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(54) Title: SUBSTANTIALLY PURE ZONULIN, A PHYSIOLOGICAL MODULATOR OF MAMMALIAN TIGHT JUNCTIONS

(57) Abstract

A substantially pure mammalian protein, hereinafter "zonulin", that is a physiological modulator of mammalian tight junctions is disclosed, as well as methods for the use of the same.
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SUBSTANTIALLY PURE ZONULIN, A PHYSIOLOGICAL MODULATOR OF MAMMALIAN TIGHT JUNCTIONS

The development of the present invention was supported by the University of Maryland, Baltimore, Maryland.

FIELD OF THE INVENTION

The present invention relates to a substantially pure mammalian protein, hereinafter "zonulin", that is a physiological modulator of mammalian tight junctions, as well as methods for the use of the same.

BACKGROUND OF THE INVENTION

I. Function and Regulation of Intestinal Tight Junctions

The tj or zonula occludens (hereinafter "ZO") are one of the hallmarks of absorptive and secretory epithelia (Madara, J. Clin. Invest., 83:1089-1094 (1989); and Madara, Textbook of Secretory Diarrhea Eds. Lebenthal et al, Chapter 11, pages 125-138 (1990). As a barrier between apical and basolateral compartments, they selectively regulate the passive diffusion of ions and water-soluble solutes through the paracellular pathway (Gumbiner, Am. J. Physiol., 253 (Cell Physiol. 22):C749-C758 (1987)). This barrier maintains any gradient generated by the activity of pathways associated with the transcellular route (Diamond, Physiologist, 20:10-18 (1977)).

Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway, since the resistances of enterocyte plasma membranes are relatively high (Madara, supra). The ZO represents the major barrier in this paracellular pathway, and the electrical resistance of epithelial tissues seems to depend on the number of transmembrane protein strands, and their complexity in the ZO, as observed by freeze-fracture electron microscopy (Madara et al, J. Cell Biol., 101:2124-2133 (1985)).

Six proteins have been identified in a cytoplasmic submembranous plaque underlying membrane contacts, but their function remains to be established (Diamond, supra). ZO-1 and ZO-2 exist as a heterodimer (Gumbiner et al, Proc. Natl. Acad. Sci., USA, 88:3460-3464 (1991)) in a detergent-stable complex with an uncharacterized 130 kD protein (ZO-3). Most immunoelectron microscopic studies have localized

To meet the many diverse physiological and pathological challenges to which epithelia are subjected, the ZO must be capable of rapid and coordinated responses that require the presence of a complex regulatory system. The precise characterization of the mechanisms involved in the assembly and regulation of the ZO is an area of current active investigation.

There is now a body of evidence that tj structural and functional linkages exist between the actin cytoskeleton and the tj complex of absorptive
cells (Gumbiner et al, supra; Madara et al, supra; and Drenchahn et al, J. Cell Biol., 107:1037-1048 (1988)). The actin cytoskeleton is composed of a complicated meshwork of microfilaments whose precise geometry is regulated by a large cadre of actin-binding proteins. An example of how the state of phosphorylation of an actin-binding protein might regulate cytoskeletal linking to the cell plasma membrane is the myristoylated alanine-rich C kinase substrate (hereinafter "MARCKS"). MARCKS is a specific protein kinase C (hereinafter "PKC") substrate that is associated with the cytoplasmic face of the plasma membrane (Aderem, Elsevier Sci. Pub. (UK), pages 438-443 (1992)). In its non-phosphorylated form, MARCKS crosslinks to the membrane actin. Thus, it is likely that the actin meshwork associated with the membrane via MARCKS is relatively rigid (Hartwig et al, Nature, 356:618-622 (1992)). Activated PKC phosphorylates MARCKS, which is released from the membrane (Rosen et al, J. Exp. Med., 172:1211-1215 (1990); and Thelen et al, Nature, 351:320-322 (1991)). The actin linked to MARCKS is likely to be spatially separated from the membrane and be more plastic. When MARCKS is dephosphorylated, it returns to the membrane where it once again crosslinks actin (Hartwig et al, supra; and Thelen et al, supra). These data suggest that the F-actin network may be rearranged by a PKC-dependent phosphorylation process that involves actin-binding proteins (MARCKS being one of them).

A variety of intracellular mediators have been shown to alter tj function and/or structure. Tight junctions of amphibian gallbladder (Duffey et al, Nature, 204:451-452 (1981)), and both goldfish (Bakker et al, Am. J. Physiol., 246:G213-G217 (1984))

II. Zonula Occludens Toxin

Most _Vibrio cholerae_ vaccine candidates constructed by deleting the ctxA gene encoding cholera toxin (CT) are able to elicit high antibody responses, but more than one-half of the vaccinees still develop mild diarrhea (Levine et al, _Infect. Immun._, 56(1):161-167 (1988)). Given the magnitude of the diarrhea induced in the absence of CT, it was hypothesized that _V. cholerae_ produce other enterotoxigenic factors, which are still present in strains deleted of the ctxA sequence (Levine et al, supra). As a result, a second toxin, zonula occludens toxin (hereinafter "ZOT") elaborated by _V. cholerae_ and which contribute to the residual diarrhea, was discovered (Fasano et al, _Proc. Natl. Acad. Sci._, _USA_, 8:5242-5246 (1991)). The zot gene is located immediately adjacent to the ctx genes. The high percent concurrence of the zot gene with the ctx genes among _V. cholerae_ strains (Johnson et al, _J. Clin. Microbiol._, 31/3:732-733 (1993); and Karasawa et al, _FEBS Microbiology Letters_, 106:143-146 (1993)) suggests a
possible synergistic role of ZOT in the causation of acute dehydrating diarrhea typical of cholera. Recently, the zot gene has also been identified in other enteric pathogens (Tschape, 2nd Asian-Pacific Symposium on Typhoid fever and other Salmonellosis, 47(Abstr.) (1994)).

It has been previously found that, when tested on rabbit ileal mucosa, ZOT increases the intestinal permeability by modulating the structure of intercellular tj (Fasano et al, supra). It has been found that as a consequence of modification of the paracellular pathway, the intestinal mucosa becomes more permeable. It also was found that ZOT does not affect Na+-glucose coupled active transport, is not cytotoxic, and fails to completely abolish the transepithelial resistance (Fasano et al, supra).

More recently, it has been found that ZOT is capable of reversibly opening tj in the intestinal mucosa, and thus ZOT, when co-administered with a therapeutic agent, is able to effect intestinal delivery of the therapeutic agent, when employed in an oral dosage composition for intestinal drug delivery (WO 96/37196; U.S. Patent Application Serial No. 08/443,864, filed May 24, 1995; and U.S. Patent Application Serial 08/598,852, filed February 9, 1996; and Fasano et al, J. Clin. Invest., 99:1158-1164 (1997); each of which is incorporated by reference herein in their entirety). It has also been found that ZOT is capable of reversibly opening tj in the nasal mucosa, and thus ZOT, when co-administered with a therapeutic agent, is able to enhance nasal absorption of a therapeutic agent (U.S. Patent Application Serial No. 08/781,057, filed January 9, 1997; which is incorporated by reference herein in its entirety).
In U.S. Patent Application Serial No. 08/803,364, filed February 20, 1997; which is incorporated by reference herein in its entirety, a ZOT receptor has been identified and purified from an intestinal cell line. The ZOT receptor represents the first step of the paracellular pathway involved in the regulation of intestinal and nasal permeability.

III. The Blood-Brain Barrier

The blood-brain barrier (BBB) is an extremely thin membranous barrier that is highly resistant to solute free diffusion, and separates blood and the brain. In molecular dimensions, the movement of drugs or solute through this membrane is essentially nil, unless the compound has access to one of several specialized enzyme-like transport mechanisms that are emebbed within the BBB membrane. The BBB is composed of multiple cells rather than a single layer of epithelial cells. Of the four different types of cells that compose the BBB (endothelial cells, pericytes, astrocytes, and neurons) the endothelial cell component of the capillaries represents the limiting factor for the permeability of the BBB. The capillary endothelium in vertebrate brain and spinal cord is endowed with tj which closes the interendothelial pores that normally exist in microvascular endothelial barriers in peripheral tissues. Ultimately, endothelial tj are responsible for the limited permeability of the BBB.

Heretofore, no efficient systems have been available to efficiently deliver compounds through the
BBB, unless they belong to one of the following categories:

1. Lipid-soluble drugs with a molecular weight less than 600 Daltons, e.g., progesterone, aldosterone, or cortisol;

2. Drugs that bind to plasma proteins, e.g., propranolol, bupivacaine, imipramine, diazepam, or cyclosporin;

3. Nutrients that bind to special carrier-mediated transport systems that are embedded within both the lumenal and antilumenal membranes of the BBB, e.g., glucose, glutamate, or phenylalanine; or

4. Peptides that bind to specific receptors that mediate the peptide transcytosis through the BBB, e.g., insulin and transferrin.

In the present invention, mammalian proteins that are immunologically and functionally related to ZOT, and that function as the physiological modulator of mammalian tight junctions, have been identified and purified. These mammalian proteins, referred to as "zonulin", are useful for enhancing absorption of therapeutic agents across TJ of intestinal and nasal mucosa, as well as across TJ of the BBB.

**SUMMARY OF THE INVENTION**

An object of the present invention is to identify a mammalian protein that is immunologically and functionally related to ZOT, and that functions as the physiological modulator of mammalian tight junctions.

Another object of the present invention is to purify and characterize said mammalian protein.
Still another object of the present invention is to use said mammalian protein as an enhancer of the delivery of a therapeutic agent across mammalian tight junctions.

Yet another object of the present invention is to use said mammalian protein as an enhancer of intestinal delivery of a therapeutic agent.

An additional object of the present invention is to use said mammalian protein as an enhancer of nasal delivery of a therapeutic agent.

Still another object of the present invention is to use said mammalian protein as an enhancer for the delivery of a therapeutic agent across the blood-brain barrier.

A further object of the present invention is to provide pharmaceutical compositions effective for delivery of a therapeutic agent.

These and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met, in one embodiment, by substantially pure zonulin having an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions.

In a preferred embodiment, the above-described objects of the present invention have been met by substantially pure zonulin having an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, and
an N-terminal amino acid sequence selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala Gly Val Thr
    Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);

(2) Glu Val Gln Leu Val Glu Ser Gly Gly Gly
    Leu Val Gln Pro Gly Gly Ser Leu Arg Leu
    (SEQ ID NO:2);

(3) Val Thr Phe Tyr Thr Asp Ala Val Ser
    (SEQ ID NO:3); and

(4) Met Leu Gln Lys Ala Glu Ser Gly Gly Val
    Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu
    (SEQ ID NO:4), or

an internal amino acid sequence selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp
    Leu (SEQ ID NO:8); and

(2) Leu Ser Glu Val Thr Ala Val Pro Ser Leu
    Asn Gly Gly (SEQ ID NO:9).

In another embodiment, the above-described objects of the present invention have been met by a pharmaceutical composition for delivery of a therapeutic agent comprising:

(A) a therapeutic agent; and

(B) an absorption enhancing effective amount of purified zonulin, wherein said zonulin has an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions, and preferably,
an N-terminal amino acid sequence
selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala
    Gly Val Thr Ala Tyr Asp Tyr
    Leu Val Ile Gln (SEQ ID NO:1);

(2) Glu Val Gln Leu Val Glu Ser
    Gly Gly Gly Leu Val Gln Pro
    Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);

(3) Val Thr Phe Tyr Thr Asp Ala
    Val Ser (SEQ ID NO:3); and

(4) Met Leu Gln Lys Ala Glu Ser
    Gly Gly Val Leu Val Gln Pro
    Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), or

an internal amino acid sequence
selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys
    Val Gly Asp Leu (SEQ ID NO:8); and

(2) Leu Ser Glu Val Thr Ala Val
    Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

In still another embodiment, the above-described objects of the present invention have been met by a method for delivery of a therapeutic agent comprising administering, to a subject in need thereof, a pharmaceutical composition comprising:

(A) a therapeutic agent; and

(B) an absorption enhancing effective amount of purified zonulin, wherein said zonulin has an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel
electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions, and preferably, an N-terminal amino acid sequence selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);

(2) Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);

(3) Val Thr Phe Tyr Thr Asp Ala Val Ser (SEQ ID NO:3); and

(4) Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), or

an internal amino acid sequence selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu (SEQ ID NO:8); and

(2) Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of zonulin purified from rabbit intestine (■), as compared to various negative controls (Fraction 2 (○); Fraction 3 (●); Fraction 4 (▲); and Fraction 5 (□) from a Q-Sepharose
column), on the tissue resistance (Rt) of CaCo2 cell monolayers.

Figure 2 shows the effect of zonulin purified from rabbit intestine (■), as compared to the negative control (□), on the tissue resistance (Rt) of rabbit ileum mounted in Ussing chambers.

Figure 3 shows the effect of zonulin purified from rabbit intestine (■), as compared to the negative controls (zonulin + anti-ZOT antibody (□); zonulin + anti-tau antibody (▲); and tau (▲)), on the tissue resistance (Rt) of rabbit ileum mounted in Ussing chambers.

Figures 4A and 4B show the effect of zonulin purified from either human heart (▲) or human brain (□), as compared to the negative control (■), on the tissue resistance (Rt) of Rhesus monkey jejunum (Figure 4A) and Rhesus monkey ileum (Figure 4B) mounted in Ussing chambers.

Figures 5A and 5B show the effect of zonulin purified from either human heart (▲) or human brain (□), as compared to the negative control (■), on the tissue resistance (Rt) of rabbit jejunum (Figure 5A) and rabbit ileum (Figure 5B) mounted in Ussing chambers.

Figure 6 shows a comparison of the N-terminal sequence of zonulin purified from rabbit and human tissues.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, in one embodiment, the above-described object of the present invention have been met by substantially pure zonulin having an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal
antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions, and preferably, an N-terminal amino acid sequence selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);
(2) Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);
(3) Val Thr Phe Tyr Thr Asp Ala Val Ser (SEQ ID NO:3); and
(4) Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Agr Leu (SEQ ID NO:4), or

an internal amino acid sequence selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu (SEQ ID NO:8); and
(2) Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

Zonulin is produced by, or found in, various mammalian cells and tissues, e.g., rabbit or human cells/tissue. The particular mammalian cell/tissue type from which zonulin is derived is not critical to the present invention. Examples of such mammalian tissue types include heart, lung, intestine, liver, brain, kidney, spleen and pancreas.

Zonulin can be obtained and purified, e.g., by affinity-purification chromatography using anti-ZOT antibodies, as described in Example 3 below.

As used herein, "zonulin biological activity" means the ability to reversibly increase permeability by modulating the structure of intercellular tj.
Thus, in another embodiment, the above-described objects of the present invention have been met by a pharmaceutical composition for delivery of a therapeutic agent comprising:

(A) a therapeutic agent; and
(B) an absorption enhancing effective amount of purified zonulin.

The pharmaceutical composition is preferably an oral dosage composition for intestinal delivery, a nasal dosage composition for nasal delivery, or an intravenous dosage composition for the delivery through the blood-brain barrier.

The oral dosage composition for intestinal delivery of a therapeutic agent comprises:

(A) a therapeutic agent; and
(B) an intestinal absorption enhancing effective amount of purified zonulin.


Tablets are made gastroresistant by the addition of, e.g., either cellulose acetate phthalate or cellulose acetate terephthalate.
Capsules are solid dosage forms in which the therapeutic agent(s) is enclosed in either a hard or soft, soluble container or shell of gelatin. The gelatin used in the manufacture of capsules is obtained from collagenous material by hydrolysis. There are two types of gelatin. Type A, derived from pork skins by acid processing, and Type B, obtained from bones and animal skins by alkaline processing. The use of hard gelatin capsules permit a choice in prescribing a single therapeutic agent or a combination thereof at the exact dosage level considered best for the individual subject. The hard gelatin capsule consists of two sections, one slipping over the other, thus completely surrounding the therapeutic agent. These capsules are filled by introducing the therapeutic agent, or gastroresistant beads containing the therapeutic agent, into the longer end of the capsule, and then slipping on the cap. Hard gelatin capsules are made largely from gelatin, FD&C colorants, and sometimes an opacifying agent, such as titanium dioxide. The USP permits the gelatin for this purpose to contain 0.15% (w/v) sulfur dioxide to prevent decomposition during manufacture.

In the context of the present invention, oral dosage compositions for small intestinal delivery also include liquid compositions which contain aqueous buffering agents that prevent the therapeutic agent and zonulin from being significantly inactivated by gastric fluids in the stomach, thereby allowing the therapeutic agent and zonulin to reach the small intestines in an active form. Examples of such aqueous buffering agents which can be employed in the present invention include bicarbonate buffer (pH 5.5 to 8.7, preferably about pH 7.4).
When the oral dosage composition is a liquid composition, it is preferable that the composition be prepared just prior to administration so as to minimize stability problems. In this case, the liquid composition can be prepared by dissolving lyophilized therapeutic agent and zonulin in the aqueous buffering agent.

The nasal dosage composition for nasal delivery of a therapeutic agent comprises:

(A) a therapeutic agent; and
(B) an nasal absorption enhancing effective amount of purified zonulin.

Nasal dosage compositions for nasal delivery are well-known in the art. Such nasal dosage compositions generally comprise water-soluble polymers that have been used extensively to prepare pharmaceutical dosage forms (Martin et al, In: Physical Chemical Principles of Pharmaceutical Sciences, 3rd Ed., pages 592-638 (1983)) that can serve as carriers for peptides for nasal administration (Davis, In: Delivery Systems for Peptide Drugs, 125:1-21 (1986)). The nasal absorption of peptides embedded in polymer matrices has been shown to be enhanced through retardation of nasal mucociliary clearance (Illum et al, Int. J. Pharm., 46:261-265 (1988)). Other possible enhancement mechanisms include an increased concentration gradient or decreased diffusion path for peptides absorption (Ting et al, Pharm. Res., 2:1330-1335 (1992)). However, reduction in mucociliary clearance rate has been predicted to be a good approach toward achievement or reproducible bioavailability of nasally administered systemic drugs (Gonda et al, Pharm. Res., 7:69-75 (1990)). Microparticles with a diameter of about 50 μm are expected to deposit in the nasal cavity (Bjork et al, Int. J. Pharm., 62:187-192

The particular water-soluble polymer employed is not critical to the present invention, and can be selected from any of the well-known water-soluble polymers employed for nasal dosage forms. A typical example of a water-soluble polymer useful for nasal delivery is polyvinyl alcohol (PVA). This material is swellable hydrophilic polymer whose physical properties depend on the molecular weight, degree of hydrolysis, cross-linking density, and crystallinity (Peppas et al, In: Hydrogels in Medicine and Pharmacy, 2:109-131 (1987)). PVA can be used in the coating of dispersed materials through phase separation, spray-drying, spray-embedding, and spray-densation (Ting et al, supra).

The intravenous dosage composition for delivery of a therapeutic agent through the blood-brain barrier comprises:

(A) a therapeutic agent; and
(B) a blood-brain barrier absorption enhancing effective amount of purified zonulin.

Intravenous dosage compositions for delivery to the brain are well-known in the art. Such intravenous dosage compositions generally comprise a physiological diluent, e.g., distilled water, or 0.9% (w/v) NaCl.

A "nasal" delivery composition differs from an "intestinal" delivery composition in that the latter
must have gastroresistant properties in order to prevent the acidic degradation of the active agents (e.g., zonulin and the therapeutic agent) in the stomach, whereas the former generally comprises water-soluble polymers with a diameter of about 50 μm in order to reduce the mucociliary clearance, and to achieve a reproducible bioavailability of the nasally administered agents. An "intravenous" delivery composition differs from both the "nasal" and intestinal" delivery compositions in that there is no need for gastroresistance or water-soluble polymers in the "intravenous" delivery composition.

In still another embodiment, the above-described objects of the present invention have been met by a method for delivery of a therapeutic agent comprising administering, to a subject in need thereof, said pharmaceutical composition.

The mode of administration is not critical to the present. Although, it is preferable that the mode of administration is orally, for the intestinal delivery composition; intranasally, for the nasal delivery composition; and intravenously for delivery through the blood-brain barrier.

The particular therapeutic agent employed is not critical to the present invention, and can be, e.g., any drug compound, biologically active peptide, vaccine, or any other moiety otherwise not absorbed through the transcellular pathway, regardless of size or charge.

Examples of drug compounds which can be employed in the present invention include drugs which act on the cardiovascular system, drugs which act on the central nervous system, antineoplastic drugs and antibiotics.
Examples of drugs which act on the cardiovascular system which can be employed in the present invention include lidocaine, adenosine, dobutamine, dopamine, epinephrine, norepinephrine and phentolamine.

Examples of drugs which act on the central nervous system which can be employed in the present invention include doxapram, alfentanil, dezocin, nalbuphine, buprenorphine, naloxone, ketorolac, midazolam, propofol, metacurine, mivacurium and succinylcholine.

Examples of antineoplastic drugs which can be employed in the present include cytarabine, mitomycin, doxorubicin, vincristine and vinblastine.

Examples of antibiotics which can be employed in the present include methicillin, mezlocillin, piperacillin, cetofoxin, cefonicid, cefmetazole and aztreonam.

Examples of biologically active peptides which can be employed in the present invention include hormones, lymphokines, globulins, and albumins.

Examples of hormones which can be employed in the present invention include testosterone, nandrolone, menotropins, progesterone, insulin and urofolitropin.

Examples of lymphokines which can be employed in the present invention include interferon-α, interferon-β, interferon-γ, interleukin-1, interleukin-2, interleukin-4 and interleukin-8.

Examples of globulins which can be employed in the present invention include α-globulins, β-globulins and γ-globulins (immunoglobulin).

Examples of immunoglobulins which can be employed in the present invention include polyvalent IgG or specific IgG, IgA and IgM, e.g., anti-tetanus antibodies.
An example of albumin which can be employed in the present invention is human serum albumin and ovalbumin.

Examples of vaccines which can be employed in the present invention include peptide antigens and attenuated microorganisms and viruses.

Examples of peptide antigens which can be employed in the present invention include the B subunit of the heat-labile enterotoxin of enterotoxigenic *E. coli*, the B subunit of cholera toxin, capsular antigens of enteric pathogens, fimbriae or pili of enteric pathogens, HIV surface antigens, dust allergens and acari allergens.


When the therapeutic agent is insulin, the pharmaceutical composition of the present invention is useful for the treatment of diabetes.

The amount of therapeutic agent employed is not critical to the present invention and will vary depending upon the particular agent selected, the
disease or condition being treated, as well as the age, weight and sex of the subject being treated.

The amount of zonulin employed is also not critical to the present invention and will vary depending upon the age, weight and sex of the subject being treated. Generally, the final concentration of zonulin employed in the present invention to enhance absorption of the therapeutic agent is in the range of about $10^{-6}$ M to $10^{-14}$ M, preferably about $10^{-6}$ M to 5.0 x $10^{-8}$ M. To achieve such a final concentration in, e.g., the intestines, nose or blood, the amount of zonulin in a single pharmaceutical composition of the present invention will generally be about 40 ng to 1000 ng, preferably about 400 ng to 800 ng.

The ratio of therapeutic agent to zonulin employed is not critical to the present invention and will vary depending upon the amount of therapeutic agent to be delivered within the selected period of time. Generally, the weight ratio of therapeutic agent to zonulin employed in the present invention is in the range of about 1:10 to 3:1, preferably about 1:5 to 2:1.

Zonulin can be also be used as an immunogen to obtain antibodies, either polyclonal or monoclonal, having binding specificity for zonulin, using techniques well-known in the art (Abrams, *Methods Enzymol.*, 121:107-119 (1986)). These antibodies can in turn can be used to assay for zonulin in body tissue or fluids, or in affinity-purification of zonulin.

The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.
EXAMPLE 1
Purification of ZOT

5000 ml of the supernatant fraction obtained after culturing V. cholerae strain CVD110
(Michalski et al., Infect. Immun., 61:4462-4468 (1993), which had been transformed with plasmid pZ14, was
concentrated 1000-fold using a lamina flow filter with a MW cutoff of 10 kDa. The construction of pZ14,
which contains the Vibrio cholera zot gene, is described in detail in, inter alia, WO 96/37196. The
resulting supernatant was then subjected to 8.0% (w/v) SDS-PAGE. Protein bands were detected by Coomassie
blue staining of the SDS-PAGE gel. No protein band corresponding to ZOT was detectable when compared to
control supernatant from strain CVD110 transformed with plasmid pTTQ181 (Amersham, Arlington Heights,
IL), and treated in the same manner. Therefore, even though the zot gene was placed behind the highly
inducible and strong tac promoter in pZ14, the level of the protein in 1000-fold concentrated pZ14
supernatant was still not detectable by the Coomassie stained SDS-PAGE gel.

Hence, to increase the amount of ZOT produced, the zot gene was fused in frame with the maltose
binding protein (hereinafter "MBP") gene to create a MBP-ZOT fusion protein.

The MBP vector pMAL-c2 (Biolab) was used to express and purify ZOT by fusing the zot gene to the
maLE gene of E. coli. This construct uses the strong, inducible tac promoter, and the maLE translation
initiation signals to give high level expression of the cloned zot gene. The vector pMAL-c2 has an exact
deletion of the maLE signal sequence, which leads to cytoplasmic expression of the fusion protein.

Affinity chromatography purification for MBP was used
to facilitate isolation of the fusion protein (Biolab).

More specifically, vector pMAL-c2 was linearized with EcoRI (that cuts at the 3' end of the maleE gene), filled in with Klenow fragment, and digested with XbaI (that has a single site in pMAL-c2 polylinker). The orf encoding ZOT was subcloned from plasmid pBB241 (Baudry et al, Infect. Immun., 60:428-434 (1992)). Plasmid pBB241 was digested with BssHII, filled in with Klenow fragment, and digested with XbaI. Then, the blunt-XbaI fragment was subcloned into pMAL-c2 to give plasmid pLC10-c. Since both the insert, and the vector had blunt and sticky ends, the correct orientation was obtained with the 3' end of maleE fused with the 5' terminus of the insert. pLC10-c was then electroporated into E. coli strain DH5α. In pBB241, the BssHII restriction site is within the zot orf. Thus, amino acids 1-8 of ZOT are missing in the MBP-ZOT fusion protein.

In order to purify the MBP-ZOT fusion protein, 10 ml of Luria Bertani broth containing 0.2% (w/v) glucose and 100 µg/ml ampicillin were inoculated with a single colony containing pLC10-c, and incubated overnight at 37°C with shaking. The culture was diluted 1:100 in 1.0 ml of the same fresh medium, and grown at 37°C while shaking, to about 1.0 x 10^8 cells/ml. 0.2 mM IPTG was then added to induce the MBP-ZOT expression, and the culture was incubated at 37°C for additional 3 hr. The bacteria were then pelleted and resuspended in 20 ml of ice cold "column buffer" comprising 20 mM Tris-HCl, 0.2 M NaCl, 1.0 mM EDTA, 10 mM 2-ME, 1.0 mM NaN3. The bacterial suspension was lysed by french press treatment and spun for 30 min at 13,000 x g at 4°C. The supernatant was collected, diluted 1:5 with column
buffer and loaded into a 1 X 10 column of amylose resin (Biolabs, MBP-fusion purification system), pre-equilibrated with column buffer. After washing the column with 5 volumes of column buffer, the MBP-ZOT fusion protein was eluted by loading 10 ml of 10 mM maltose in column buffer. The typical yield from 1.0 ml of culture was 2-3 mg of protein.

The MBP fusion partner of the purified MBP-ZOT fusion protein was then cleaved off using 1.0 µg of Factor Xa protease (Biolabs) per 20 µg of MBP-ZOT. Factor Xa protease cleaves just before the amino terminus of ZOT. The ZOT protein so obtained was run on a 8.0% (w/v) SDS-PAGE gel, and electroeluted from the gel using an electroseparation chamber (Schleicher & Schuell, Keene, NH).

When tested in Ussing chambers, the resulting purified ZOT induced a dose-dependent decrease of Rt, with an ED\textsubscript{50} of 7.5 x 10\textsuperscript{-8} M.

**EXAMPLE 2**

**Production of Affinity-Purified Anti-ZOT Antibodies**

To obtain specific antiserum, a chimeric glutathione S-transferase (GST)-ZOT protein was expressed and purified.

More specifically, oligonucleotide primers were used to amplify the zot orf by polymerase chain reaction (PCR) using plasmid pBB241 (Baudry et al, supra) as template DNA. The forward primer (TCATCACGGC CGGCCAGG, SEQ ID NO:5) corresponded to nucleotides 15-32 of zot orf, and the reverse primer (GGAGSTCTTAG AATCTGCCCG AT, SEQ ID NO:6) corresponded to the 5' end of ctxA orf. Therefore, amino acids 1-5 of ZOT were missing in the resulting fusion protein. The amplification product was inserted into the polylinker (SmaI site) located at the end of the GST
gene in pGEX-2T (Pharmacia, Milwaukee, WI). pGEX-2T is a fusion-protein expression vector that expresses a cloned gene as a fusion protein with GST of *Schistosoma japonicum*. The fusion gene is under the control of the tac promoter. Upon induction with IPTG, derepression occurs and GST fusion protein is expressed.

The resulting recombinant plasmid, named pLC11, was electroporated in *E. coli* DH5α. In order to purify GST-ZOT fusion protein, 10 ml of Luria Bertani broth containing 100 µg/ml ampicillin were inoculated with a single colony containing pLC11, and incubated overnight at 37°C with shaking. The culture was diluted 1:100 in 1.0 ml of the same fresh medium and grown at 37°C while shaking, to about 1.0 x 10^8 cells/ml. 0.2 mM IPTG was then added to induce the GST-ZOT expression, and the culture was incubated at 37°C for additional 3 hr. The bacteria were then pelleted, resuspended in 20 ml of ice cold PBS (pH 7.4), and lysed by the french press method. The GST-ZOT fusion protein was not soluble under these conditions as it sedimented with the bacterial pellet fraction. Therefore, the pellet was resuspended in Laemli lysis buffer comprising 0.00625 M Tris-HCl (pH 6.8), 0.2 M 2-ME, 2.0% (w/v) SDS, 0.025% (w/v) bromophenol blue and 10% (v/v) glycerol, and subjected to electrophoresis on a 8.0% (w/v) PAGE-SDS gel, and stained with Coomassie brilliant blue. A band of about 70 kDa (26 kDa of GST + 44 kDa of ZOT), corresponding to the fusion protein, was electroeluted from the gel using an electroseparation chamber (Schleicher & Schuell, Keene, NH).

10 µg of the resulting eluted protein (10-20 µg) was injected into a rabbit mixed with an equal volume of Freund's complete adjuvant. Two booster doses were
administered with Freund's incomplete adjuvant four and eight weeks later. One month later the rabbit was bled.

To determine the production of specific antibodies, 10^{-10} M of ZOT, along with the two fusion proteins MBP-ZOT and GST-ZOT, was transferred onto a nylon membrane and incubated with a 1:5000 dilution of the rabbit antiserum overnight at 4{\degree}C with moderate shaking. The filter was then washed 15 min 4 times with PBS containing 0.05% (v/v) Tween 20 (hereinafter "PBS-T"), and incubated with a 1:30,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 2 hr at room temperature. The filter was washed again for 15 min 4 times with PBS containing 0.1% (v/v) Tween, and immunoreactive bands were detected using enhanced chemiluminescence (Amersham).

On immunoblot, the rabbit antiserum was found to recognize ZOT, as well as MBP-ZOT and GST-ZOT fusion proteins, but not the MBP negative control.

Moreover, to confirm the production of appropriate anti-ZOT antibodies, neutralization experiments were conducted in Ussing chambers. When pre-incubated with pZ14 supernatant at 37{\degree}C for 60 min, the ZOT-specific antiserum (1:100 dilution), was able to completely neutralize the decrease in {R} induced by ZOT on rabbit ileum mounted in Ussing chambers.

Next, the anti-ZOT antibodies were affinity-purified using an MBP-ZOT affinity column. More specifically, a MBP-ZOT affinity column was prepared by immobilizing, overnight at room temperature, 1.0 mg of purified MBP-ZOT, obtained as described in Example 1 above, to a pre-activated gel (Aminolink, Pierce). The column was washed with PBS,
and then loaded with 2.0 ml of anti-ZOT rabbit antiserum. After a 90 min incubation at room temperature, the column was washed with 14 ml of PBS, and the specific anti-ZOT antibodies were eluted from the column with 4.0 ml of a solution comprising 50 mM glycine (pH 2.5), 150 mM NaCl, and 0.1% (v/v) Triton X-100. The pH of the 1.0 ml eluted fractions was immediately neutralized with 1.0 N NaOH.

EXAMPLE 3

Purification of Zonulin

Based upon the observation in U.S. Patent Application Serial No. 08/803,364, filed February 20, 1997, that ZOT interacts with a specific epithelial surface receptor, with subsequent activation of a complex intracellular cascade of events that regulate tj permeability, it was postulated in the present invention that ZOT may mimic the effect of a physiological modulator of mammalian tj. It was postulated in the present invention that ZOT, and its physiological analog (zonulin), would be functionally and immunologically related. Therefore, affinity-purified anti-ZOT antibodies and the Ussing chamber assay were used in combination to search for zonulin in various rabbit and human tissues.

A. Rabbit Tissues

Initially, zonulin was purified from rabbit intestine. The tissue was disrupted by homogenization in PBS. The resulting cell preparations were then centrifuged at 40,000 rpm for 30 min, the supernatant collected and lyophilized. The resulting lyophilized product was subsequently reconstituted in PBS (10:1 (v/v)), filtered through a 0.45 mm membrane filter, loaded onto a Sephadex G-50 chromatographic
column, and eluted with PBS. Then, 2.0 ml fractions obtained from the column were subjected to standard Western immunoblotting using the affinity-purified anti-ZOT antibodies obtained as described in Example 2 above.

Positive fractions, i.e., those to which the anti-ZOT antibodies bound, were combined, lyophilized, reconstituted in PBS (1:1 (v/v)), and subjected to salt gradient chromatography through a Q-Sepharose column. The salt gradient was 0 - 100% (w/v) NaCl in 50 mM Tris buffer (pH 8.0). Five 20 ml fractions were collected, and subjected to standard Western immunoblotting using the affinity-purified anti-ZOT antibodies obtained as described in Example 2 above. Fraction 1 (20% (w/v) NaCl) was the only fraction that was found to be positive in the Western immunoblot assay.

The fractions obtained from the Q-Sepharose column were then tested for their tissue resistance effects on both CaCo2 monolayers, and rabbit small intestine in Ussing chambers.

More specifically, CaCo2 cells were grown in cell-culture flasks (Falcon) under humidified atmosphere of 95% O₂/5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal-calf serum, 40 μg/l penicillin and 90 μg/l streptomycin. The cells were subcultured at a surface ratio of 1:5 after trypsin treatment every 5 days, when they had reached 70-80% confluence. The passage number of the cells used in the this study varied between 15 and 30.

The CaCo2 monolayers were grown to confluence (12-14 days after plating at a 1:2.5 surface ratio) on tissue-culture-treated polycarbonate filters firmly attached to a polystyrene ring (6.4 mm diameter,
Transwell Costar). The filters were placed in a tightly fitting insert separating the serosal and mucosal compartment of a modified Ussing chamber, and the experiments were carried out as described by Fasano et al, *Proc. Natl. Acad. Sci., USA*, 8:5242-5246 (1991), for rabbit intestines in Ussing chambers. The results are shown in Figure 1.

As shown in Figure 1, the zonulin-containing fraction induced a significant reduction of CaCo2 monolayers' resistance, as compared to zonulin-negative fractions.

Next, Ussing chamber assays were carried out using ileum from 2-3 kg adult male New Zealand white rabbits, which were sacrificed by cervical dislocation. A 20 cm segment of ileum was removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa so prepared were then mounted in lucite Ussing chambers (1.12 cm³ opening), connected to a voltage clamp apparatus (EVC 4000 WPI, Saratosa, FL), and bathed with freshly prepared Ringer's solution comprising 53 mM NaCl, 5.0 mM KCl, 30.5 mM mannitol, 1.69 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 1.25 mM CaCl₂, 1.1 mM MgCl₂, and 25 mM NaHCO₃. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O₂/5% CO₂.

100 μl of zonulin purified from rabbit intestine was added to the mucosal side. The potential difference (PD) was measured every 10 min, and the short-circuit current (Isc) and tissue resistance (Rt) were calculated as described by Fasano et al, supra. Because of tissue variability, data were calculated as
ΔRt (Rt at time x) - (Rt at time 0). The results are shown in Figure 2.

As shown in Figure 2, the zonulin-containing fraction induced a significant reduction in rabbit small intestinal resistance, as compared to a zonulin-negative fraction. This effect was completely reversible once zonulin was withdrawn from the reservoir.

The zonulin-positive fraction was also subjected to 8.0% (w/v) SDS-PAGE, followed by Western immunoblotting using the anti-ZOT antibodies. The protein bands separated by SDS-PAGE were then transferred onto PVDF filter (Millipore) using CAPS buffer comprising 100 ml of (3-[cyclohexylaminoc]-1 propanesulfonic acid) 10x, 100 ml of methanol, 800 ml of distilled water. The protein that aligned to a single band that was detected by Western immunoblotting had an apparent molecular weight of about 47 kDa. This band was cut out from the PVDF filter, and subjected to N-terminal sequencing as described by Hunkapiller, In: Methods of Protein Microcharacterization, Ed. Shibley, Chapters 11-12, Humana Press, pages 315-334 (1985), using a Perkin-Elmer Applied Biosystems Apparatus Model 494. The N-terminal sequence of zonulin purified from rabbit intestine is shown in SEQ ID NO:1.

The rabbit zonulin N-terminal sequence was compared to other protein sequences by BLAST search analysis. The result of this analysis revealed that the N-terminal sequence of rabbit zonulin is 85% identical, and 100% similar, to the N-terminal sequence of tau protein from Homo sapiens.

As a result, to determine whether rabbit zonulin and tau are the same moiety, cross-neutralization experiments were conducted in Ussing chambers. More
specifically, 10 μl/ml of rabbit zonulin was added to the mucosal side of rabbit ileum either untreated or pre-incubated for 60 min at 37°C with anti-tau antibodies (dilution 1:10) (Sigma). Both 10 μl/ml of rabbit zonulin pre-incubated with anti-ZOT antibodies (dilution 1:10) (Example 2); and 0.4 μg/ml of purified tau (Sigma), were used as controls. The results are shown in Figure 3.

As shown in Figure 3, rabbit zonulin induced the typical decrease of tissue resistance that was readily reversible once the protein was withdrawn from the Ussing chambers. This activity was completely neutralized by pre-treatment with anti-ZOT antibodies, but not by pre-treatment with anti-tau antibodies. On the other hand, there was no significant effect on tissue resistance in tissues exposed to tau protein.

Rabbit zonulin was also detected in various other rabbit tissues, i.e., rabbit heart, brain, muscle, stomach, spleen, lung, kidney, as well as various portions of rabbits intestines, i.e., distal jejunum, proximal jejunum, ileum, caecum and colon. That is, when these rabbit tissues were processed in the same manner as the rabbit intestine, discussed above, and subjected to 8.0% (w/v) SDS-PAGE, followed by Western immunoblotting using affinity-purified anti-ZOT antibodies obtained as described in Example 2 above, a single band of approximately 47 kDa in size was detected in all of the tissues tested.

B. Human Tissues

Zonulin was also purified from several human tissues, including intestine, heart, and brain. Both fetal and adult tissues were used. The tissues were disrupted by homogenization in PBS. The resulting cell preparations were then centrifuged at 40,000 rpm
for 30 min, the supernatant collected and lyophilized. The resulting lyophilized product was subsequently reconstituted in PBS (10:1 (v/v)), filtered through a 0.45 mm membrane filter, loaded onto a Sephadex G-50 chromatographic column, and eluted with PBS. Then, 2.0 ml fractions obtained from the column were subjected to standard Western immunoblotting using the affinity-purified anti-ZOT antibodies obtained as described in Example 2 above.

Positive fractions, i.e., those to which the anti-ZOT antibodies bound, were combined, lyophilized, reconstituted in PBS (1:1 (v/v)), and subjected to salt gradient chromatography through a Q-Sepharose column. The salt gradient was 0 - 100% (w/v) NaCl in 50 mM Tris buffer (pH 7.4). Five 20 ml fractions were collected, and subjected to standard Western immunoblotting using the affinity-purified anti-ZOT antibodies obtained as described in Example 2 above. Fraction 1 (20% (w/v) NaCl) showed a single band of 47 kDa in size in the Western immunoblot assay. Fraction 2 (40% (w/v) NaCl) showed two additional bands of 35 kDa and 15 kDa in size in the Western immunoblot assay. Fraction 3 (60% (w/v) NaCl) and Fraction 4 (80% (w/v) showed only the 35 kDa and 15 kDa bands. These results suggest that zonulin may be subjected to degradation by proteases, probably present in the human tissues used, and that the breakdown products elute from the column at higher salt concentrations as compared to the holoprotein.

Fraction 1 (from both human heart and brain tissues) and Fraction 4 (from heart tissue) obtained from the Q-Sepharose column were then tested for their tissue resistance effects on both rabbit intestine and Rhesus monkey intestine in Ussing chambers.
Ussing chamber assays were carried out using different tracts of intestine, including jejunum, ileum, or colon from either 2-3 kg adult male New Zealand white rabbits, or 5-6 kg adult male Rhesus monkeys. After the animals were sacrificed, different segments of intestine, including jejunum, ileum, and colon, were removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa so prepared (three jejunum, three ileum, and two colon) were then mounted in lucite Ussing chambers (1.12 cm² opening), connected to a voltage clamp apparatus (EVC 4000 WPI, Saratosa, FL), and bathed with freshly prepared Ringer's solution comprising 53 mM NaCl, 5.0 mM KCl, 30.5 mM mannitol, 1.69 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 1.25 mM CaCl₂, 1.1 mM MgCl₂, and 25 mM NaHCO₃. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O₂/5% CO₂.

100 µl of Fraction 1 of zonulin purified from human heart or Fraction 1 of zonulin purified from human brain, or Fraction 4 purified from human heart, was added to the mucosal side. The potential difference (PD) was measured every 10 min, and the short-circuit current (Isc) and tissue resistance (Rt) were calculated as described by Fasano et al, supra. Because of tissue variability, data were calculated as ΔRt (Rt at time x) − (Rt at time 0). The results are shown in Figures 4A and 4B (monkey intestine) and Figures 5A and 5B (rabbit intestine).

As shown in Figures 4A and 4B, zonulin purified from human heart (Fraction 1) induced a significant reduction in monkey intestinal resistance (both jejunum (Figure 4A) and ileum (Figure 4B), as compared
to the PBS negative control. No significant changes were observed when zonulin purified from human heart was tested in the colon. Figures 4A and 4B also show that no significant effect on both monkey jejunum (Figure 4A) and monkey ileum (Figure 4B) was observed when zonulin purified from human brain (Fraction 1) was tested. Fraction 4 of zonulin purified from human heart also induced a significant decrease in monkey small intestinal tissue resistance.

As shown in Figures 5A and 5B, similar results were obtained when rabbit intestine was used. That is, zonulin purified from human heart (Fraction 1) showed a significant effect on tissue resistance both in the rabbit jejunum (Figure 5A) and rabbit ileum (Figure 5B), but not in the colon. Figures 5A and 5B also show that no significant effect on both rabbit jejunum (Figure 5A) and rabbit ileum (Figure 5B) was observed when zonulin purified from human brain (Fraction 1) was tested.

To establish whether zonulin increases the oral delivery of insulin, in vitro model experiments using rabbit intestine were performed. Briefly, adult male New Zealand white rabbits (2-3 kg) were sacrificed by cervical dislocation. Segments of rabbit small intestine (either jejunum or ileum) were removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa so prepared were then mounted in Lucite Ussing chambers (1.12 cm² opening), connected to a voltage clamp apparatus (EVC 4000 WPI, Sarasota, FL), and bathed with freshly prepared buffer containing 53 mM NaCl, 5.0 mM KCl, 30.5 mM mannitol, 1.69 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 1.25 mM CaCl₂, 1.1 mM MgCl₂, and 25 mM NaHCO₃. The bathing solution was maintained at 37°C with
water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O₂/5% CO₂. Potential difference (PD) was measured, and short-circuit current (Isc) and tissue resistance (Rt) were calculated. Once the tissues reached a steady state condition, paired tissues, matched on the basis of their resistance, were exposed luminally to 10⁻¹¹ M ¹²⁵I-insulin (Amersham, Arlington Heights, IL; 2.0 μCi = 10⁻¹² M), alone or in the presence of 100 μl of heart zonulin from Fraction 1. A 1.0 ml aliquot from the serosal side and a 50 μl aliquot from the mucosal side were immediately obtained to establish baseline values. Samples from the serosal side were then collected at 20 min intervals for the following 100 min.

It was found that heart zonulin increased the intestinal absorption of insulin both in the jejunum (0.058 ± 0.003 fmol/cm².min vs. 0.12 ± 0.005 fmol/cm².min, untreated vs. zonulin-treated tissues, respectively, p=0.001), and in the ileum (0.006 ± 0.0002 fmol/cm².min vs. 0.018 ± 0.005 fmol/cm².min, untreated vs. zonulin-treated tissues, respectively, p=0.05) in a time-dependent manner.

Fraction 1 of zonulin purified from human heart and Fraction 1 of zonulin purified from human brain were also subjected to 8.0% (w/v) SDS-PAGE, followed by Western immunoblotting using the anti-ZOT antibodies obtained as described in Example 2 above. The protein bands separated by SDS-PAGE were then transferred onto PVDF filter using CAPS buffer comprising 100 ml of (3-[cyclohexylamino]-1 propanesulfonic acid) 10x, 100 ml of methanol, 800 ml of distilled water. The protein that aligned to a single band that was detected by Western immunoblotting had an apparent molecular weight of
about 47 kDa. This band was cut out from the PDVF filter, and subjected to N-terminal sequencing as described by Hunkapiller, In: Methods of Protein Microcharacterization, Ed. Shibley, Chapters 11-12, Humana Press, pages 315-334 (1985), using a Perkin-Elmer Applied Biosystems Apparatus Model 494. The N-terminal sequence of zonulin purified from adult human heart is shown in SEQ ID NO:2, and the N-terminal sequence of zonulin purified from adult human brain is shown in SEQ ID NO:3.

The first nine amino acids from the N-terminal sequence of zonulin purified from adult human intestine (SEQ ID NO:7) were also sequenced, and found to be identical to the first nine amino acids of zonulin purified from human heart shown in SEQ ID NO:2 (see Figure 6). The first twenty amino acids from the N-terminal sequence of zonulin purified from human fetal intestine were also sequenced: Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), and found to be almost identical to the amino acid sequence of zonulin purified from human heart shown in SEQ ID NO:2 (see Figure 6).

The N-terminal sequence of zonulin purified from adult human brain (SEQ ID NO:3) was completely different than the N-terminal of zonulin purified from both heart and intestine (SEQ ID NO:2) (see Figure 6). This difference is believed to explain the tissue-specificity of zonulin in determining the permeability of tissues, such as the intestine, demonstrated above.

The N-terminal sequences of human zonulin purified from heart, intestine, and brain, all differ from the N-terminal sequence of zonulin purified from rabbit intestine (Figure 6). To establish whether
these proteins represent different isoforms of a tau-related family of proteins, tissues from both rabbit and human were subjected to 8.0% (w/v) SDS-PAGE, followed by Western immunoblotting using either anti-ZOT or anti-tau antibodies. The 47 kDa zonulin bands purified from both rabbit and human tissues (including brain, intestine, and heart) which were found to be recognized by the anti-ZOT antibodies, were also found to cross-react with anti-tau antibodies. The different fractions of zonulin purified from human brain obtained by salt chromatography were also subjected to Western immunoblotting using either anti-ZOT antibodies or anti-tau antibodies. While anti-ZOT antibodies recognized the intact 47 kDa protein and both of the 35 kDa and 15 kDa breakdown fragments, the anti-tau antibodies only recognized the intact 47 kDa protein and the 35 kDa fragment, while the anti-tau antibodies did not recognize the 15 kDa fragment. To establish whether the 35 kDa fragment includes the N-terminus or the C-terminus of zonulin, the N-terminal sequence of the 35 kDa band was obtained and found to be: Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu (SEQ ID NO:8). This sequence is different from the N-terminal sequence of the intact human brain zonulin (SEQ ID NO:3). These results suggest that the 15 kDa fragment represents the N-terminal portion of zonulin, while the 35 kDa fragment represents the C-terminal portion of zonulin.

Combined together, these results suggest that the zonulin domain recognized by the anti-tau antibodies is toward the C-terminus of the protein, is common to the different isoforms of zonulin from either human or rabbit tissues (while the N-terminal portion may vary), and is probably involved in the permeabilizing
effect of the protein (based on the observation that tau binds to β-tubulin with subsequent rearrangement of the cell cytoskeleton, and the effect of Fraction 4 on monkey small intestinal tissue resistance).

The N-terminal sequence of human zonulin purified from both the heart and intestine was compared to other protein sequences by BLAST search analysis. The result of this analysis revealed that the N-terminal sequence of human zonulin is 95% identical to the N-terminal sequence of the heavy variable chain of IgM from Homo sapiens.

As a result, to determine whether human zonulin purified from heart and human IgM are the same moiety, a partial digestion of the human zonulin was performed to obtain an internal fragment, which was then sequenced.

More specifically, 1.0 mm of the PVDF filter containing zonulin purified from human heart was placed in a plastic tube previously washed with 0.1% (w/v) trifluoracetic acid (TFA), and rinsed with methanol. 75 µl of a buffer solution comprising 100 mM Tris (pH 8.2), 10% (v/v) CH₃CN, and 1.0% (v/v) dehydrogenated Triton X-100 was added, and incubated with the membrane at 37°C for 60 min. 150 ng of trypsin was then added, and an additional 24 h incubation period at 37°C was carried out. The resulting solution was sonicated for 10 min, and the supernatant decanted. 75 µl of 0.1% (w/v) TFA was then added, the solution was sonicated for additional 10 min, and the supernatant decanted. Both aliquots were loaded on a 0.5 mm x 250 mm C₁₈ column, 5.0 µm particle size, 300 Å pore size. A gradient from 0.1% (w/v) TFA to 45% CH₃CN water + 0.1% (w/v) TFA was
developed for 2 h and 15 min. The peaks were finally collected and sequenced.

The internal sequence of human zonulin purified from adult human heart was found to be: Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

The human zonulin internal sequence was compared to other protein sequences by BLAST search analysis. The result of this analysis revealed that the internal sequence of human zonulin has 0% identity to any internal sequence of the heavy variable chain of IgM from Homo sapiens.

The results in Example 3 above demonstrate that (1) zonulin represents the physiological modulator of the paracellular pathway; (2) the N-terminal sequence of rabbit zonulin is highly homologous to the N-terminal sequence of the tau protein; (3) zonulin and tau are two distinct moieties that are immunologically related, yet functionally different; (4) the N-terminal sequence of human zonulin obtained from heart and intestine is highly homologous to the N-terminal sequence of the heavy chain of the variable region of IgM; (5) human zonulin and IgM are two distinct moieties that are structurally related, yet functionally different; and (6) zonulin represents a family of tau-related proteins with common, active C-terminal sequences, and variable N-terminal sequences.

While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: PASANO, Alessio
(ii) TITLE OF INVENTION: SUBSTANTIALLY PURE ZONULIN, A PHYSIOLOGICAL MODULATOR OF MAMMALIAN TIGHT JUNCTIONS
(iii) NUMBER OF SEQUENCES: 9
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SUGHRUE, MION, ZINN, MACPEAK & SEAS
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(C) CITY: Washington, D.C.
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20037
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 21 MAY 1997
(C) CLASSIFICATION:
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KIT, Gordon
(B) REGISTRATION NUMBER: 30,764
(C) REFERENCE/DOCKET NUMBER: A-6901
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 293-7060
(B) TELEFAX: (202) 293-7860

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr
   1     5    10

Asp Tyr Leu Val Ile Gln
   15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val
   1     5    10

Gln Pro Gly Gly Ser Leu Arg Leu
   15    20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Val Thr Phe Tyr Thr Asp Ala Val Ser
   1     5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

SUBSTITUTE SHEET (RULE 26)
(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val
1     5
Gln Pro Gly Xaa Ser Asn Arg Leu
15    20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCATCACGGC GCGCCAGG
18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGGTCTAG AATCTGCCCG AT
22
(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 11 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Val Gln Leu Val Glu Ser Gly Gly Xaa Leu
1  5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 11 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu
1  5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 13 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly
1  5

**WHAT IS CLAIMED:**

Claim 1. Substantially pure zonulin having an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions.

Claim 2. The substantially pure zonulin as claimed in Claim 1, wherein said zonulin has:

an N-terminal amino acid sequence selected from the group consisting of:

1. Asn Gln Arg Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);
2. Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);
3. Val Thr Phe Tyr Thr Asp Ala Val Ser (SEQ ID NO:3); and
4. Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), or

an internal amino acid sequence selected from the group consisting of:

1. Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu (SEQ ID NO:8); and
2. Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

Claim 3. A pharmaceutical composition for delivery of a therapeutic agent comprising:

(A) a therapeutic agent; and

(B) an absorption enhancing effective amount of purified zonulin, wherein said zonulin has an apparent molecular
weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions.

Claim 4. The pharmaceutical composition of Claim 3, wherein said zonulin has:

an N-terminal amino acid sequence selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);
(2) Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);
(3) Val Thr Phe Tyr Thr Asp Ala Val Ser (SEQ ID NO:3); and
(4) Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), or

an internal amino acid sequence selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu (SEQ ID NO:8); and
(2) Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

Claim 5. The pharmaceutical composition of Claim 3, wherein said therapeutic agent is selected
from the group consisting of a drug compound, biologically active peptide and vaccine.

Claim 6. The pharmaceutical composition of Claim 5, wherein said drug compound is selected from the group consisting of a drug which acts on the cardiovascular system, a drug which acts on the central nervous system, an antineoplastic drug and antibiotics.

Claim 7. The pharmaceutical composition of Claim 6, wherein said drug which acts on the cardiovascular system is selected from the group consisting of lidocaine, adenosine, dobutamine, dopamine, epinephrine, norepinephrine and phenolamine.

Claim 8. The pharmaceutical composition of Claim 6, wherein said drug which acts on the central nervous system is selected from the group consisting of doxapram, alfentanil, dezocin, nalbuphine, buprenorphine, naloxone, ketorolac, midazolam, propofol, metacurine, mivacurium and succinylcholine.

Claim 9. The pharmaceutical composition of Claim 6, wherein said antineoplastic drug is selected from the group consisting of cytarabine, mitomycin, doxorubicin, vincristine and vinblastine.

Claim 10. The pharmaceutical composition of Claim 6, wherein said antibiotic is selected from the group consisting of methicillin, mezlocillin, piperacillin, cetoxin, cefonicid, cefmetazole and aztreonam.

Claim 11. The pharmaceutical composition of Claim 5, wherein said biologically active peptide is selected from the group consisting of a hormone, lymphokine, globulin and albumin.

Claim 12. The pharmaceutical composition of Claim 11, wherein said hormone is selected from the
group consisting of testosterone, nandrolene, menotropins, insulin and urofolltropin.

Claim 13. The pharmaceutical composition of Claim 11, wherein said lymphokine is selected from the group consisting of interferon-α, interferon-β, interferon-γ, interleukin-1, interleukin-2, interleukin-4 and interleukin-8.

Claim 14. The pharmaceutical composition of Claim 11, wherein said globulin is an immunoglobulin selected from the group consisting of polyvalent IgG, and specific IgG, IgA or IgM.

Claim 15. The pharmaceutical composition of Claim 3, wherein the ratio of therapeutic agent to zonulin is in the range of about 1:10 to 3:1.

Claim 16. The pharmaceutical composition of Claim 15, wherein the ratio of therapeutic agent to zonulin is in the range of about 1:5 to 2:1.

Claim 17. The pharmaceutical composition of Claim 3, wherein zonulin is present in the composition in an amount of from about 40 ng to 1000 ng.

Claim 18. The pharmaceutical composition of Claim 17, wherein zonulin is present in the composition in an amount of from about 400 ng to 800 ng.

Claim 19. The pharmaceutical composition of Claim 3, wherein said composition is an oral dosage composition for intestinal delivery of said therapeutic agent.

Claim 20. The pharmaceutical composition of Claim 3, wherein said composition is a nasal dosage composition for nasal delivery of said therapeutic agent.

Claim 21. The pharmaceutical composition of Claim 3, wherein said composition is an intravenous
dosage composition for delivery of said therapeutic agent through the blood-brain barrier.

Claim 22. A method for delivery of a therapeutic agent comprising administering, to a subject in need thereof, a pharmaceutical composition comprising:

(A) a therapeutic agent; and

(B) an absorption enhancing effective amount of purified zonulin, wherein said zonulin has an apparent molecular weight of about 47 kDa, as determined by SDS-polyacrylamide gel electrophoresis, which is recognized by both anti-tau polyclonal antibody and by anti-ZOT polyclonal antibody, and is capable of reversibly opening mammalian tight junctions.

Claim 23. The method of Claim 22, wherein said zonulin has:

an N-terminal amino acid sequence selected from the group consisting of:

(1) Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln (SEQ ID NO:1);

(2) Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu (SEQ ID NO:2);

(3) Val Thr Phe Tyr Thr Asp Ala Val Ser (SEQ ID NO:3); and

(4) Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu (SEQ ID NO:4), or
an internal amino acid sequence
selected from the group consisting of:

(1) Xaa Xaa Asp Gly Thr Gly Lys
    Val Gly Asp Leu (SEQ ID NO:8); and

(2) Leu Ser Glu Val Thr Ala Val
    Pro Ser Leu Asn Gly Gly (SEQ ID NO:9).

Claim 24. The method of Claim 22, wherein said therapeutic agent is selected from the group consisting of a drug compound, biologically active peptide and vaccine.

Claim 25. The method of Claim 24, wherein said drug compound is selected from the group consisting of a drug which acts on the cardiovascular system, a drug which acts on the central nervous system, an antineoplastic drug and antibiotics.

Claim 26. The method of Claim 25, wherein said drug which acts on the cardiovascular system is selected from the group consisting of lidocaine, adenosine, dobutamine, dopamine, epinephrine, norepinephrine and phentolamine.

Claim 27. The method of Claim 25, wherein said drug which acts on the central nervous system is selected from the group consisting of doxapram, alfentanil, dezocin, nalbuphine, buprenorphine, naloxone, ketorolac, midazolam, propofol, metacurine, mivacurium and succinylcholine.

Claim 28. The method of Claim 25, wherein said antineoplastic drug is selected from the group consisting of cytarabine, mitomycin, doxorubicin, vincristine and vinblastine.

Claim 29. The method of Claim 25, wherein said antibiotic is selected from the group consisting of
methicillin, mezlocillin, piperacillin, cetoxitin, cefonicid, cefmetazole and aztreonam.

Claim 30. The method of Claim 24, wherein said biologically active peptide is selected from the group consisting of a hormone, lymphokine, globulin and albumin.

Claim 31. The method of Claim 30, wherein said hormone is selected from the group consisting of testosterone, nandrolone, menotropins, insulin and urofolltropin.

Claim 32. The method of Claim 30, wherein said lymphokine is selected from the group consisting of interferon-α, interferon-β, interferon-γ, interleukin-1, interleukin-2, interleukin-4 and interleukin-8.

Claim 33. The method of Claim 30, wherein said globulin is an immunoglobulin selected from the group consisting of polyvalent IgG, and specific IgG, IgA or IgM.

Claim 34. The method of Claim 22, wherein the ratio of therapeutic agent to zonulin is in the range of about 1:10 to 3:1.

Claim 35. The method of Claim 34, wherein the ratio of therapeutic agent to zonulin is in the range of about 1:5 to 2:1.

Claim 36. The method of Claim 22, wherein zonulin is present in the composition in an amount of from about 40 ng to 1000 ng.

Claim 37. The method of Claim 36, wherein zonulin is present in the composition in an amount of from about 400 ng to 800 ng.

Claim 38. The method of Claim 22, wherein the zonulin is administered in an amount such that the final concentration is in the range of about $10^{-5}$ M to $10^{-14}$ M.
Claim 39. The method of Claim 38, wherein the zonulin is administered in an amount such that the final concentration is in the range of about $10^{-6}$ M to $5.0 \times 10^{-8}$ M.

Claim 40. The method of Claim 32, wherein said composition is an oral dosage composition for intestinal delivery of said therapeutic agent, and said administering is by oral administration.

Claim 41. The method of Claim 32, wherein said composition is a nasal dosage composition for nasal delivery of said therapeutic agent, and said administering is by nasal administration.

Claim 42. The method of Claim 32, wherein said composition is an intravenous dosage composition for delivery of said therapeutic agent through the blood-brain barrier, and said administering is by intravenous administration.
FIG. 1

![Graph showing the change in Rt (ohms/cm²) over time for different fractions of Zonulin.](graph.png)

- **ZONULIN**
- **FRACTION 5**
- **FRACTION 4**
- **FRACTION 3**
- **FRACTION 2**
FIG. 3

SAMPLE REMOVED

SAMPLE ADDED

0 20 40 60 80 100 120 140 160
TIME (min)

4 3 2 1 0
RT (m/s/cm²)

\[ \n\]

ZONULIN
ZONULIN + Zot Ab
ZONULIN + Tau Ab
Tau

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FIG. 6

RABBIT INTESTINE -
( SEQ ID NO: 1 )
Asn Gln Arg Pro Pro Pro Ala Gly Val Thr Ala Tyr Asp Tyr Leu Val Ile Gln

HUMAN ADULT INTESTINE -
( SEQ ID NO: 7 )
Glu Val Gln Leu Val Glu Ser Gly Gly Xaa Leu

HUMAN FETAL INTESTINE -
( SEQ ID NO: 4 )
Met Leu Gln Lys Ala Glu Ser Gly Gly Val Leu Val Gln Pro Gly Xaa Ser Asn Arg Leu

HUMAN ADULT HEART -
( SEQ ID NO: 2 )
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu

HUMAN ADULT BRAIN -
( SEQ ID NO: 3 )
Val Thr Phe Tyr Thr Asp Ala Val Ser

INTERNAL SEQUENCE
HUMAN ADULT HEART -
( SEQ ID NO: 9 )
Leu Ser Glu Val Thr Ala Val Pro Ser Leu Asn Gly Gly

HUMAN ADULT BRAIN 35kDa
FRAGMENT -
( SEQ ID NO: 8 )
Xaa Xaa Asp Gly Thr Gly Lys Val Gly Asp Leu
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07636

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 37/18; A61K 38/00, 38/28; C07K 1/00, 14/00, 17/00
US CL. :530/350; 514/2, 3, 4, 12
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)
U.S. : 530/350; 514/2, 3, 4, 12

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)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search
02 SEPTEMBER 1998

Date of mailing of the international search report
OCT 13 1998

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Form PCT/ISA/210 (second sheet)(July 1992)
B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):