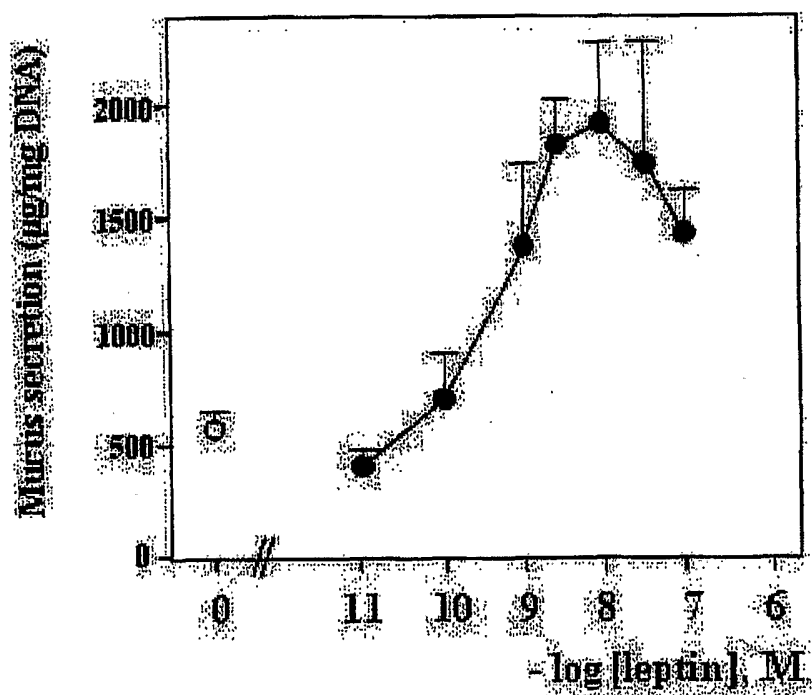
1/5

FIGURE 1

1A

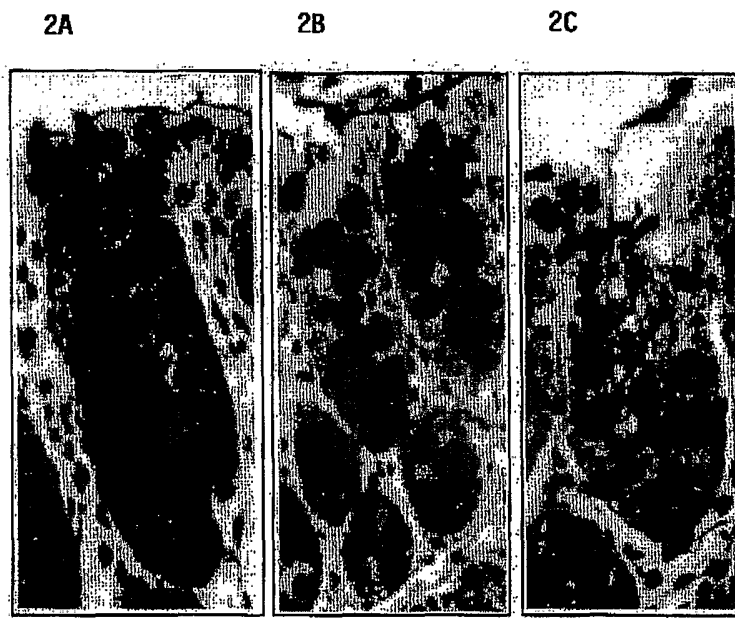


1B

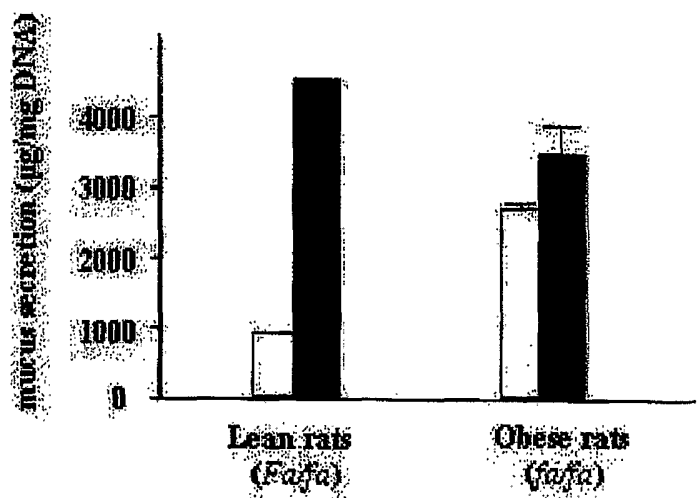


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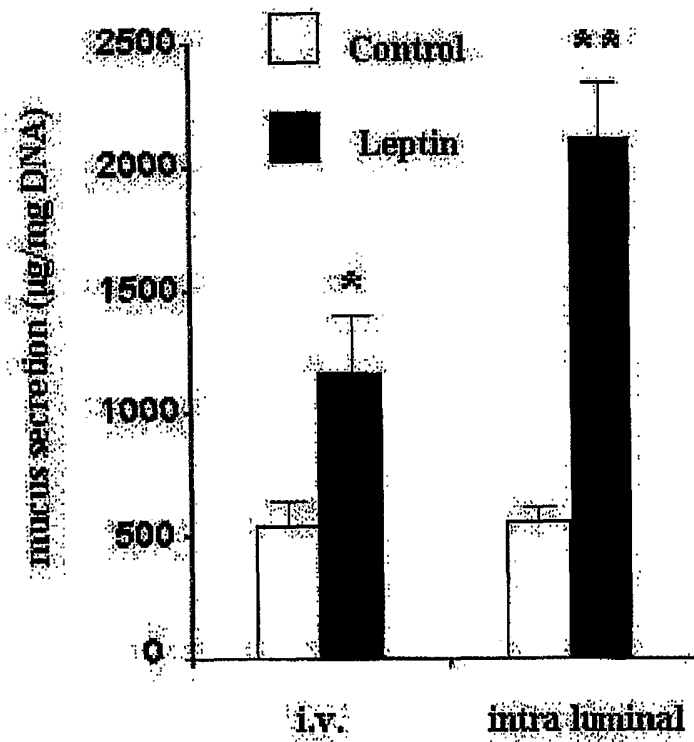
FIGURE 2



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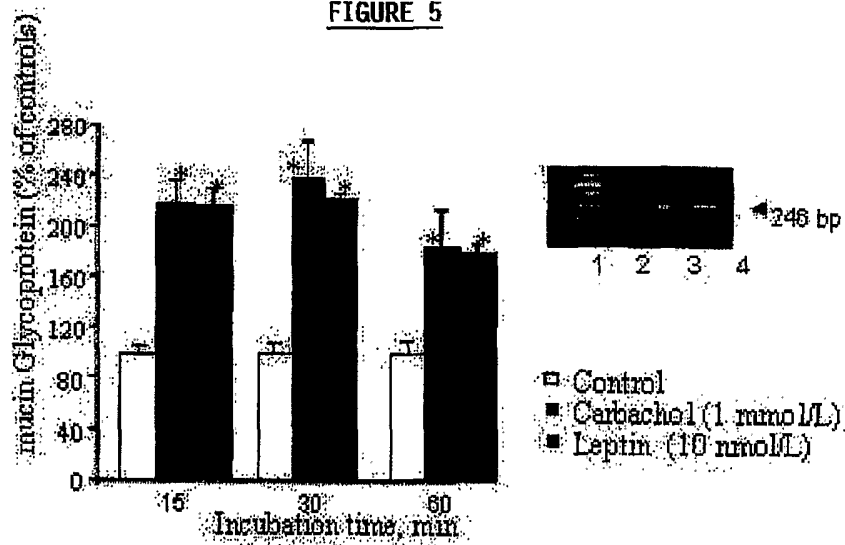
FIGURE 3

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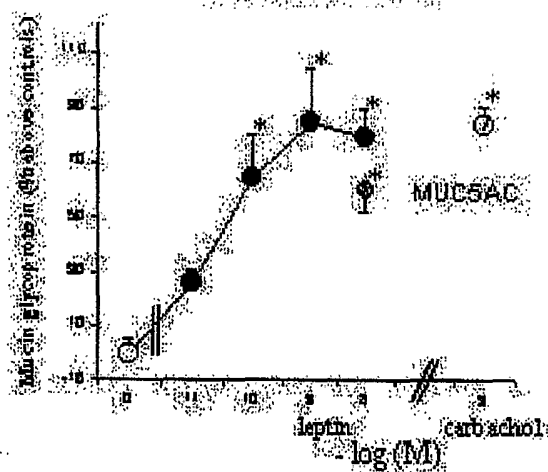
FIGURE 4

5/5
FIGURE 5

5 A



5 B



INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/003604

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/22 A61P1/04

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)

IPC 7 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 practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 630 444 B1 (SCHWARTZ MARSHALL ET AL) 7 October 2003 (2003-10-07) column 3, line 1 - line 5 column 4, line 10 - line 30 -----	1-37
X	WO 02/085302 A (BETH ISRAEL DEACONESS MEDICAL CENTER, INC; POTHOUKAKIS, CHARALABOS; MA) 31 October 2002 (2002-10-31) page 11, line 14 - line 30; claims 1,6,31 ----- -/--	1-6,8-25



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

18 July 2005

Date of mailing of the international search report

29/07/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Bochelen, D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/003604

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRZOWSKI T ET AL: "Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions" JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, vol. 52, no. 4 Part 1, December 2001 (2001-12), pages 583-602, XP002336473 ISSN: 0867-5910 page 588 - page 590; tables 1,2 -----	1,2, 7-14,17, 18
A	GUILMEAU S ET AL: "Gastric leptin: a new manager of gastrointestinal function" CURRENT OPINION IN PHARMACOLOGY, ELSEVIER SCIENCE PUBLISHERS,, NL, vol. 4, no. 6, 3 October 2004 (2004-10-03), pages 561-566, XP004624568 ISSN: 1471-4892 the whole document -----	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2004/003604

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: —
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB2004/003604

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6630444	B1	07-10-2003	NONE
WO 02085302	A	31-10-2002	WO 02085302 A2 31-10-2002
		US 2004229783	A1 18-11-2004