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Phosphoglycan Messengers and Their Medical Uses (Lipidemia)

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(54) Title: PHOSPHOGLYCAN MESSENGERS AND THEIR MEDICAL USES

(57) Abstract: Phosphoglycan messengers (PGMs) which are carbohydrate derivatised phosphatidyl cyclitols are disclosed, together with the finding that these substances are biologically active, e.g. in lowering blood glucose levels. These compounds are distinct from GPI-anchors disclosed in the prior art as these GPI-anchors are protein linked and are not biologically active.

Phosphoglycan Messengers and Their Medical Uses

Field of the Invention

The present invention relates to phosphoglycan messengers (PGMs) and their medical uses, and in particular to PGMs comprising one or more lipid moieties.

Background of the Invention

Glycosylphosphatidylinositols (GPIs) are essential components in the plasma membrane of cells (Thomas et al, 1990) including malaria parasites, both as membrane anchors for proteins and as the sole class of free glycolipids (Gerold et al,1997), and, in their role of precursors of IPGs, they may also play a role in insulin signal transduction (Saltiel & Cuatrecasas, 1986). Binding of insulin to its receptor leads to phosphatidylinositol-specific phospholipase cleavage of GPI and generation of two extracellular signals, diacyl glycerol and inositol phosphoglycans (IPGs).

Inositol phosphoglycans (IPGs) which resemble the polar core glycan head of free GPIs, mimic several insulin actions and have been suggested to constitute a unique insulin second messenger system (Rademacher et al, 1994; Varela-Nieto et al, 1996). IPG are released in a biologically active form by the cleavage of GPI precursors by the action of the enzyme GPI-PLD. In mammalian cells, two chemically and functionally distinct classes of IPGs denominated IPG-A and IPG-P, are tissue-specific (Kunjara et al, 1994). IPG-A mediators mimic the lipogenic activity of insulin in adipocytes and inhibit cAMP-dependent protein kinase (Caro et al, 1997). IPG-P mediators mimic the glycogenic activity of insulin

phosphatase (PDH). WO98/11116 and WO98/11117 (Rademacher Group Limited) disclose the purification, isolation and characterisation of IPG-P and IPG-A from human tissue. Prior to these applications, it had not been possible to isolate single components from the tissue derived IPG fractions, much less in sufficient quantities to allow structural characterisation.

Although IPGs have been isolated from different sources including mammals, yeast and Trypanosomes, and have been extensively tested for their insulin mimetic activity *in vitro*, they have so far only been tested *in vivo* in streptozotocin (STZ)-diabetic rats (Huang et al, 1993; Fonteles et al, 1996). IPG-P from malaria parasites has been shown to have insulin mimetic activities *in vitro* (Caro et al, 1996). No IPGs or IPG-related compounds from any source have been studied in murine models of Type 2 diabetes. GPIs purified from malaria parasites have been tested *in vivo* only in normal mice pretreated with thioglycollate (Schofield & Hackett, 1993) in experiments demonstrating that some components of parasitized erythrocytes trigger the release of toxins such as TNF from activated macrophages.

Summary of the Invention

The present invention is based on the finding that phosphoglycan messengers (PGMs), and in particular PGMs comprising lipid groups, are biologically active and therefore have therapeutic utility. These observations arose from the experiments described herein in which GPIs and IPGs obtained from malaria parasites were studied in murine models of Type 2 diabetes. Experiments in which

the hypoglycaemic effect of *Plasmodium yoelii* GPIs and IPGs on STZ-diabetic mice are also disclosed and other insulin mimetic activities are explored *in vitro*, showing that malaria parasites can be used as a source of biologically active GPIs and further providing data to enable the development of new drugs for the clinical management of Type 2 diabetes. The PGMs of the present invention can be contrasted with the GPI-anchors purified in the prior art as the latter are protein linked.

Thus, in contrast to the prior art view that GPIs are biologically inactive and merely serve as the precursor of biologically active IPGs (Rademacher et al, 1994; Varela-Nieto et al, 1996), or else induce the production of malaria toxins such as TNF, the experiments described herein demonstrate for the first time that PGMs comprising lipid groups, such as GPIs, are active molecules in their own right and may be useful in the treatment of various medical conditions ameliorated by PGM second messengers, for example Type 1 and Type 2 diabetes. These and other uses are discussed in more detail below.

Accordingly, in a first aspect, the present invention provides a substance which is a carbohydrate derivatised phosphatidyl cyclitol. In one preferred embodiment, the substance comprises one or more lipid moieties attached to the cyclitol group or the phosphatidyl group via one or more ester and/or ether linkages. Preferably, the substance has one or more properties selected from reducing blood glucose in a diabetic *ob/ob* and *db/db* murine model, regulating lipogenesis, stimulating

pyruvate dehydrogenase phosphatase, inhibiting cAMP dependent protein kinase, inhibiting fructose-1,6-bisphosphatase, inhibiting glucose-6-phosphatase, and/or modulating plasma cholesterol, plasma triglycerides and/or high density lipoprotein levels or modulating, and in particular lowering, the LDL:HDL ratio. These and other properties of the PGMs are discussed below. As mentioned above, preferred substances are devoid of protein.

In a further aspect, the present invention provides one or more of the substances described above for use in method of medical treatment. As set out above, as PGMs comprising lipids were previously thought to be the precursor of biologically active IPGs or to stimulate the release of toxins, the finding that they can be used in the treatment of medical disorders requiring the administration of PGMs such as GPIs and IPGs is highly surprising.

In a further aspect, the present invention provides the use of one or more of the above substances for the preparation of a medicament for the treatment of a condition ameliorated by administration of a PGM second messenger. Examples of such conditions are described below.

In a further aspect, the present invention provides an isolated PGM as obtainable from malaria parasites. In one embodiment, the PGMs are obtainable using a sequence of steps comprising:

(a) making an extract by heat and acid treatment of

red blood cells parasitized with *Plasmodium yoelii*;

(b) after centrifugation and charcoal treatment, allowing the resulting solution to interact overnight with an AG1-X8 (formate form) anion exchange resin;

(c) collecting a fraction having A-type IPG activity obtained by eluting the column with 50 mM HCl;

(d) neutralising to pH4 (pH not to exceed 7.8) and lyophilising the fraction to isolate the substance;

(e) descending paper chromatography using 4/1/1 butanol/ethanol/water as solvent;

(f) purification using high-voltage paper electrophoresis in pyridine/acetic acid/water; and,

(g) purification using Dionex anion exchange chromatography, or purification and isolation using Vydac HPLC chromatography.

The experiments described herein demonstrate for the first time that infection with *Plasmodium yoelii YM* malaria and *Plasmodium chabaudi*, or injection of extracts from malaria-parasitized red cells, induces hypoglycaemia in normal mice and normalizes the hyperglycaemia in streptozotocin (STZ)-diabetic mice. *Plasmodium* glycosylphosphatidyl inositols (GPIs) which are believed to be the precursor of inositol phosphoglycan (IPGs) were extracted in chloroform:methanol:water (10:10:3), purified in high performance thin layer chromatography (HPTLC) and tested for their insulin-mimetic activities. The experiments show that:

(a) A single i.v. injection of *Plasmodium yoelii* GPI significantly ($p<0.0001$) lowered the blood glucose in STZ-diabetic mice from 17.8 ± 1.1 mmol to 8.9 ± 0.65 mmol

after 4 hours, an effect which is associated with increased plasma insulin ($p<0.001$). Both hepatic glycogen content and plasma lactate was significantly decreased in STZ-mice pretreated with GPI.

(b) The C57BL/KsJ-*db/db* offer a good model for studies on human obesity and Type 2 diabetes. Intravenous injection of GPIs (8 and 30 nM expressed as phosphate content) induced a significant drop in blood glucose in a dose-related ($p<0.001$). When given orally as a single dose, GPI significantly lowered blood glucose in db/db mice ($p<0.01$). GPI stimulated lipogenesis in rat adipocytes in a dose-dependent manner in the presence and absence of maximal concentrations of insulin (10^{-8} M) ($p<0.01$).

(c) GPI stimulated PDH-Pase and inhibited both cAMP-dependent protein kinase A (PKA), fructose-1,6 bisphosphatase and glucose-6-phosphatase.

(d) ^{3}H-myo-inositol was incorporated in biologically active GPI and compositional analysis of GPI demonstrated the presence of phosphate, *myo*-inositol, glucosamine, galactosamine, mannose, galactose, glucose, fucose and xylose.

(e) GPI administered to diabetic mice normalised levels of plasma cholesterol and plasma triglycerides, and lowered the LDL:HDL ratio.

In addition, inositol phosphoglycans (IPGs) are released outside cells by hydrolysis of membrane bound

glycosylphosphatidyl inositols (GPIs), and act as second messengers mediating insulin action. The C57BL/KsJ-*db/db* and C57BL/6J-*ob/ob* mice offer good models for studies on human obesity and Type 2 diabetes (NIDDM). These experiments show that:

(a) A single i.v. injection of IPG-A or IPG-P extracted from *Plasmodium yoelii* significantly ($p<0.02$) lowers the blood glucose in STZ-diabetic, *db/db* and in *ob/ob* mice for at least 4-6 hours.

(b) Using rat white adipocytes, IPG-P increased lipogenesis by 20-30% in the presence and absence of maximal concentrations of insulin (10^{-8} M) ($p<0.01$) and stimulated pyruvate dehydrogenase (PDH) phosphatase in a dose-related manner.

(c) Both IPG-A and IPG-P inhibited c-AMP-dependent protein kinase (PKA) in a dose- related manner.

(d) Compositional analysis revealed the presence of *myo*-inositol and phosphorus in both IPG- A and IPG-P. IPG-A contains glucosamine, while IPG-P contains glucosamine, galactosamine and galactose.

This is the first investigation reporting the hypoglycaemic effect of PGMs in murine models of Type 2 diabetes and the information disclosed herein relating to GPIs and IPGs isolated from *Plasmodium yoelii* YM therefore provides new treatments for conditions ameliorated by PGMs and provides structural information for the synthesis of new drugs, e.g. for the management

of both Type 1 and Type 2 diabetes.

Embodiments of the present invention will now be described in more detail by way of example and not limitation with reference to the accompanying figures.

Brief Description of the Figures

Figure 1. Blood glucose concentrations of STZ-diabetic mice given a single i.v. injection of *Plasmodium* GPI (10 nmole/mouse) (●); or saline (O). Values are Means ± S.E.M; n=10 *p<0.02, **p<0.0001 vs. saline.

Figure 2. Blood glucose concentrations of *db/db* diabetic mice given a single i.v. injection of *Plasmodium* GPI (9 nmole/mouse, n=5) (●); *Plasmodium* GPI (30 nmole/mouse, n=2) (▲); or saline (O), n=7. Values are Means ± S.E.M; *p<0.001, **p<0.0001 vs. saline.

Figure 3. Blood glucose concentrations of *db/db* diabetic mice given a single oral dose or *Plasmodium* GPI (2.7 µmole/mouse, n=6) (●); or saline (O), n=8. Values are Means ± S.E.M; *p<0.01 vs. saline.

Figure 4. Blood glucose and insulin concentrations in normal mice treated with saline and STZ-diabetic mice 6 hours after given a single i.v. injection of *Plasmodium* GPI (10 nmole/mouse); or saline. Value are Means ± S.E.M; n=4-8; *p<0.05, **p<0.001 vs saline.

Figure 5. Plasma lactate concentrations in normal mice treated with saline and STZ- diabetic mice 6 hours after given a single i.v. injection of *Plasmodium* GPI (10

nmole/mouse); or saline. Values are Means ± S.E.M; n=4-8, **p<0.0001 vs saline.

Figure 6. Dose response of two different batches of *Plasmodium* GPI (A & B) on lipgenesis in rat adipocytes in absence of insulin, (6A); and in the presence of maximal dose of insulin (10^{-8} M), (6B). Values are Means ± S.E.M; n=4.

Figure 7. Effect of different concentrations of *Plasmodium* GPI on activity of PDH-phosphatase, n=2.

Figure 8. Effect of different concentrations of *Plasmidium* GPI on the activity cAMP dependent protein kinase A (PKA), n=2.

Figure 9. Effect of different concentrations of *Plasmodium* GPI on the activity of fructose-1,6-bisphosphatase.

Figure 10. Effect of *Plasmodium* GPI, *Plasmodium* IPGs eluted from a cellulose column, and anhydromannitol on the activity of fructose-6-bisphosphatase.

Figure 11. Dixon plot of *Plasmodium* GPI (1.4-7 µM) on the activity of glucose-6-phosphatase, using 2 mM glucose-6-phosphate (11A) and 10 mM glucose-6-phosphate (11B) as substrate.

Figure 12. Effect of *Plasmodium* GPI (10 nmole/mouse i.v.) on total plasma cholesterol (A) and plasma triglycerides (B) in STZ-diabetic mice. Values are Means

± S.E.M.; n=6-7, *p<0.05, **p<0.01 vs saline.

Figure 13. Effect of *Plasmodium* GPI (10 nmole/mouse i.v.) on plasma HDL cholesterol.

Figure 14A. Blood glucose concentrations of STZ-diabetic mice given a single i.v. injection of IPG-A (●); or saline (O). Values are Means ± S.E.M; n=5. *p<0.02, **p<0.01 vs. Control.

Figure 14B. Blood glucose concentrations of *ob/ob* diabetic mice given a single i.v. injection of IPG-A (●); or saline (O). Values are Means ± S.E.M; n=5. *p<0.01 vs. control.

Figure 14C. Blood glucose concentrations of *ob/ob* diabetic mice given a single i.v. injection of IPG-P (●); or saline (O). Values are Means ± S.E.M; n=5; **p<0.01 vs. control.

Figure 14D. Blood glucose concentrations of *db/db* diabetic mice given a single i.v. injection of IPG-P (●); or saline (O). Values are Means ± S.E.M; n=6; *p<0.02, **p<0.01 vs control.

Figure 15. Inhibitory effect of different concentrations of IPGs eluted from a cellulose column on the activity of PKA. IPG-A water (--▵--), IPG-A acid (--▲--); IPG-P water (O); IPG-P acid (●). Values are the mean of two experiments.

Figure 16. Effect of different concentrations of IPG-P

acid fraction eluted from a cellulose column on PDH-phosphatase, n=2.

Figure 17. High pH anion exchange chromatograms of IPG-A, showing separation of sugars in Carbopac™ PA 10 column (a and b) and separation of sugar alditols in Carbopac™ MA1 column (c). IPG-A hydrolysed in 6N HCl for 24 hours (a and c); IPG-A hydrolysed in 4N HCl for 4 hours (b).

Figure 18. High pH anion exchange chromatograms of IPG-P, showing separation of sugars in Carbopac™ PA 10 column (a and b) and separation of sugar alditols in Carbopac™ MA1 column (c). IPG-P hydrolysed in 6N HCl for 24 hours (a and c); IPG-P hydrolysed in 4N HCl for 4 hours (b).

Detailed Description

Phosphoglycan Messengers (PGMs)

In the present application, "phosphoglycan messengers" or PGMs denote a class of substances comprising inositol phosphoglycans (IPGs) and glycosyl phosphatidyl inositols (GPIs). IPGs are examples of a family of PGMs which may comprise lipidic groups, whereas GPIs are examples of a family of PGMs comprising one or more lipidic groups attached to the cyclitol. As discussed above, free IPGs are produced from GPIs by cleavage by enzymes such as GPI-PLD which removes the lipid groups from the parent GPI. In contrast, the GPI anchors described in the prior art are protein linked.

In a preferred embodiment, the present invention concerns

PGM substances which are carbohydrate derivatised phosphatidyl cyclitols. In these PGMs, the phosphatidyl group comprises one or more lipid moieties attached to the cyclitol group or the phosphatidyl group via one or more ester and/or ether linkages.

Preferably, the cyclitol is *chiro*-inositol, *myo*-inositol, or derivatives thereof, such as substituted derivatives thereof, including pinitol (*3-O*-methyl-*chiro*-inositol).

In some embodiments, the lipid moieties are linked to the phosphatidyl group. Preferably this group is linked to one or two oxygen linked lipid moieties. The lipid moieties can be selected from the group consisting of diacyl, dialkyl, acyl-alkyl, lyso-acyl, lyso-alkyl, lyso, acyl or alkyl lipids. Examples of lipidic moieties include 1-*O*-(C16:0)*lyso*-alkylglycerol; (C16:0))*lyso*-acylglycerol; (C18:0))*lyso*-acylglycerol; (C20:0))*lyso*-acylglycerol; (C22:0))*lyso*-acylglycerol; ceramide, (C16:0)fatty acid-(C18:1)sphingosine; ceramide, (C16:0)fatty acid-(C18:0)sphinganine; ceramide, (C24:0)fatty acid-(C18:1)sphingosine; ceramide, (C24:0)fatty acid-(C18:0)sphinganine; 1-*O*-(C16:0)alkyl-2-*O*-(C16:0)acylglycerol; 1-*O*-(C16:0)alkyl-2-*O*-(C18:2)acylglycerol; 1-*O*-(C16:0)alkyl-2-*O*-(C18:1)acylglycerol; 1-*O*-(C16:0)alkyl-2-*O*-(C18:0)acylglycerol; (C16:0)-alkyl-(C16:0)acyl-glycerol (AAG) and (C16:0)mono(lyso)-alkyl-glycerol (MAG).

Preferably, the carbohydrate group is a hexose, and more preferably is selected from glucosamine, galactosamine, galactose, mannose, glucose, fucose or xylose including

substituted derivatives thereof.

Preferably, the substances of the invention have one or more properties selected from lowering blood glucose, lowering plasma cholesterol or plasma triglycerides and/or normalising the ratio of low: high density lipoproteins (LDL:HDL ratio), for example by reducing the level of low density lipoprotein. These properties can be readily assessed in a suitable animal model such as a diabetic *ob/ob* and *db/db* mice fed with a high fat diet or in STZ-diabetic mice. The substances may also have one or more of the properties attributed to IPGs in the prior art, such as regulating lipogenesis, stimulating pyruvate dehydrogenase phosphatase, inhibiting cAMP dependent protein kinase, inhibiting fructose-1,6-bisphosphatase and/or inhibiting glucose-6-phosphatase.

IPG-A mediators modulate the activity of a number of insulin-dependent enzymes such as cAMP dependent protein kinase (inhibits), adenylate cyclase (inhibits) and cAMP phospho-diesterases (stimulates). In contrast, IPG-P mediators modulate the activity of insulin-dependent enzymes such as pyruvate dehydrogenase phosphatase (stimulates) and glycogen synthase phosphatase (stimulates). The IPG-A mediators mimic the lipogenic activity of insulin on adipocytes, whereas the IPG-P mediators mimic the glycogenic activity of insulin on muscle. Both IPG-A and IPG-P mediators are mitogenic when added to fibroblasts in serum free media. The ability of the mediators to stimulate fibroblast proliferation is enhanced if the cells are transfected with the EGF-receptor. IPG-A mediators can stimulate

cell proliferation in the chick cochleovestibular ganglia.

Soluble IPG fractions having IPG-A and IPG-P activity have been obtained from a variety of animal tissues including rat tissues (liver, kidney, muscle, brain, adipose, heart) and bovine liver. IPG-A and IPG-P biological activity has also been detected in human liver and placenta, malaria parasitized RBC and mycobacteria. The ability of an anti-inositolglycan antibody to inhibit insulin action on human placental cytotrophoblasts and BC3H1 myocytes or bovine-derived IPG action on rat diaphragm and chick cochleovestibular ganglia suggests cross-species conservation of many structural features. However, it is important to note that although the prior art includes these reports of IPG-A and IPG-P activity in some biological fractions, the purification or characterisation of the agents responsible for the activity is not disclosed.

IPG-A substances are cyclitol-containing carbohydrates, also containing Zn^{2+} ion and optionally phosphate and having the properties of regulating lipogenic activity and inhibiting cAMP dependent protein kinase. They may also inhibit adenylate cyclase, be mitogenic when added to EGF-transfected fibroblasts in serum free medium, and stimulate lipogenesis in adipocytes.

IPG-P substances are cyclitol-containing carbohydrates, also containing Mn^{2+} and/or Zn^{2+} ions and optionally phosphate and having the properties of regulating glycogen metabolism and activating pyruvate dehydrogenase

phosphatase. They may also stimulate the activity of glycogen synthase phosphatase, be mitogenic when added to fibroblasts in serum free medium, and stimulate pyruvate dehydrogenase phosphatase.

Methods for obtaining IPG-A and IPG-P and for determining characteristic activites of these substances and GPIs are set out in Caro et al, 1997, and in WO98/11116 and WO98/11117.

Drug Formulation

The PGMs of the invention may be derivatised in various ways. As used herein "derivatives" of the PGMs includes salts, coordination complexes with metal ions such as Mn^{2+} and Zn^{2+}, esters such as *in vivo* hydrolysable esters, free acids or bases, hydrates, prodrugs or lipids, coupling partners.

Salts of the PGM compounds of the invention are preferably physiologically well tolerated and non toxic. Many examples of salts are known to those skilled in the art. Compounds having acidic groups, such as phosphates or sulfates, can form salts with alkaline or alkaline earth metals such as Na, K, Mg and Ca, and with organic amines such as triethylamine and Tris (2-hydroxyethyl)amine. Salts can be formed between compounds with basic groups, e.g. amines, with inorganic acids such as hydrochloric acid, phosphoric acid or sulfuric acid, or organic acids such as acetic acid, citric acid, benzoic acid, fumaric acid, or tartaric acid. Compounds having both acidic and basic groups can form internal salts.

Esters can be formed between hydroxyl or carboxylic acid groups present in the compound and an appropriate carboxylic acid or alcohol reaction partner, using techniques well known in the art.

Derivatives which as prodrugs of the PGM compounds are convertible *in vivo* or *in vitro* into one of the active PGMs. Typically, at least one of the biological activities of compound will be reduced in the prodrug form of the compound, and can be activated by conversion of the prodrug to release the compound or a metabolite of it.

Other derivatives include coupling partners of the compounds in which the compounds is linked to a coupling partner, e.g. by being chemically coupled to the compound or physically associated with it. Examples of coupling partners include a label or reporter molecule, a supporting substrate, a carrier or transport molecule, an effector, a drug, an antibody or an inhibitor. Coupling partners can be covalently linked to compounds of the invention via an appropriate functional group on the compound such as a hydroxyl group, a carboxyl group or an amino group. Other derivatives include formulating the compounds with liposomes.

Pharmaceutical Compositions

The PGMs described herein or their derivatives can be formulated in pharmaceutical compositions, and administered to patients in a variety of forms, in particular to treat conditions which are ameliorated by

the administration of inositol phosphoglycan second messengers.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant or an inert diluent. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Such compositions and preparations generally contain at least 0.1wt% of the compound.

Parenteral administration includes administration by the following routes: intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraocular, transepithelial, intraperitoneal and topical (including dermal, ocular, rectal, nasal, inhalation and aerosol), and rectal systemic routes. For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, solutions of the compounds or a derivative thereof, e.g. in physiological saline, a dispersion prepared with glycerol, liquid polyethylene glycol or oils.

In addition to one or more of the compounds, optionally

in combination with other active ingredient, the compositions can comprise one or more of a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, isotonicizing agent, preservative or anti-oxidant or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. orally or parenterally.

Liquid pharmaceutical compositions are typically formulated to have a pH between about 3.0 and 9.0, more preferably between about 4.5 and 8.5 and still more preferably between about 5.0 and 8.0. The pH of a composition can be maintained by the use of a buffer such as acetate, citrate, phosphate, succinate, Tris or histidine, typically employed in the range from about 1 mM to 50 mM. The pH of compositions can otherwise be adjusted by using physiologically acceptable acids or bases.

Preservatives are generally included in pharmaceutical compositions to retard microbial growth, extending the shelf life of the compositions and allowing multiple use packaging. Examples of preservatives include phenol, meta-cresol, benzyl alcohol, para-hydroxybenzoic acid and its esters, methyl paraben, propyl paraben, benzalconium chloride and benzethonium chloride. Preservatives are typically employed in the range of about 0.1 to 1.0 % (w/v).

Preferably, the pharmaceutically compositions are given to an individual in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. Typically, this will be to cause a therapeutically useful activity providing benefit to the individual. The actual amount of the compounds administered, and rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980. By way of example, and the compositions are preferably administered to patients in dosages of between about 0.01 and 100mg of active compound per kg of body weight, and more preferably between about 0.5 and 10mg/kg of body weight.

The composition may further comprise one or more other pharmaceutically active agents, either further compounds of the invention, inositol phosphoglycans, growth factors such as insulin, NGF or other growth factors listed below, or other drugs, e.g. those in use for the treatment of diabetes or other conditions set out below.

Medical Uses

The PGM compounds of the invention can be used in the preparation of medicaments for the treatment of conditions mediated (i.e. ameliorated) by administration of a PGM second messenger. PGMs are second messengers for a range of different growth factors, including insulin, nerve growth factor, hepatocyte growth factor, insulin-like growth factor I (IGF-I), fibroblast growth factor, transforming growth factor β, the action of IL-2 on B-cells and T-cells, ACTH signalling of adrenocortical cells, IgE, FSH and hCG stimulation of granulosa cells, thyrotropin stimulation of thyroid cells, cell proliferation in the early developing ear and rat mammary gland. Consequently, PGMs or their antagonists can be used in the treatment or amelioration of disorders mediated by the growth factors or to mimic specific growth factor biological activities.

In particular, the results show that the PGMs disclosed herein can be used in the treatment and management of diabetes as they are capable of normalising elevated blood sugar levels *in vivo*. The forms of diabetes that may be treated include diabetes due to insulin resistance, insulin resistance in Type 1 diabetes and brittle diabetes, Type 2 diabetes (both obese and lean forms), dyslipidemia associated with diabetes and diabetic complications, and of conditions associated with insulin resistance or insulin underproduction, such as neurotrophic disorders or polycystic ovary syndrome, age-related memory loss, and post-ischaemic damage secondary to stroke or post-transplant complications.

Abbreviations

NIDDM, Non-insulin-dependent diabetes mellitus.

P. yoelii, *Plasmodium yoelii*.

TNF, Tumour necrosis factor.

IPGs, Inositol phosphoglycans.

GPI, Glycosylphosphatidyl inositol.

PGM, Phosphoglycan messenger.

PKA, cAMP-dependent-protein kinase A.

STZ, Streptozotocin.

HPAE, High-performance Anion Exchange Chromatography.

PAD, Pulsed Amperometric Detection.

PDH, Pyruvate dehydrogenase.

FBPase, Fructose-1,6-Bisphosphatase.

G6Pase, Glucose-6-phosphatase.

Materials and Methods

Streptozotocin, activated charcoal, ascorbic acid, ammonium molybdate, *myo*-inositol and bovine serum albumin (BSA) were obtained from Sigma, Poole, UK. D-[3-^{3}H] glucose (18 Ci/mmol) was obtained from Amersham Pharmacia, Bucks, UK. Collagenase and insulin were obtained from (Boehringer Mannheim GmbH, Germany). Silica gel 60 HPTLC aluminium sheets were obtained from (Merck, Darmstadt, Germany). 50% Sodium hydroxide was obtained from (Fisher Scientific, Loughborough, UK). Ion exchange resin AG1-X8 (HO^-, 20-50 mesh) was obtained from Bio-Rad Laboratories (Hemel Hempsted, UK). Cellulose microcrystalline was from (Merck, Germany).

MonoStandard™ was obtained from Dionex Corporation, Sunnyvale, CA, USA. All other materials were of high purity and were obtained from BDH.

Mice

The normal (C57BlxBalb/c) F_1 mice and obese diabetic (C57BL/Ks - *db/db*) were bred in our animal colony from parental strains obtained from the National Institute for Medical Research, Mill Hill, London, UK. Wistar rats (120-150 g) and obese diabetic (C57BL/6J-*ob/ob*) mice were obtained from Harlan Olac Ltd, Bicester, UK. Male mice 8-12 weeks old were used, when both their blood glucose and insulin levels were markedly raised. Mice were allowed to acclimatize for at least 7 days before being used. All animals had free access to water and were fed ad libitum with normal laboratory chow.

Induction of diabetes with Streptozotocin (STZ)

Mice were made diabetic by 3 daily i.p. injections of 100mg/kg bw STZ dissolved in 0.01N citrate buffer (pH 4.5) within 5 min of solubilization. Control groups of mice received injections of equivalent volumes of sodium citrate buffer. Blood glucose rose to 15-20 mmol/l and remained stable for 3 weeks. The syndrome induced in mice or rats by STZ injection closely resembles that observed in patients with Type 1 diabetes.

Measurement of blood glucose

Blood glucose concentrations were determined enzymatically on 10 µl volumes of tail blood, collected between 10 a.m. and midday, or at intervals thereafter as indicated below, using Glucotide™ strips and Glucometer 4 (Bayer plc, Newbury, UK). Results, in mmol/l, are expressed as mean ± SEM.

Measurement of immunoreactive insulin

Blood was collected into heparinised tubes from the trunk after decapitation. Plasma was separated by centrifugation and frozen at -20°C. Immunoreactive insulin (IRI) concentrations were determined in 50 µl volumes of plasma by a double-antibody radioimmunoassay (kit supplied by ICN Biomedical, Irvine, CA). Results, in µUnits/ml, are expressed as means ± SEM.

Parasites

The lethal YM line of *P. yoelii* strain 17X (from Dr. A. Holder, NIMR, London, UK), was maintained in (C57BLxBalb/c) F_1 mice by blood passage of parasitized red blood cells. Mice were bled 5-7 days after intravenous infection with 10^4 parasites and parasitaemia was determined from blood films stained with Giemsa.

Extraction and purification of GPIs

Plasmodium yoelii was maintained by blood passage of 10^4 parasitised red cells in F_1 mice. Mice were bled 5-7 days after infection. Parasitized blood (>90% parasitaemia) was washed x 3 with sterile saline by centrifugation at 3000 rpm at 4°C, and the pellet was lysed by incubation in 0.01 % saponin for 3 minutes at room temperature. Parasites were washed x 3 with sterile saline by centrifugation at 3000 rpm at 4°C. The pellet was suspended in 5 ml saline, sonicated for 12 seconds and freeze dried. GPIs were extracted following a procedure described before with some modifications (Gerold et al, 1994). Briefly, parasites (9 x 10^{10}) from 10 mice (0.1 gram dry weight) were extracted twice with 10 ml of Chloroform: Methanol: Water (10:10:3)(CMW) and

centrifuged for 15 minutes at 1800 rpm. The CMW extracted GPIs were pooled, dried in Speed-Vac and suspended in 5 ml of water-saturated n-butanol. An equal volume of water was added, thoroughly mixed, and centrifuged for 10 minutes at 1800 rpm. The organic top layer was removed by aspiration and the bottom layer of water was back-extracted with an equal volume of water-saturated n-butanol. The organic phases were pooled and dried. Non-lipidic material was removed following a procedure described before with some modifications (Zawadski et al, 1998). Briefly GPIs were dried and suspended in 1 ml of 5% 1-propanol containing 0.1M ammonium acetate and applied to C_8 Bond Elute™ cartridge equilibrated in 5% 1-propanol containing 0.1M ammonium acetate. The cartridge was then washed with 10 ml 5% 1-propanol containing 0.1M ammonium acetate. GPIs were eluted with 10 ml of 40% 1-propanol and 10 ml of 60% 1-propanol. Eluates containing glycolipids were pooled and dried in a rotary evaporator.

Isolation of inositol phosphoglycans (IPGs)

Plasmodium yoelii IPGs were extracted from parasitized erythrocytes as described before (Caro et al, 1996) with some modifications. Briefly, mice infected with the lethal YM line of *P. yoelii* were bled 5 -7 days after infection into tubes containing heparinised saline. Parasitized erythrocytes were pelleted and washed twice with saline by centrifugation at 3000 rpm at 4°C for 20 min. Parasitized erythrocytes (>90% parasitaemia) containing 20-30 $x10^9$ parasites were homogenised and boiled for 5 minutes in 25 ml of a solution of 50 mM formic acid, 1 mM EDTA and 1mM 2-mercaptoethanol. The

extract was centrifuged at 18,000 x g for 90 minutes at 4°C. The supernatant was stirred for 10 minutes on ice with charcoal (10 mg/ml) and centrifuged at 18,000 x g for 30 minutes at 4°C. The supernatant was then diluted with 10 volumes of distilled water, adjusted to pH 6 with 10% NH_4OH, and shaken overnight at 4°C with AG1X8 ion exchange resin (formate form). The resin was poured into a column and washed with 2 bed volumes of water and 2 bed volumes of 1 mM HCl (pH 3). The resin was sequentially eluted with 5 bed volumes of 10 mM HCl (pH 2) to yield IPG-P, followed by 5 bed volumes of 50 mM HCl (pH 1.3) to yield IPG-A respectively. The two fractions were concentrated and freeze dried twice to remove residual HCl and stored at -20°C. IPGs were injected i.v. in 0.2 ml saline. Control preparations derived from the same number of normal red cell ghosts were made as described above.

Cellulose column chromatography

Plasmodium yoelii IPGs eluted from the anion exchange resin were subjected to cellulose chromatography. IPGs were dissolved in 1 ml of a solvent containing n-butanol, ethanol, and water [4:1:1] (B:E:W) and applied slowly onto a cellulose column (1 ml) pre-equilibrated in B:E:W [4:1:1]. The column was sequentially eluted with 5 ml of B:E:W, 5 ml methanol, 5 ml water, and 5 ml 50 mM HCl (pH 1.3). Different fractions were concentrated and freeze dried.

Metabolic labelling of *P. yoelii* schizonts

Inositol-free MEM tissue culture medium was used. The medium was supplemented with 0.001% (w/v) Para-amino

benzoic acid; L-methionine, 14.92 mg/ml; L-glutamine, 292 mg/ml; L-cystine, 24.02 mg/ml; L-arginine 126.4 mg/ml; L-leucine, 52.46 mg/ml, 5% foetal calf serum and 5.5 mM glucose. Parasites were obtained from 4 infected 12 wk old male (C57/Bl6 x Balb/c)F_1 mice. Parasitaemias were 90-95% comprising mainly trophozoites and schizonts in roughly equal number. Parasitized RBCs (PRBCs) were collected aseptically in heparanized tissue culture medium and washed once by centrifugation at 2500 rpm for 10 minutes. The pellet was resuspended in 10 ml culture medium (1 x10^9 parasites/ml) and incubated with 250 µCi of *myo*-[^{3}H]inositol in 25 ml Falcon TC flask for 3 hours at 37°C with 5% CO_2. Giemsa-stained blood smears showed that the majority of the late trophozoites had transformed into schizonts. Parasites were viable during the course of labelling. Labelled PRBCs were washed and lysed with saponin and GPIs were extracted from labelled PRBCs as described above.

Thin layer chromatography

GPIs were dissolved in CMW (10:10:3) and applied to silica gel HPTLC plates. These were developed twice in a solvent containing Chloroform: Acetone: Methanol: glacial acetic acid: Water (50:20:10:10:5). GPIs were detected using orcinol and ninhydrin reagents. Biologically active glycolipids that remained at the origin, were scraped and eluted with 2 x 20 ml of methanol, filtered and dried using a rotary evaporator. Control preparations derived from the same number of normal red cell ghosts were made as described earlier. Radioalabelled GPIs were monitored by fluorography after HPTLC sheets were sprayed with En3H enhancer (New England

Nuclear) and exposed to BioMax Mj Film (Kodak) at -80°C for 10 days.

Assay of inorganic phosphate

Inorganic phosphate in malaria GPIs and IPGs was determined following the standard procedure with some modifications (Bartlett, 1958). Disodium hydrogen phosphate was used as standard (0-2µM). Briefly, 10 µl of samples and standards were dried and hydrolysed with 90 µl perchloric acid (70%) at 180°C for 45 minutes. Distilled water (600 µl), 5% ammonium molybdate (50 µl) and 10% ascorbic acid (50 µl) were added and incubated at 95°C for 15 minutes. Absorbance was measured at 830 nm in an OPTI max™ microplate reader (Molecular Device Corporation, Sunnyvale, CA, USA).

Assay of hexosamines

The hexosamine content of *Plasmodium yoelii* of malaria GPIs and IPGs were measured following a procedure described before (Bosworth et al, 1994). Briefly, 100 µl samples were deacetylated with 100 µl 5.5 N HCl at 105°C and adjusted to pH 3 with 100 µl 6M potassium acetate. 100 µl of 10 % acetic acid and 50 µl $NaNO_2$ were added to the mixture on ice. After 30 minutes 100 µl of ammonium sulfamate was added. Fluorescence was developed after coupling with 100 µl of 0.8 % (w/v) 3,5-diaminobenzoic acid. To the mixture, 20 µl of 12 N HCl was added and diluted five fold in water. Fluorescence was measured at 514 nm, the excitation wavelength being 422 nm.

Acid hydrolysis of GPIs

Plasmodium yoelii GPIs were hydrolysed at 100°C in

Teflon-lined screw-capped tubes using100 µl of 4N HCl for 4 hours or 6N HCl for 24 hours. Hydrolysates were centrifuged at 2000 rpm for 20 minutes at 4°C and the supernatants dried in a rotary evaporator. 100 µl of methanol was added and dried twice and the samples were dissolved in 200-400 µl water and filtered through a 0.2 mm PTFE filter. Sugars released by acid hydrolysis were separated by strong anion exchange HPLC on Carbopak TM PA10 (4 x 250 mm, Dionex) and Carbopak TM MA1 (4 x 250 mm, Dionex) analytical columns. Retention times were compared with a set of authentic standards.

Acid hydrolysis of IPGs

Plasmodium yoelii IPGs were hydrolysed at 100°C in Teflon-lined screw-capped tubes using 100 µl of 4N HCl for 4 hours or 6N HCl for 24 hours. Hydrolysates were centrifuged at 2000 rpm for 20 minutes at 4°C and the supernatants were dried in a Speed Vac. To remove residual acid, 100 µl of methanol was added and dried, this treatment was repeated twice. Samples were dissolved in 200-300 µl water and passed through a 0.2 µm PTFE filtered (Whatman, NJ, USA) and stored at -20°C.

Carbohydrate chromatography

Dionex 500 HPLC system carbohydrate analyser from Dionex Corporation, Sunnyvale, CA, USA. It consists of an eluant degassing module, GP40 gradient pump module, AS 50 Autosampler, ED40 amperometric detector with a working gold electrode (Ag/Ag Cl reference electrode) and PeakNet chromatography workstation software was used. Monosaccharides and monosaccharide-alditols of *Plasmodium yoelii* IPGs hydrolysates were analysed by HPLC using

Carbopac™ PA10 and Carbopac™ MA1 columns respectively. An AminoTrap guard column was used to eliminate any possible interference of amino acids and peptides.

Carbopak™ PA10 column chromatography

A Carbopak™ PA10 column (4 x 250 mm) with a guard column (4 x 50 mm) was used for the separation of sugars as recommended by the supplier (Weitzhandler et al, 1996). Before injecting each sample, columns were regenerated by elution with 200 mM sodium hydroxide for 15 minutes and were equilibrated for 15 minutes with 18 mM sodium hydroxide at a flow rate of 1 ml/minute. The monosaccharides were resolved by isocratic elution with 18 mM sodium hydroxide for 20 minutes. Standard carbohydrate detection waveform (E1/2/3= +0.05/ +0.75/ -0.15 V and Time1/2/3 = 0.0-0.4/ 0.41-0.6/ 0.61-1 s) was applied to the ED40 working electrode for pulsed amperometric detection (PAD). 10 µl of a cocktail of monosaccharide standards, MonoStandard™ (fucose, galactosamine, glucosamine, galactose, glucose, mannose) was used for calibration.

Carbopak™ MA1 column chromatography

A Carbopak™ MA1 column (4 x 250 mm) with a guard column (4 x 50 mm) was used for the separation of monosaccharide-alditol (*chiro*-inositol and *myo*-inositol) as recommended by the supplier. 612 mM NaOH was used for regeneration and re-equilibration of columns. *Chiro*-inositol and *myo*-inositol were resolved using an isocratic system of NaOH 612 mM (0.4 ml/minute) for 30 minutes. ED40 operating parameters were E1= +0.05 V, E2= +0.65 V, E3= -0.10 V. *Myo*-inositol, *chiro*-inositol,

mannitol, fructose and glucose were used to calibrate the column.

Lipogenesis assay

Male Wistar rats (120-150 g) were killed by cervical dislocation and adipocytes were obtained from the epididymal adipose tissue and prepared by digestion with collagenase according to the method of Rodbell (1964) with some modifications. Fat pads from two rats were dissected and placed in Krebs Ringer Hepes (KRH) buffer containing 9.2 mM Hepes, 2.2 mM $NaH_2PO_4.2H_2O$, 10 mM $NaHCO_3$, 132 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2.6H_2O$, 2% BSA and 5 mM glucose, pH 7.4. Fat pads were finely minced with scissors and incubated in10 ml KRH buffer containing 20 mg collagenase for 20-30 minutes at 37°C in a shaking water bath with continuous gassing with 95% O_2 and 5% CO_2.

Lipogenesis was determined as the incorporation of [3-^{3}H] glucose into toluene-extractable lipids. Briefly, into a 96-multiwell plate, 100 µl of adipocyte suspension (3.5 x 10^5/ml) was incubated for 30 minutes at 37°C in a CO_2 incubator with 2 µl of various concentrations of GPIs or IPGs. Lipogenesis was initiated by the addition of 100 µl KRH containing 0.2 µCi D-[3-^{3}H] glucose, and the incubation continued for 2 hours. Adipocytes were harvested onto glass-fibre filter mats using a cell harvester, and rinsed with 5 mM glucose in 0.154M NaCl. 3 ml of a toluene-based scintillation cocktail were added to each filter disc for counting the radioactivity incorporated into lipids.

Effects on pyruvate dehydrogenase phosphatase (PDH)

HPTLC-purified GPI was sonicated in 100 µl of water and tested for its effect on bovine heart PDH-phosphatase. GPIs were sonicated in water before assay. Different fractions of IPGs eluted from cellulose columns were tested for their effects on bovine heart PDH-phosphatase. 2-16 µl of IPGs (containing 1-2 nmol phosphate/ml) were used to stimulate PDH phosphatase. Activated PDH was determined spectrophotometrically following the procedure described earlier (Caro et al, 1997) by measuring the rate of production of NADH at 340nm (Jasco V560 spectrophotometer, Jasco corporation, Tokyo, Japan).

Inhibition of cAMP-dependent protein kinase activity (PKA)

GPIs were sonicated in dilution buffer. The ability of GPIs and IPGs to inhibit PKA activity was determined using a colourimetric assay kit and a standard PKA preparation (Pierce, Rockford, IL, USA). Kemptide labelled with a fluorescent probe was used as PKA substrate. Phosphorylated Kemptide was detected by measuring absorbance at 570nm in OPTI max™ microplate reader (Molecular Device Corporation, Sunnyvale, CA, USA).

Determination of plasma and liver lactate

During the hypoglycaemia effect of *Plasmodium yoelii* GPI (4-6 hours after injection) blood was collected from the trunk as described above for lactate measurement. Liver was immediately homogenised in 0.5 M $HClO_4$ (1:10) and centrifuged at 10000 g for 10 minutes. The supernatant was neutralised with KOH and centrifuged for 5 minutes at

10000 g. Lactate in the supernatant and plasma was determined enzymatically following the standard procedure (Gutman & Wahlefed, 1974) using a kit supplied by Sigma Poole, UK.

Determination of liver glycogen

Liver glycogen content was determined enzymatically following standard procedure (Keppler & Dekker, 1974) with some modification. Briefly, frozen liver tissue was homogenised with 5 parts by weight ice-cold 70% perchloric acid and centrifuged at 4°C for 10 minutes. 200 µl supernatant was neutralized with 1M $KHCO_3$ and incubated with 2 ml acetate buffer (pH 4.8) containing 5 units of amyloglucosidase for 2 hours at 40°C. Glucose was determined enzymatically using glucose oxidase and peroxidase (Bergmeyer & Berndt, 1974) (kit supplied by Sigma).

Fructose-1,6-bisphosphatase (F-1,6-BPase)

Frozen livers obtained from *db/db* mice was used as a source of F-1,6-BPase. Livers were cut into small pieces and homogenised in 10 volumes (w/v) of cold 0.1 M Tris-HCl buffer solution, pH 7.5, containing 0.15 M KCl, 5 mM EDTA, 5 mM dithiothreitol and 5 mM $MgSO_4$. The homogenate was centrifuged at 105000 x g for one hour at 4°C. The supernatant was aliquoted and store at -80°C and 5-10 µl was used for the assay of F-1,6-Bpase activity. The precipitate was suspended in 1 ml buffer and used for determination of the activity of glucose-6-phosphate.

F-1,6-BPase activity was measured using a method described before (Ulm et al, 1975). Assay mixture

contained 0.0625 M- Tris-HCl (pH 7.4), 5mM EDTA, 5 mM $MgSO_4$, 0.25 mM NADPH, glucose-6-phosphate dehydrogenase (5 units/ml), phosphoglucose isomerase (5 units/ml) and 5 mM fructose-1,6-bisphosphate. The F-1,6-Bpase activity was measured in the absence and presence of IPGs or 2,5 anhydro-D-mannitol by measuring the rate of production of NADH at 340nm (Jasco V560 spectrophotometer, Jasco Corporation, Tokyo, Japan).

Glucose-6-Phosphatase (G6Pase)

Frozen livers obtained from STZ-diabetic mice was used as a source of glucose-6-phosphatase. Livers (7.5 g) were cut into small pieces and homogenised in 10 volumes (w/v) of cold 50 mM Tris- HIC buffer solution, pH 7.5, containing 250 mM sucrose and 0.2 mM EDTA. The homogenate was centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was centrifuged at 105,000 x g for 60 min at 4°C. The resulting sediments were suspended in 8 ml homogenizing buffer and used for the assay of G6Pase. G6Pase activity was measured using an assay mixture containing 900 µl Trinder reagent, 200 µg/ml liver microsomes, 0.1-0.5 M glucose-6-phosphate and *Plasmodium* GPI (1.4-7 µM) or water. The G6Pase activity was measured in the absence and presence of *Plasmodium* GPI by measuring the rate of production of quinoneimine at 510 nm and 28°C. (Jasco V560 spectrophotometer, Jasco corporation, Tokyo, Japan).

Measurement of plasma cholesterol and triglycerides

Blood was collected from the trunk after decapitation into heparinised tubes. Plasma was separated by centrifugation and frozen -20°C. Plasma was assayed for

total cholesterol and total triglycerides using kits from Sigma diagnostic. HDL cholesterol was assayed after precipitation of LDL cholesterol using phosphotungistic acid supplied by Sigma Diagnostics. Results, in mmol/l, are expressed as means ± SEM.

Statistical analysis

Statistical significance was assessed using ANOVA or Student's t-test for unpaired samples as appropriate. Values of $P<0.05$ were considered to be statistically significant.

Results

Effect of GPIs on the blood glucose of STZ-diabetic mice

A single i.v. injection of GPI (10 nmole) induced a higly significant drop in blood glucose from 17.8 ± 1.1 to 8.9 ± 0.65 mmol/l within 4 hours, an effect lasted for more than 6 hours (Fig.1 $p<0.0001$, $n=10$). The blood glucose of STZ-diabetic mice receiving saline remained above 19 mmol/l during the course of the experiment (Figure 1).

Effect GPIs on blood glucose of *db/db* diabetic mice

Obese diabetic (C57 Bl/Ks - *db/db*) mice were used at less than 15 weeks of age when they were hyperglycaemic and hyperinsulinaemic. GPI (8 nmole/mouse) induced a significant drop in blood glucose from 26.85 ± 1.5 mmol/l to 15.1 ± 0.5 mmol/l within 4 hours ($p<0.001$, $n=7$, Figure 2). A higher dose of GPI (30 nmole/mouse, i.v.) induced a highly significant drop in blood glucose from 23.2 (1.3 mmol/l to 8.3 ± 1.34 ($p<0.0001$, $n=6$, Figure 2). Blood glucose in saline-treated mice remained between 25 and 30 mmol/l during the course of the experiment (Figure

2). In a different experiments, GPI extracted from 5 x 10^8 parasites induced a significant drop in blood glucose ($p<0.01$, n=6) when administered orally in C57 B1/Ks - *db/db* obese diabetic mice (Figure 3).

Effect of GPIs on plasma insulin

GPI was injected in STZ-diabetic mice and after 6 hours blood was collected and plasma insulin was measured. GPI significantly increased the level of plasma insulin in STZ-diabetic mice compared with saline treated mice (Figure 4).

Effects of GPIs on plasma lactate of STZ-diabetic mice

Plasma lactate was measured in STZ-diabetic mice 6 hours after injection of GPI. There was an increased levels of plasma lactate in STZ-diabetic mice compared with normal mice (Figure 5). Intravenous injection of GPI significantly reduced plasma lactate in STZ-diabetic mice compared with STZ-diabetic mice treated with saline ($p<0.001$, n=8, Figure 5).

Effects on lipogenesis

Figure 6A shows the effect of two different batches of GPI on lipogenesis in rat adipocytes. GPI increased lipogenesis by more than 5-fold. In addition, different concentrations of GPI (0.062-1 µM, equivalent of phosphate) increased lipogenesis in the presence of a maximal dose of insulin (10^{-8} M) (Figure 6B).

Effects of GPIs on pyruvate dehydrogenase phosphatase (PDH-Pase)

Different concentration of GPIs (0.5-4.5 x 10^{-7} M)

stimulated PDH-Pase in a dose dependent manner (Figure 7). GPIs stimulated PDH-Pase by more than 95% above the base line value.

Effects on of GPIs on cAMP-dependent protein kinase activity (PKA)

Different concentrations of GPIs (0.012-12.2 $x10^{-6}$ M) inhibited PKA in a dose dependent manner (Figure 8) GPI (12.2 $x10^{-6}$ M) inhibited PKA by 25% (Figure 7). A different experiment using a different batch of GPIs (2 and 6.25 $x10^{-6}$ M) inhibited PKA by 23 and 40% respectively (Figure 9).

Effect of GPIs on Fructose-1,6-Bisphosphatase (F-1,6-BPase)

GPI (2 x 10^{-5} M) inhibited the activity of F-1,6-Bpaes by 25% (Figure 10). Figure 11 shows the effects of IPGs, anhydromannitol and GPIs on F-1,6-Bpase activities.

Effect of GPIs on total cholesterol and plasma triglycerides

The effects of *Plasmodium* GPI on plasma cholesterol and triglycerides level in STZ-diabetic mice are shown in Figures 12A and 12B. 6 hours after injection of GPI (10 nmole/mouse, i.v.) there was a significant drop in total plasma cholesterol compared to saline treated mice (Figure12A, $p<0.01$, $n=7$). 6 hours after injection of GPI (10 nmole/mouse, i.v.) there was a significant drop in plasma triglycerides compared to saline treated mice(Figure 12B, $p<0.05$, $n=6$).

Effect of *Plasmodium yoelii* GPI on plasma cholesterol in

STZ-diabetic mice

The effects of *Plasmodium yoelii* GPI on plasma cholesterol and the ratio of LDL:HDL lipids is shown in Figure 13 and Table 1.

Table 1:

Treatment	Total Cholesterol (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	Ratio of LDL/HDL
STZ-mice + GPI	2.460	2.456	0.004	0.002
STZ-mice + saline	3.838	3.025	0.813	0.269
Normal F1 mice	2.725	2.506	0.219	0.087

Chemical composition of *Plasmodium yoelii* GPIs

Initial characterisation of the total biologically active *Plasmodium yoelii*-derived GPIs anion revealed the presence of phosphorus and hexosamines. Using a Dionex-HPLC chromatography with Carbopac TM MA1 column on acid hydrolysates of GPIs after 24h hydrolysis in 6N HCl demonstrated the presence of *myo*-inositol and glycerol. Analysis on Carbopac TM PA10 column showed the presence of glucosamine, galactose, mannose, glucose and galactosamine. *Plasmodium yoelii* GPI was found to be labelled with H^3-*myo*-inositol.

Effect of *Plasmodium yoelii* IPGs on blood glucose in

STZ-diabetic mice

IPGs extracted from malaria parasites (5-10 x 10^9 /kg bw) were injected i.v. into STZ-diabetic mice, and blood glucose was monitored every 2 h for up to 8 h.

IPG-A induced a significant drop in blood glucose from 17.6 ± 0.9 to 12.1 ± 0.1 mmol/l an effect which lasted for more than 4 hour (Figure 14A $p<0.01$, $n=5$). The blood glucose of STZ-diabetic mice receiving saline remained above 19 mmol/l (Figure 14A). The hypoglycaemic effect of IPG-A was also tested in STZ-diabetic rats where it induced a 30 % drop in the blood glucose after 4-6 hours, with no effect on insulin concentrations (data not shown). IPG-P was fractionated on cellulose chromatography columns and the different eluates were tested in STZ-diabetic mice. Only IPG-P eluted with water induced a drop in blood glucose from 18.4 to 12.34 and 13 mmol/l in 4 hours and 6 hrs respectively.

Effect of *Plasmodium yoelii* IPGs on blood glucose of *ob/ob* diabetic mice

Obese diabetic mice (C57 BL/6J-*ob/ob*) were used at the time of hyperglycaemia and hyperinsulinaemia. IPG-A induced a rapid and significant drop in the blood glucose from 12.8 ± 0.7 mmol/l to the euglycaemic value of 7.6 ± 0.7 mmol/l within 2 hours ($p<0.02$, Figure 14B). After 6 hours there was still a significant difference ($p<0.02$, Figure 14B) in the blood glucose of IPG-A treated mice compared to saline treated animals (10.24 ± 1.5 versus 17.5 ± 1.3 mmol/l, respectively). IPG-P also induced a significant drop in the blood glucose of *ob/ob* mice from 17.3 ± 1 to 9.4 ± 0.7 mmol/l within 4 hours ($p<0.01$, Figure 14C). The blood glucose in ob/ob mice receiving

saline remained high around 18 mmol/l after 4 hours.

Effect of IPG-P on blood glucose of *db/db* diabetic mice

Obese diabetic (C57BL/Ks - *db/db*) mice were used at less than 15 weeks of age when they were hyperglycaemic and hyperinsulinaemic. IPG-P induced a significant drop in blood glucose from 26.85 ± 1.5 mmol/l to 15.1 ± 0.3 mmol/l within 4 hours ($p<0.01$, Figure 14D). Blood glucose in saline- treated mice remained between 25 and 30 mmol/l during the course of the experiment (Figure 14D).

Effect of *Plasmodium yoelii* IPGs on cAMP-dependent protein kinase activity (PKA)

The ability of *Plasmodium yoelii* IPGs to inhibit PKA activity was determined after fractionation of IPGs on cellulose chromatography columns (Figure 15). Different concentrations (0.001-7 µM) of acid and water eluates were tested. Both IPG-P and IPG-A induced a dose-related inhibition of cAMP-dependent protein kinase activity, with IPG-P being more inhibitory. Acid eluates of IPG-A and IPG-P inhibited PKA by more than 95%. 50% inhibitory dose concentrations (IC_{50}) for the acid eluates of IPG-A and IPG-P were 0.9 and 0.09 µM respectively.

Effect of *Plasmodium yoelii* IPGs on lipogenesis

IPG-P and IPG-A eluted from the anion exchange resin were subjected to cellulose chromatography column (1 ml) and eluted with different solvents (refer to the method section) and 1 µM of IPGs (equivalent of phosphate) was used for the lipogenesis assay. IPG-P from mammalian sources normally has no significant lipogenic activities.

Plasmodium IPG-P increased lipogenesis in adipocytes by 20-30% from basal (Table 2, with methanol fraction stimulation was statistically significant, $p<0.01$). In the presence of a maximal dose of insulin (10^{-8} M), IPG-P increased lipogenesis by 20-30% (Table 1, with acid fraction stimulation was statistically significant, $p<0.01$). However, different fractions of IPG-A had no significant lipogenic activity (Table 2).

Table 2. Effect of IPG-A and IPG-P fractions (1µM) eluted from a cellulose column on lipogenesis in absence of insulin and in presence of a maximal concentration of insulin (10^{-8}M). Values are mean of percentage stimulation ± SD, *$p<0.01$ vs basal, n=4.

Fraction	IPG-P (1µM)		IPG-A (µM)	
	(-) insulin	(+) insulin	(-) insulin	(+) insulin
B:E:W	13.1 ± 4.6	20.2 ± 6.0	-21.2 ± 2.3	-20.5 ± 3.4
Methanol	23.9 ± 0.4*	23.0 ± 5.4	-3.4 ± 0.4	-6.4 ± 1.3
Water	31.9 ± 6.8	30.9 ± 7.0	4.4 ± 0.5	1.8 ± 0.4
Acid	21.6 ± 5.9	30.2 ± 1.7*	2.2 ± 0.2	5.7 ± 0.7

Effects on pyruvate dehydrogenase phosphatase (PDH)

The ability of *Plasmodium yoelii* IPGs to stimulate PDH was determined after fractionation of IPGs on cellulose chromatography columns. Only IPG-P (acid fraction) stimulated PDH-phosphatase. Different concentrations of IPG-P (0.006, 0.0125 and 0.02 µM) stimulated PDH-phosphatase by 60, 100 and 130% above the base line value and at 0.05 µM by 101% (Figure 16). 50% effective dose concentration of IPG-P (ED_{50}) was 0.005 µM. IPG-A fractions had no effect on PDH phosphatase (data not shown).

Chemical composition of *Plasmodium yoelii* IPGs

Initial characterisation of the total biologically active *Plasmodium*-derived molecules recovered from the anion-exchange resin revealed the presence of phosphorus and hexosamines. Thus the *Plasmodium yoelii* IPGs from 15 infected mice contained 0.568 µmole and 0.265 µmole inorganic phosphate in IPG-A and IPG-P respectively. Hexosamine concentrations in IPG-A and IPG-P were 0.117 µmole and 0.106 µmole respectively. Using a Dionex-HPLC chromatography with Carbopac TM PA10 column on acid hydrolysates of IPG-A (Figure 16a) and IPG-P (Figure 17a) after 24 h hydrolysis in 6N HCl demonstrated the presence of galactosamine, glucosamine and glucose (Figure 17a). However, there was a difference in the composition of sugar released after 4 h hydrolysis of IPGs in 4N HCl acid (Figures 17b and 18b). After 4 h hydrolysis of IPG-A, galactosamine, galactose, mannose and glucose were released (Figure 17b). However, after 4 h hydrolysis of IPG-P, in addition of galactosamine, galactose, mannose and glucose, galactosamine was also released. (Figure

17b). Analysis on Carbopac™ MA1 column showed the presence of *myo*-inositol and glycerol in IPG-A (Figure 17c) and (Figure 18c) after hydrolysis in 6N HCl for 24h.

Discussion

These results establish that phosphoglycan messengers (PGMs) comprising lipid groups have one or more of the biological activities previously thought to be associated with IPGs. These observations arose from the experiments described herein in which GPIs and IPGs obtained from malaria parasites were studied in murine models of Type 2 diabetes by administering these mediators to diabetic *db/db* and monitored their blood glucose. Experiments in which the hypoglycaemic effect of *Plasmodium yoelii* GPIs and IPGs on STZ-diabetic mice are also disclosed and other insulin mimetic activities are explored *in vitro*, showing that *Plasmodium* parasites can be used as a source of biologically active GPIs and further providing data to enable the development of new drugs for the clinical management of diabetes including Type 2 diabetes. These observations are important as there is a continuing need for new antidiabetic agents, since biguanides have undesirable side-effects while sulphonylurea is ineffective in patients with severely impaired islet cell function. Long term treatment with these agents may result in secondary failure of efficacy with enhancement of obesity in 50% of patients.

The substances disclosed herein which have been isolated from malaria parasites, GPIs, yields molecules that can induce lipogenesis both in the absence and even in the presence of maximal doses of insulin (10^{-8} M). This

suggests that malaria parasites GPIs either sensitise cells to insulin action or act through a pathway different from that used by insulin. It seems likely that the main effect of the *Plasmodium yoelii* IPGs is to reduce insulin resistance in *db/db* mice.

In addition, the present results are the first to establish that malaria parasites GPIs and IPGs have antidiabetic actions in murine models of Type 2 diabetes. The diabetic mouse strains C57BL/Ks-db/db and C57BL/6J-ob/ob employed in these experiments exhibit many of the classical metabolic disturbances of human Type 2 diabetes, including hyperglycaemia, obesity, and early hyperinsulinaemia. Since these diabetic mice were used at the time of peak hyperinsulinaemia, the major effect of malaria parasites GPIs and IPGs are to amplify insulin action, possibly through sensitisation.

The molecular nature of the *Plasmodium yoelii*-derived GPIs active in these experiments may be the precursor of inositol phosphoglycans. Characterization of these molecules described herein shows that they are ninhydrin and orcinol positive indicating the presence of amino groups and sugars respectively, they contain phosphate, and from Dionex analysis, they contain *myo*-inositol, glycerol, glucosamine, galactosamine, galactose and mannose.

The rate of gluconeogenesis increases in the liver of db/db mice. The present study demonstrated that GPIs and IPGs inhibited the activity of the gluconeogenic enzyme Fructose-1,6-Bisphosphatase and glucose-6-phosphatase *in*

vitro. This observation contributes to the hypoglycaemic activities of GPIs. However, in view of the hyperinsulinaemia during infection with *Plasmodium yoelii* parasites, the release of insulin and the blood glucose lowering activities of GPI in STZ-diabetic mice can be partially explained by the increase in insulin secretion.

The acid eluate of cellulose column chromatography of IPG-P increased lipogenesis and significantly stimulated pyruvate dehydrogenase phosphatase. This finding agrees with previous studies using IPGs from different sources showing that IPG-A and IPG-P are functionally distinct. Though both *Plasmodium yoelii* IPG-A and IPG-P lowered blood glucose in the different murine models of diabetes and inhibited PKA, they differed in their abilities to modulate lipogenesis and PDH phosphatase.

Plasmodium yoelii IPGs contain phosphate and are ninhydrin and orcinol positive indicating the presence of free amino groups and sugars respectively. The carbohydrate composition analysis of biologically active malaria derived IPGs type A and P has demonstrated the presence of IPG marker molecules such as inositol and glucosamine. *Plasmodium yoelii* IPGs are structurally different to IPGs extracted from liver since malaria IPGs do not contain *chiro*-inositol, and only *myo*-inositol was demonstrated both in A and P types. In addition malaria IPG-P contains glucosamine, galactosamine and galactose. The structure of *Plasmodium yoelii* IPGs may explain the reason why there was no reported data with IPGs extracted from liver in murine models of Type 2 diabetes.

References

The references mentioned herein are all incorporated by reference.

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Claims:

1. A substance which is a carbohydrate derivatised phosphatidyl cyclitol.

2. The substance of claim 1, wherein the substance comprises one or more lipid moieties attached to the cyclitol group or the phosphatidyl group via one or more ester and/or ether linkages.

3. The substance of claim 1 or claim 2, wherein the substance has one or more properties selected from reducing blood glucose in a diabetic *ob/ob* or *db/db* murine model, regulating lipogenesis, stimulating pyruvate dehydrogenase phosphatase, inhibiting cAMP dependent protein kinase, inhibiting fructose-1,6-biphosphatase or inhibiting glucose-6-phosphatase.

4. The substance of any one of claims 1 to 3, wherein the cyclitol is *chiro*-inositol, *myo*-inositol or derivatives thereof.

5. The substance of claim 4, wherein the cyclitol is pinitol, or a derivative thereof.

6. The substance of any one of the preceding claims, wherein the phosphatidyl group has one oxygen linked lipid moieties.

7. The substance of any one of claims 1 to 6, wherein the phosphatidyl group has two oxygen linked lipid moieties.

8. The substance of claim 6 or claim 7, wherein the lipid(s) attached phosphatidyl group is or are diacyl, dialkyl, acyl-alkyl, alkyl-acyl, lysol-acyl, lysol-alkyl, acyl-lysol or alkyl-lysol lipids.

9. The substance of any one of the preceding claims, wherein the carbohydrate group is a hexose.

10. The substance of claim 9, wherein the carbohydrate is glucosamine, galactosamine, galactose, mannose or glucose.

11. A substance which is a carbohydrate derivatised phosphatidyl cyclitol which is devoid of protein.

12. The substance of any one of the preceding claims for use in method of medical treatment.

13. Use of a substance which is a carbohydrate derivatised phosphatidyl cyclitol of any one of claims 1 to 11 for the preparation of a medicament for the treatment of a conditions ameliorated by administration of a PGM second messenger.

14. The use of claim 13, wherein the substance has the property of reducing blood glucose levels.

15. The use of claim 13 or claim 14, wherein the medicament for the treatment diabetes or a related condition.

16. The use of any one of claims 13 to 15, wherein the

diabetes or related condition is diabetes due to insulin resistance, insulin resistance in Type 1 diabetes and brittle diabetes, Type 2 diabetes (both obese and lean forms), diabetic complications or conditions associated with insulin resistance or insulin underproduction, such as neurotrophic disorders or polycystic ovary syndrome, age-related memory loss, and post-ischaemic damage secondary to stroke or post-transplant complications, and dyslipidemia resulting from diabetes or diabetic complications.

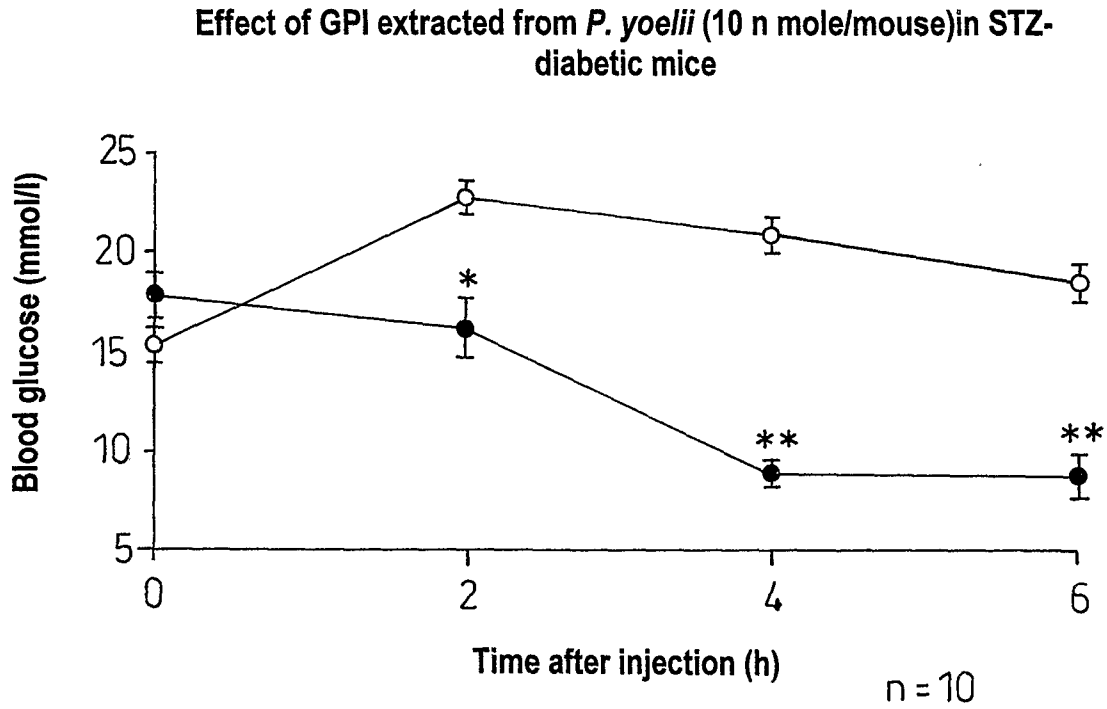
Effect of GPI extracted from P. yoelii (10 n mole/mouse)in STZ-diabetic mice
Blood glucose (mmol/l)
25
20
15
10
5
0
2
4
6
*
**
**
Time after injection (h)
n = 10

Fig. 1

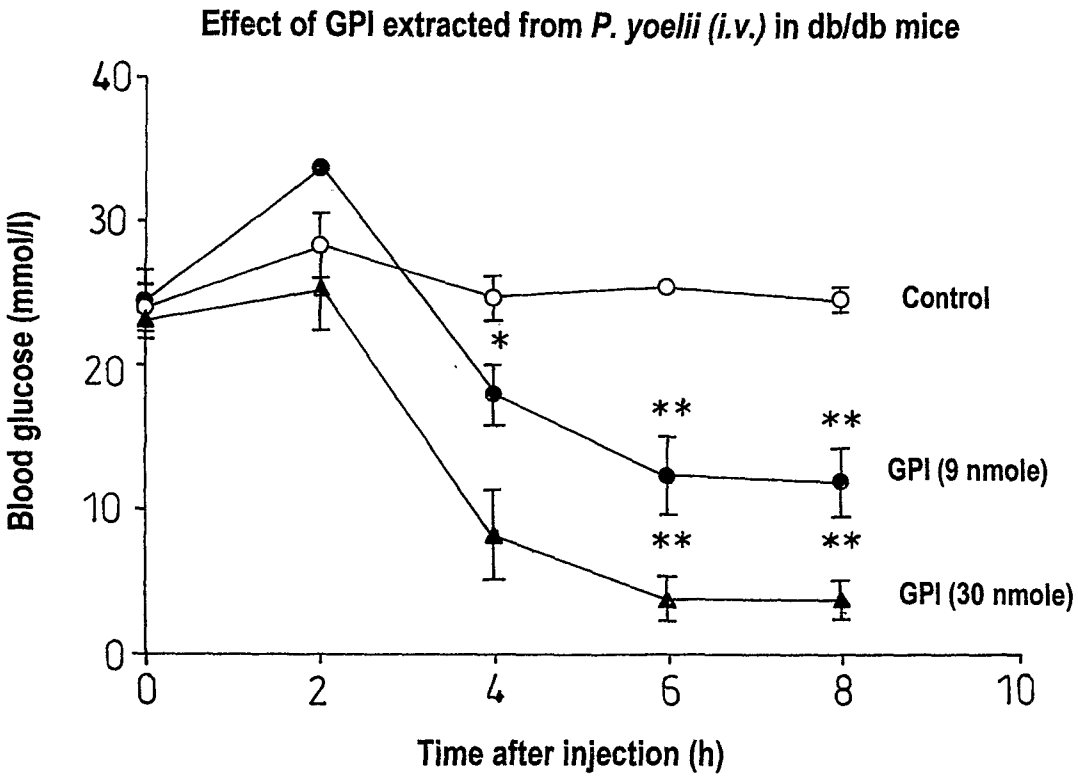
Effect of GPI extracted from P. yoelii (i.v.) in db/db mice
Blood glucose (mmol/l)
40
30
20
10
0
0
2
4
6
8
10
Control
GPI (9 nmole)
GPI (30 nmole)
*
**
**
**
**
Time after injection (h)

Fig. 2

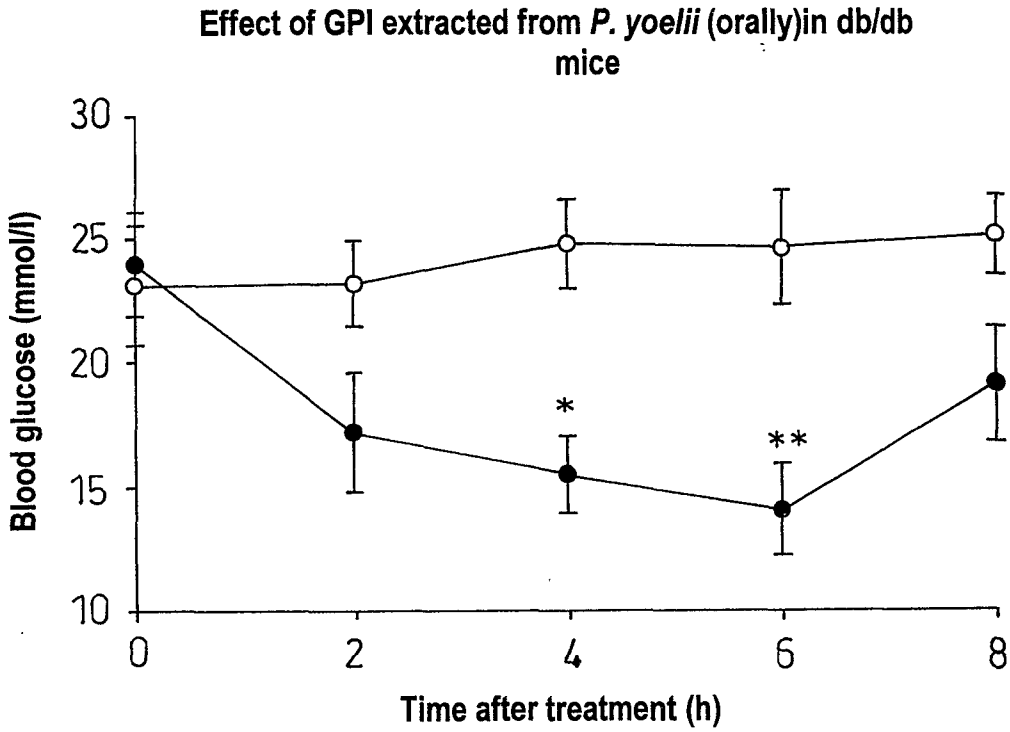
Effect of GPI extracted from P. yoelii (orally)in db/db mice
Blood glucose (mmol/l)
30
25
20
15
10
0
2
4
6
8
*
**
Time after treatment (h)

Fig. 3

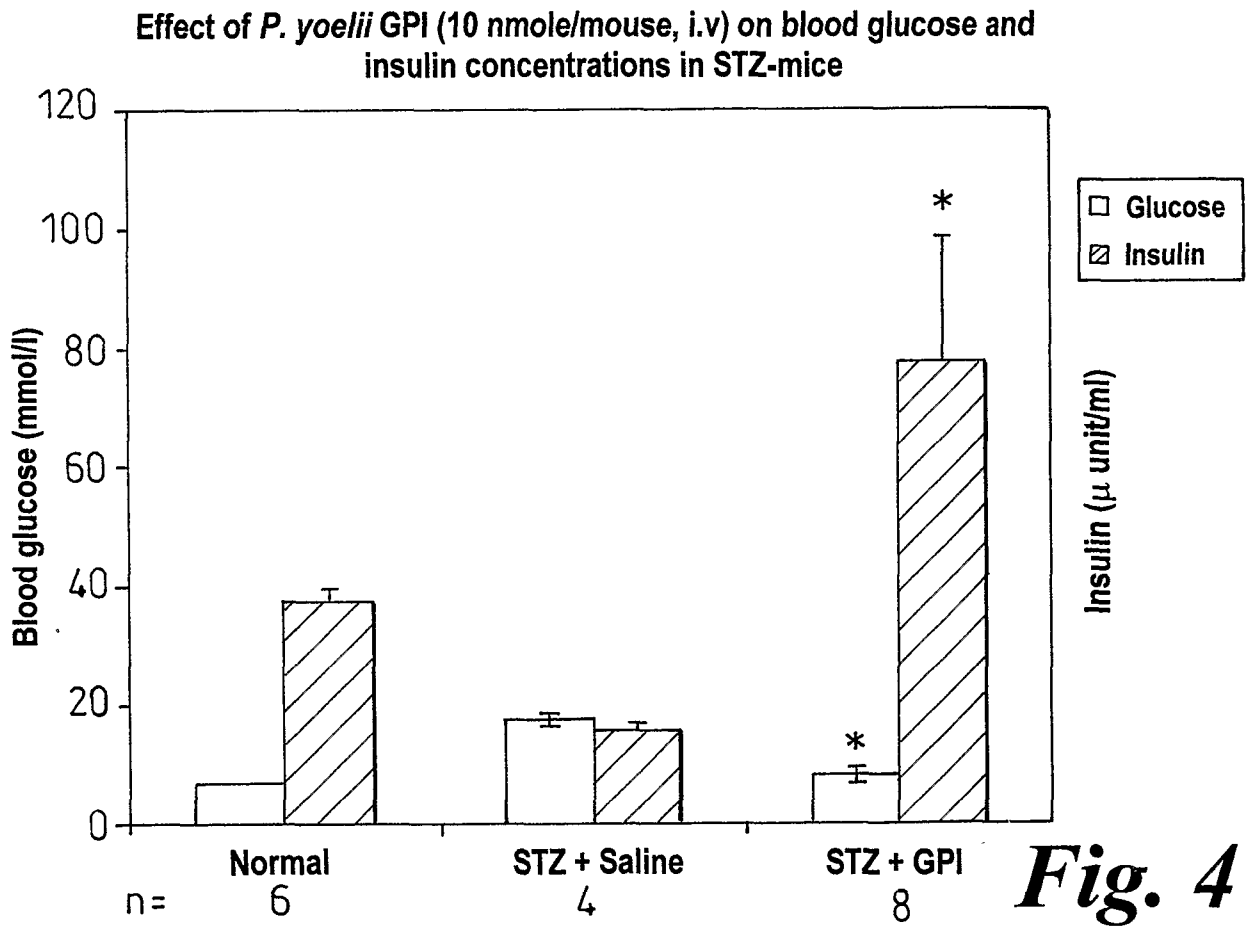
Effect of P. yoelii GPI (10 nmole/mouse, i.v) on blood glucose and insulin concentrations in STZ-mice
Blood glucose (mmol/l)
Insulin (μ unit/ml)
120
100
80
60
40
20
0
Glucose
Insulin
*
*
Normal
STZ + Saline
STZ + GPI
n=
6
4
8

Fig. 4

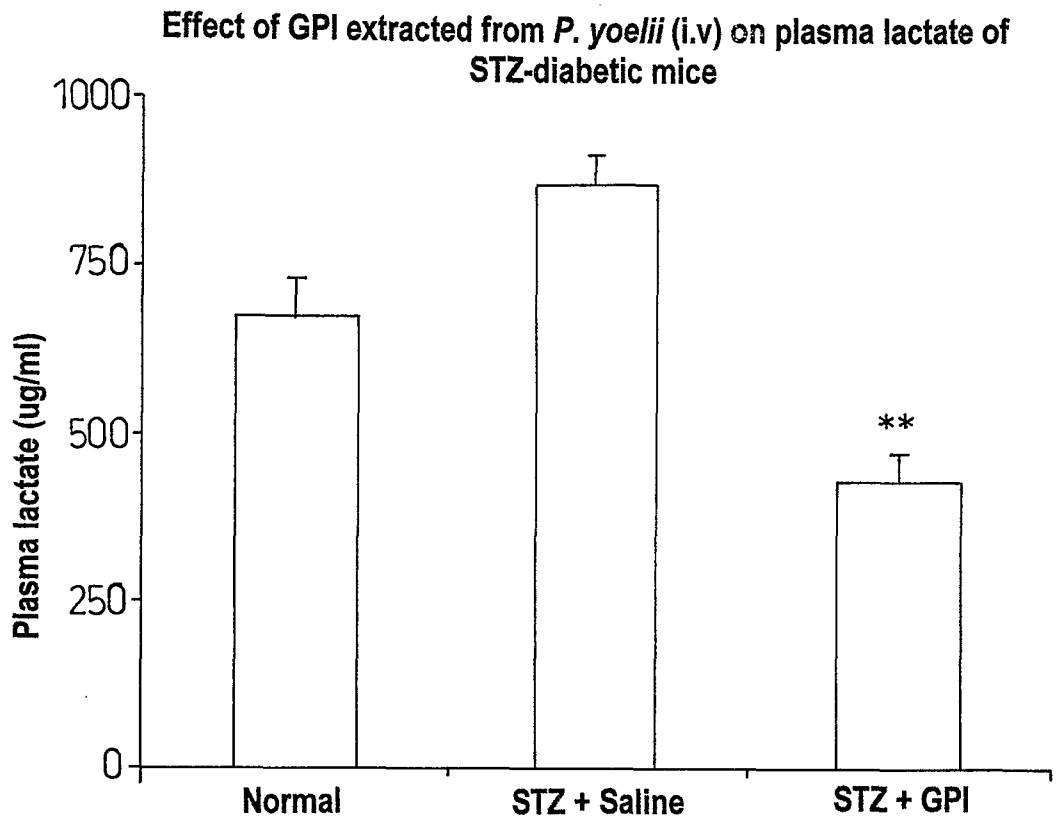
Effect of GPI extracted from P. yoelii (i.v) on plasma lactate of STZ-diabetic mice
Plasma lactate (ug/ml)
1000
750
500
250
0
**
Normal
STZ + Saline
STZ + GPI

Fig. 5

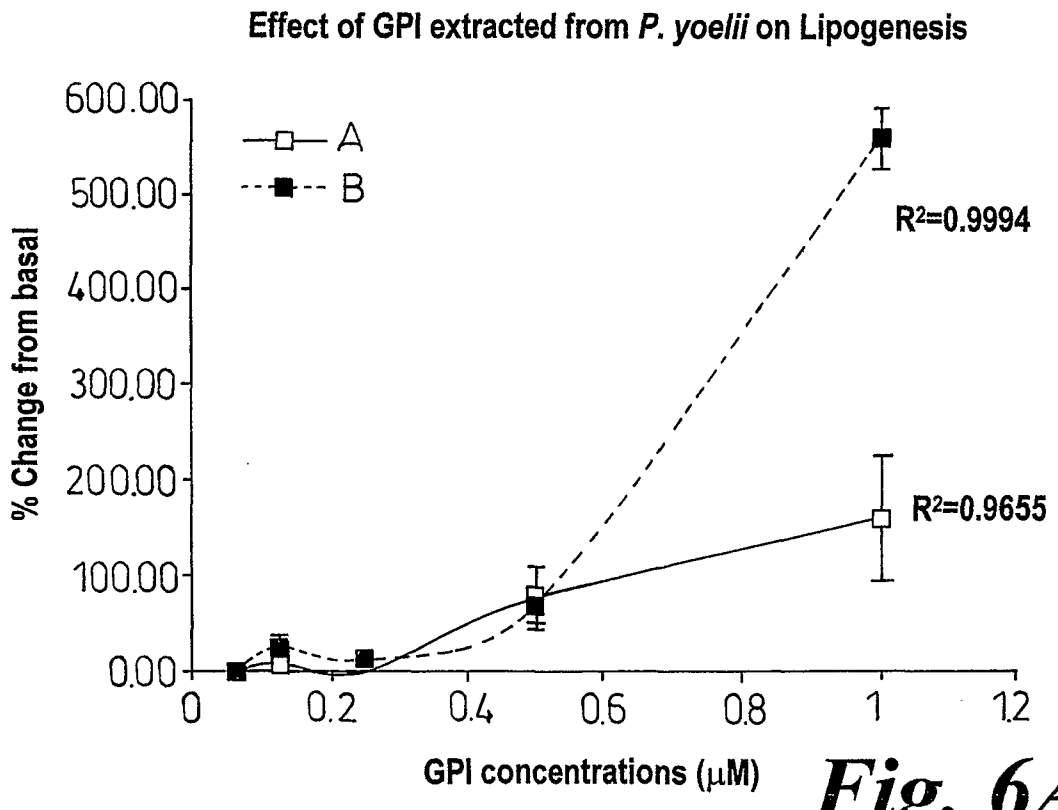
Effect of GPI extracted from P. yoelii on Lipogenesis
% Change from basal
600.00
500.00
400.00
300.00
200.00
100.00
0.00
A
B
R²=0.9994
R²=0.9655
0
0.2
0.4
0.6
0.8
1
1.2
GPI concentrations (μM)

Fig. 6A

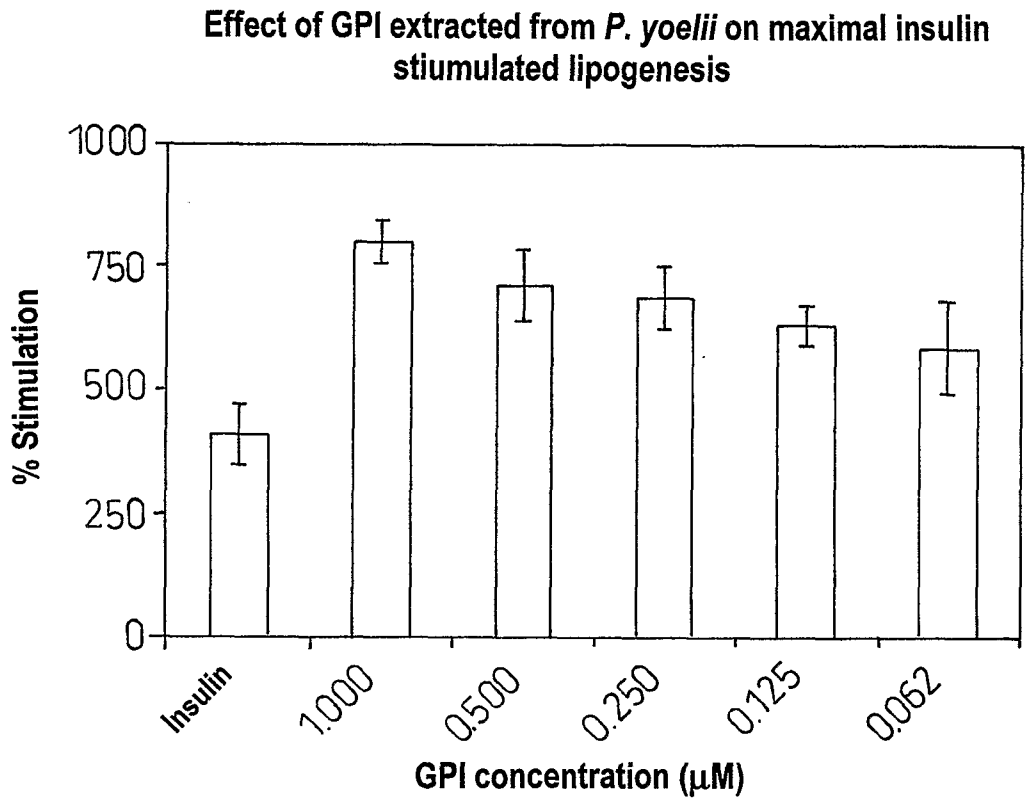
Effect of GPI extracted from P. yoelii on maximal insulin stiumulated lipogenesis
% Stimulation
1000
750
500
250
0
Insulin
1.000
0.500
0.250
0.125
0.062
GPI concentration (μM)

Fig. 6B

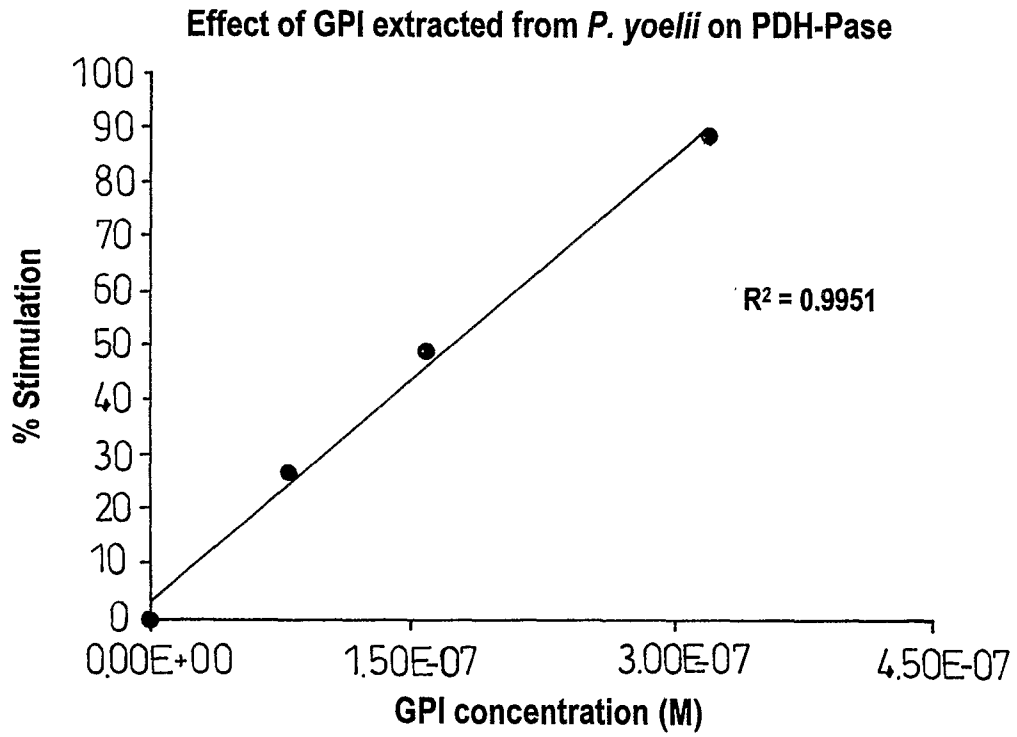
Effect of GPI extracted from P. yoelii on PDH-Pase
% Stimulation
100
90
80
70
60
50
40
30
20
10
0
R² = 0.9951
0.00E+00
1.50E-07
3.00E-07
4.50E-07
GPI concentration (M)

Fig. 7

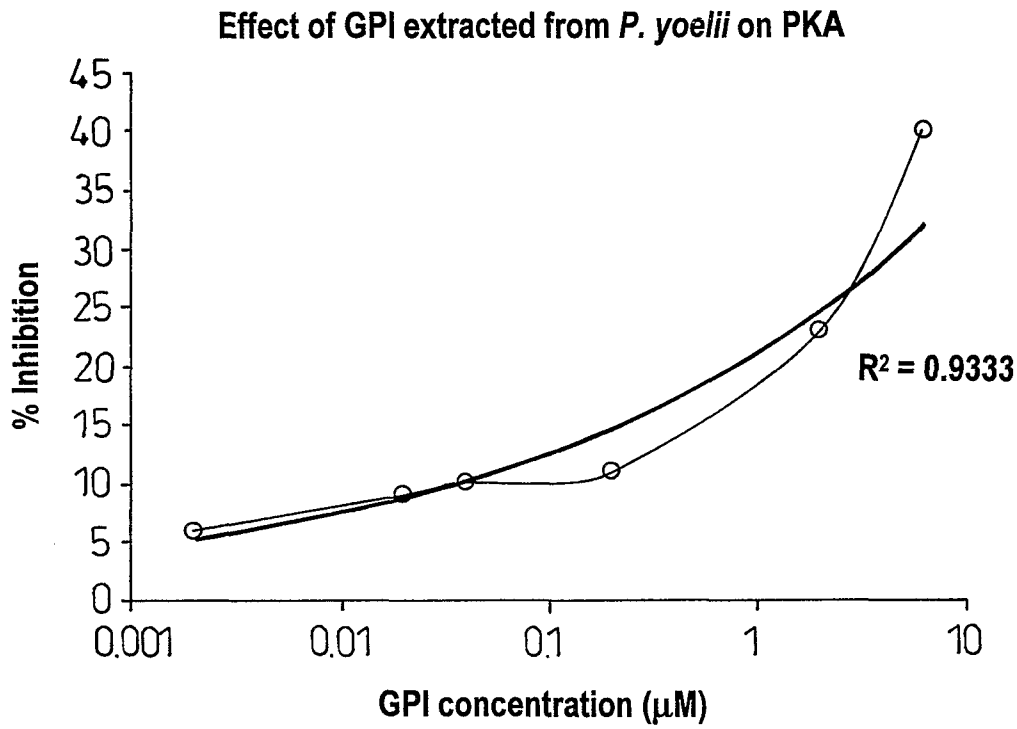
Effect of GPI extracted from P. yoelii on PKA
% Inhibition
45
40
35
30
25
20
15
10
5
0
0.001
0.01
0.1
1
10
R² = 0.9333
GPI concentration (µM)

Fig. 8

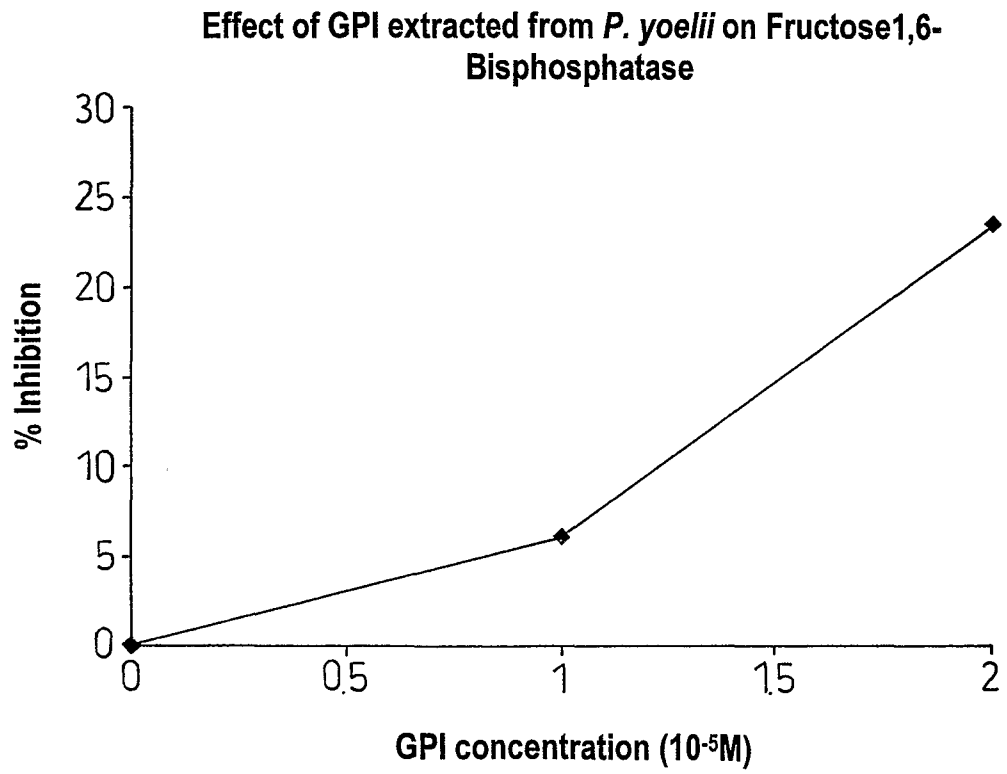
Effect of GPI extracted from P. yoelii on Fructose1,6-Bisphosphatase
% Inhibition
30
25
20
15
10
5
0
0
0.5
1
1.5
2
GPI concentration (10-5M)

Fig. 9

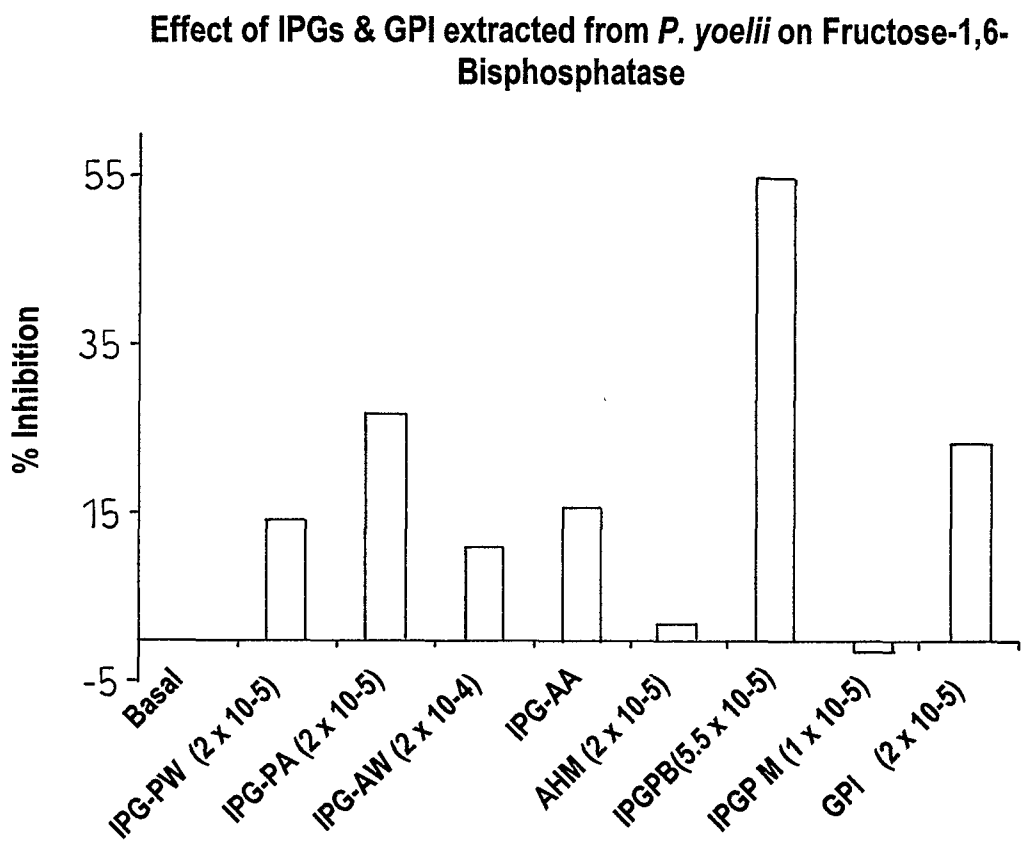
Effect of IPGs & GPI extracted from P. yoelii on Fructose-1,6-Bisphosphatase
% Inhibition
55
35
15
-5
Basal
IPG-PW (2 x 10-5)
IPG-PA (2 x 10-5)
IPG-AW (2 x 10-4)
IPG-AA
AHM (2 x 10-5)
IPGPB(5.5 x 10-5)
IPGP M (1 x 10-5)
GPI (2 x 10-5)

Fig. 10

Effect of *P. yoelii* GPI on Glucose-6-Phosphatase using 2 mM Glucose-6-phosphate as substrate

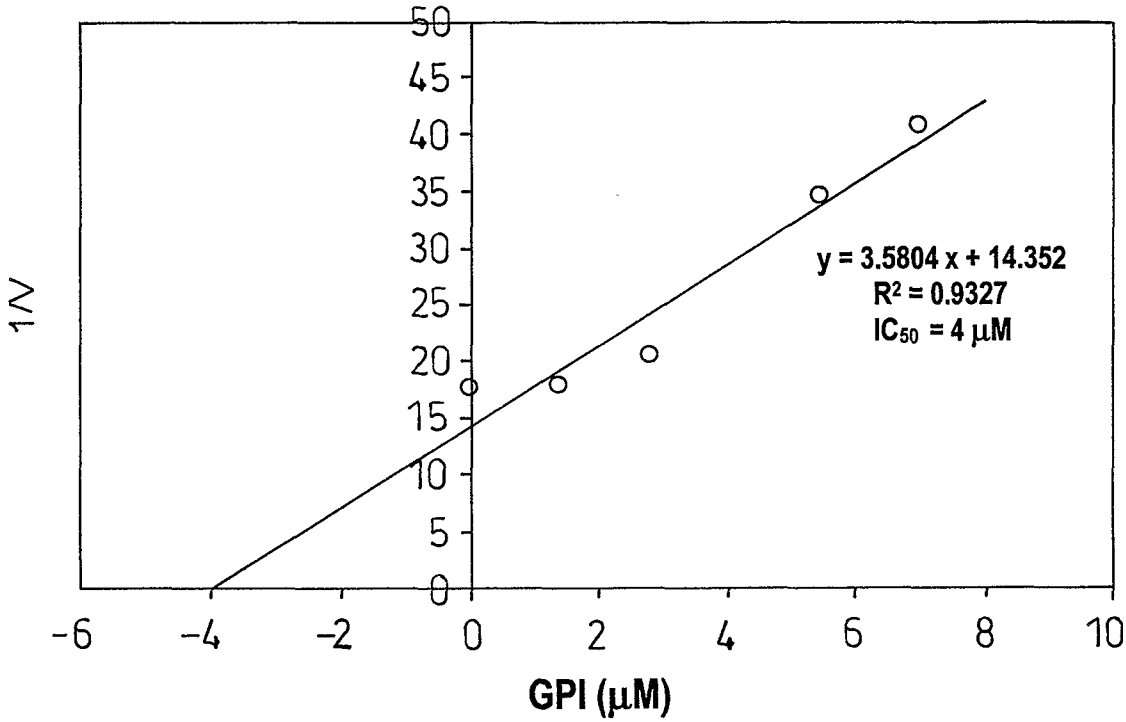

Fig. 11A

Effect of *P. yoelii* GPI on Glucose-6-Phosphatase using 10mM Glucose-6-phosphate as substrate

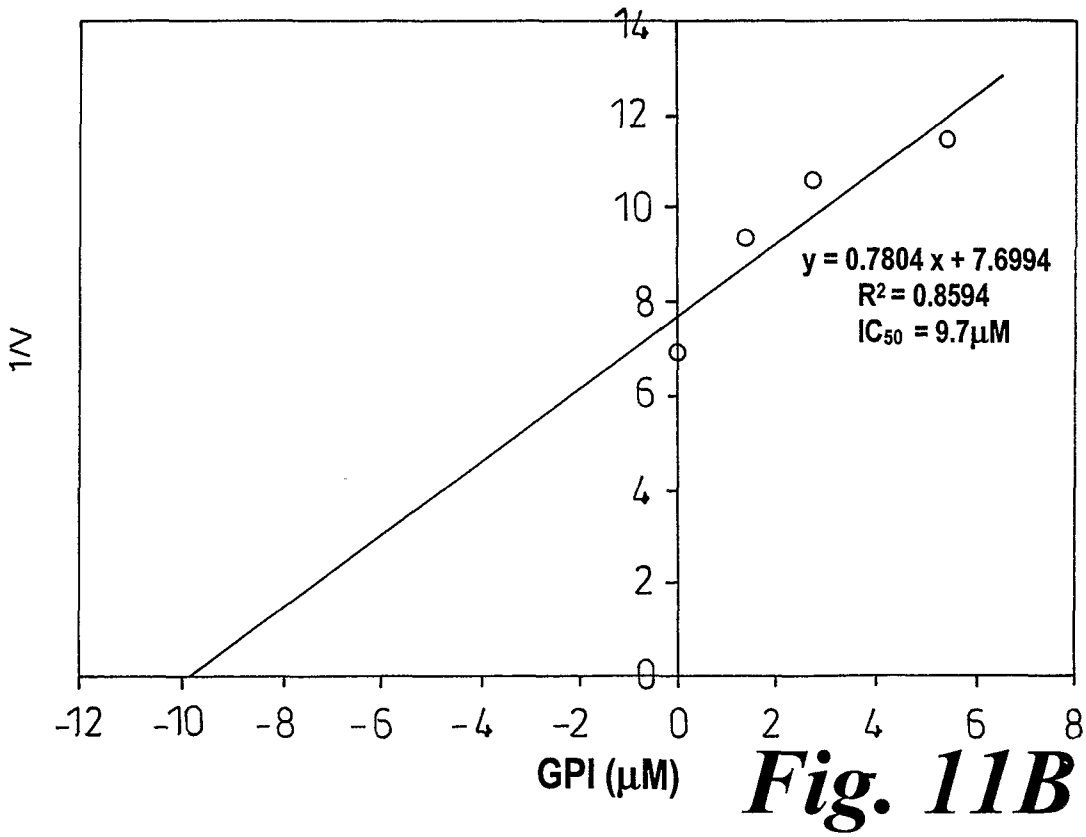

Fig. 11B

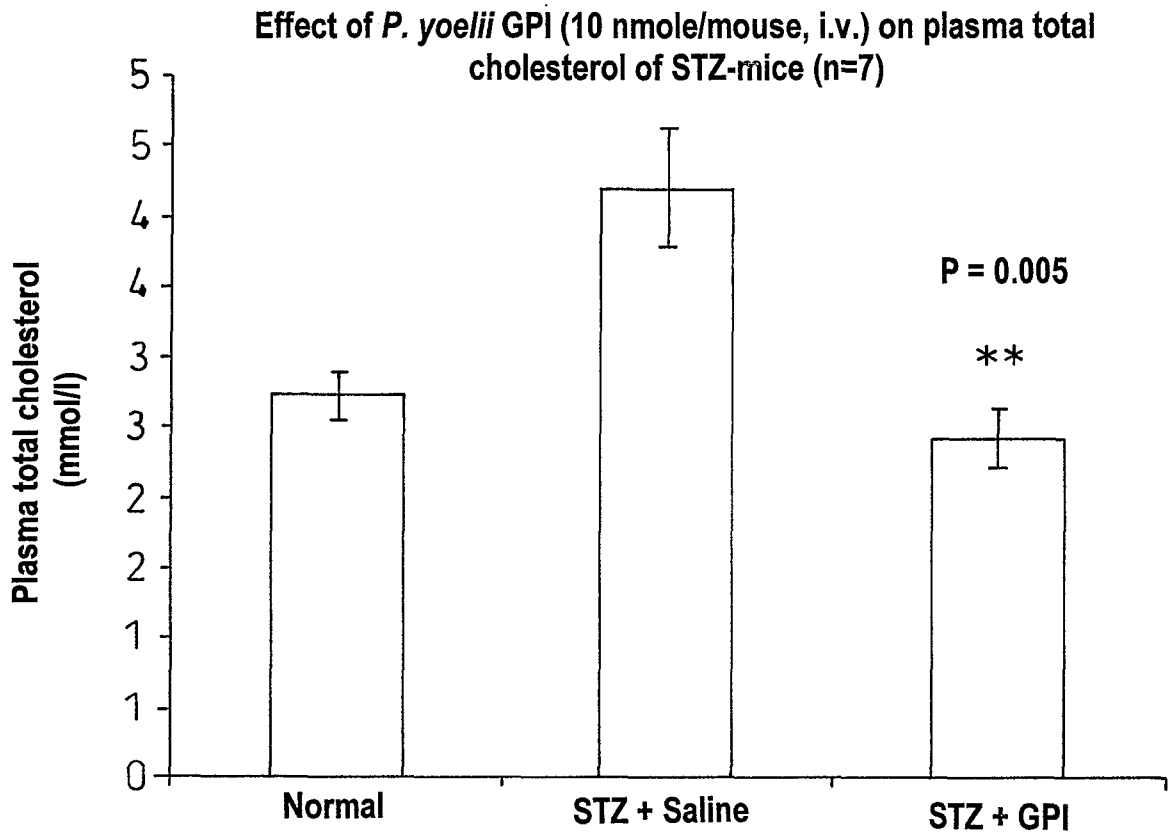

Fig. 12A

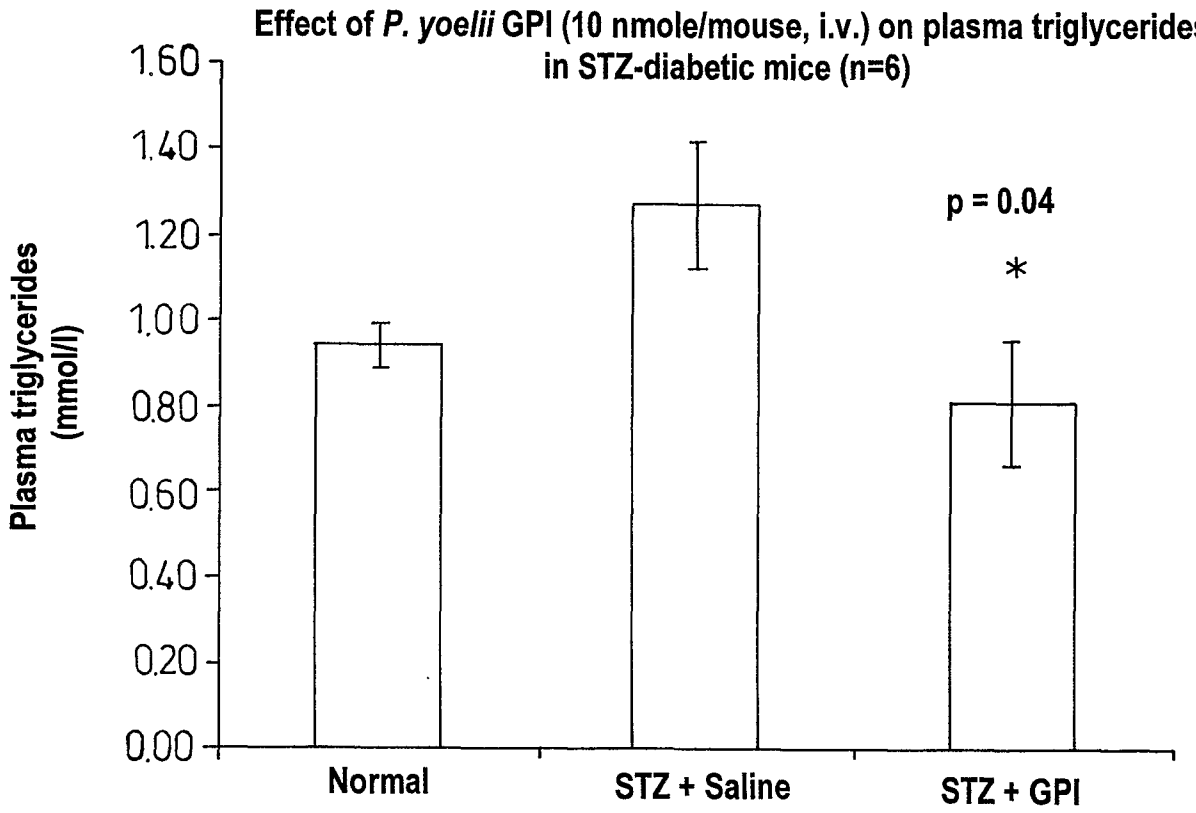

Fig. 12B

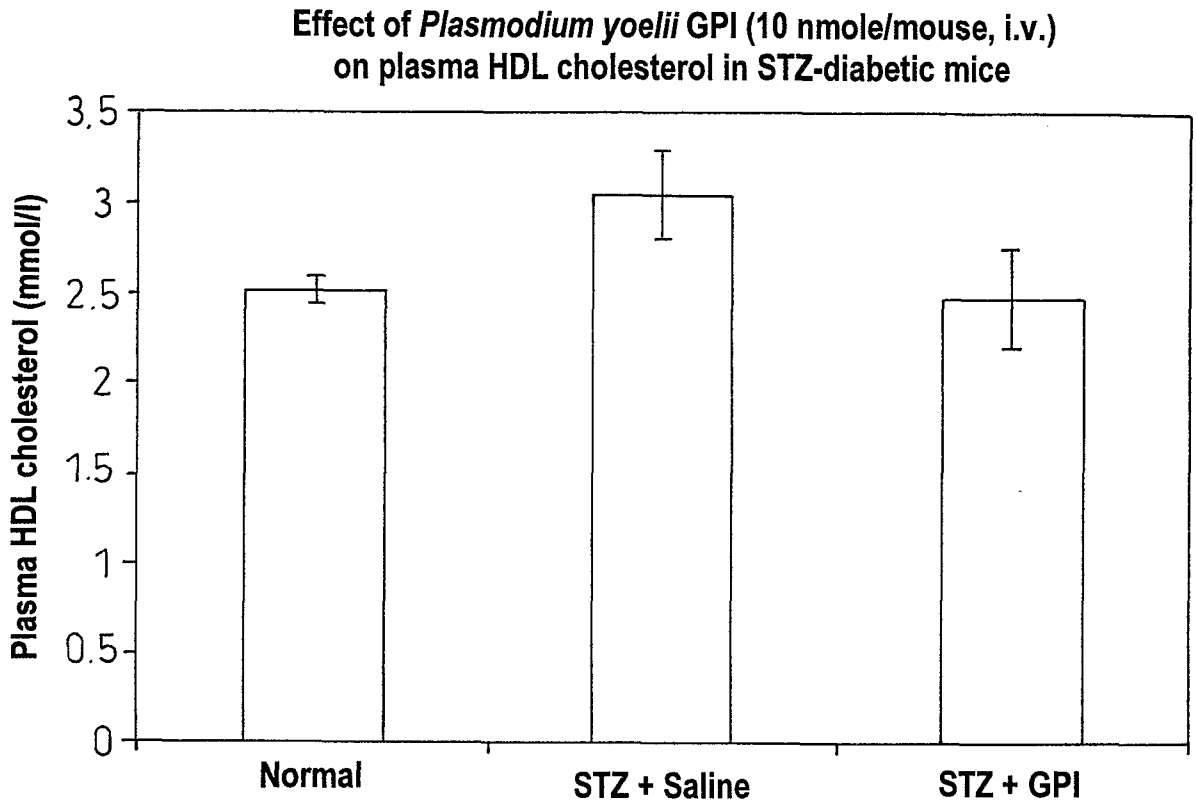

Fig. 13

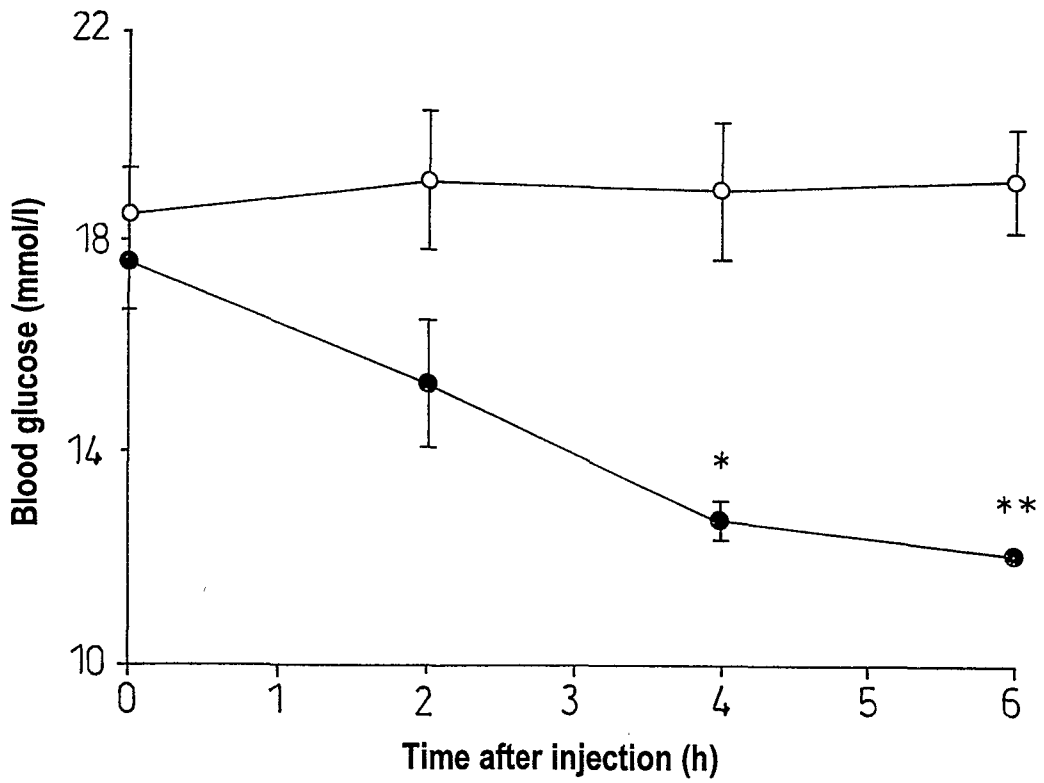

Fig. 14A

10/14

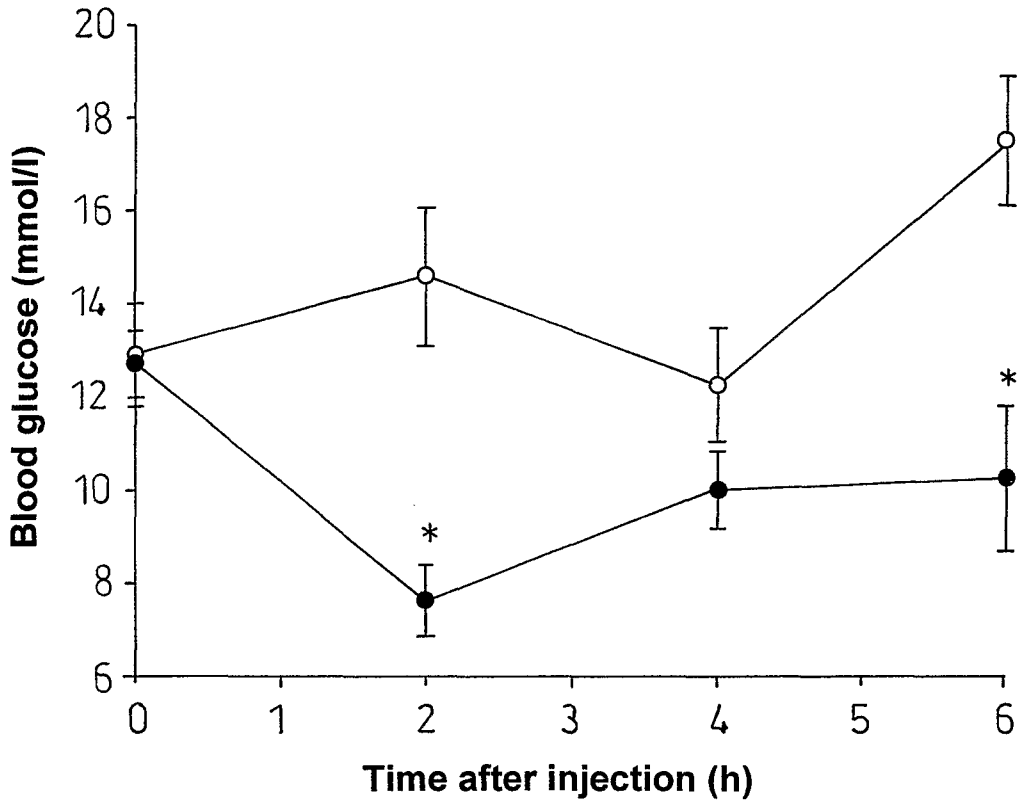

Fig. 14B

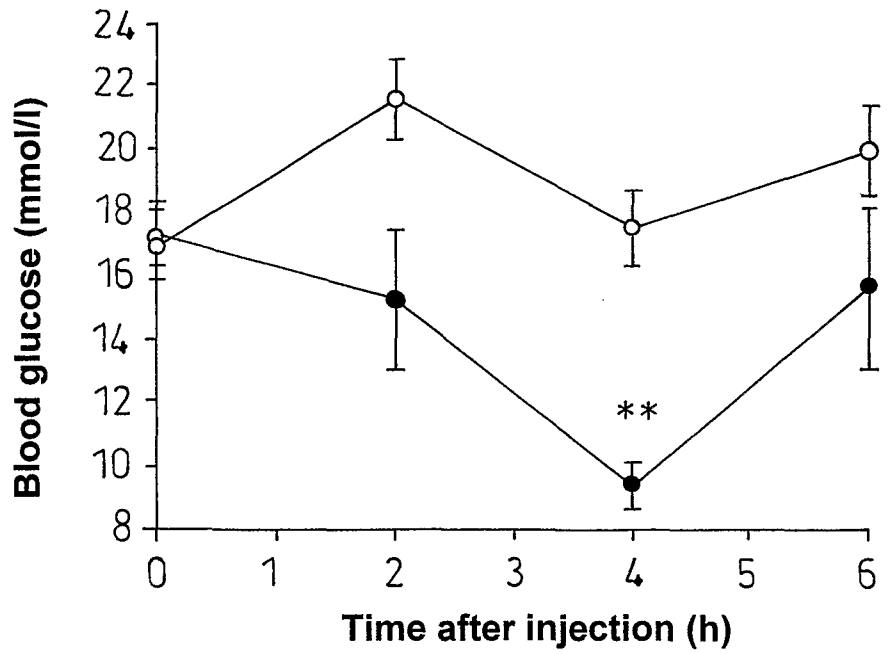

Fig. 14C

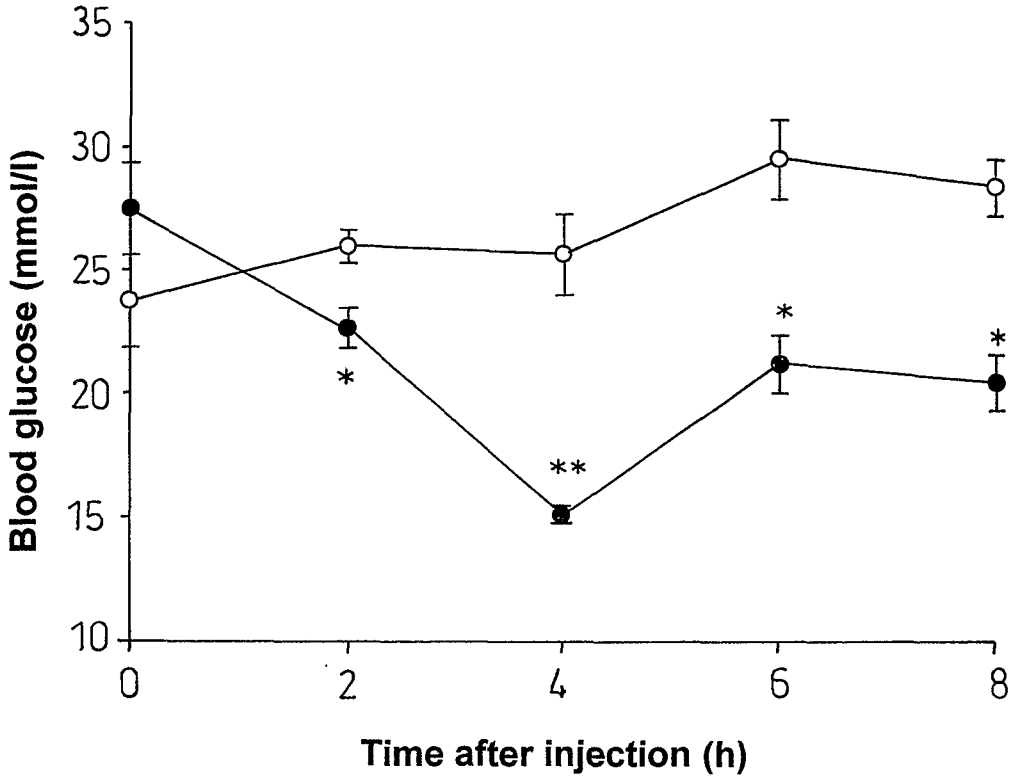

Fig. 14D

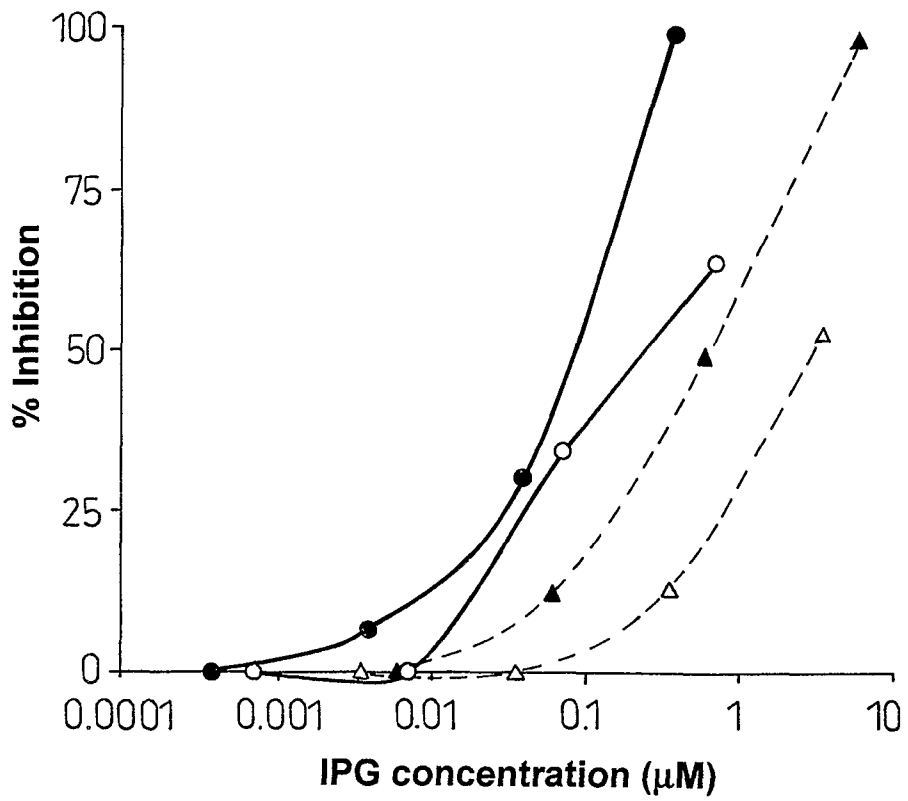

Fig. 15

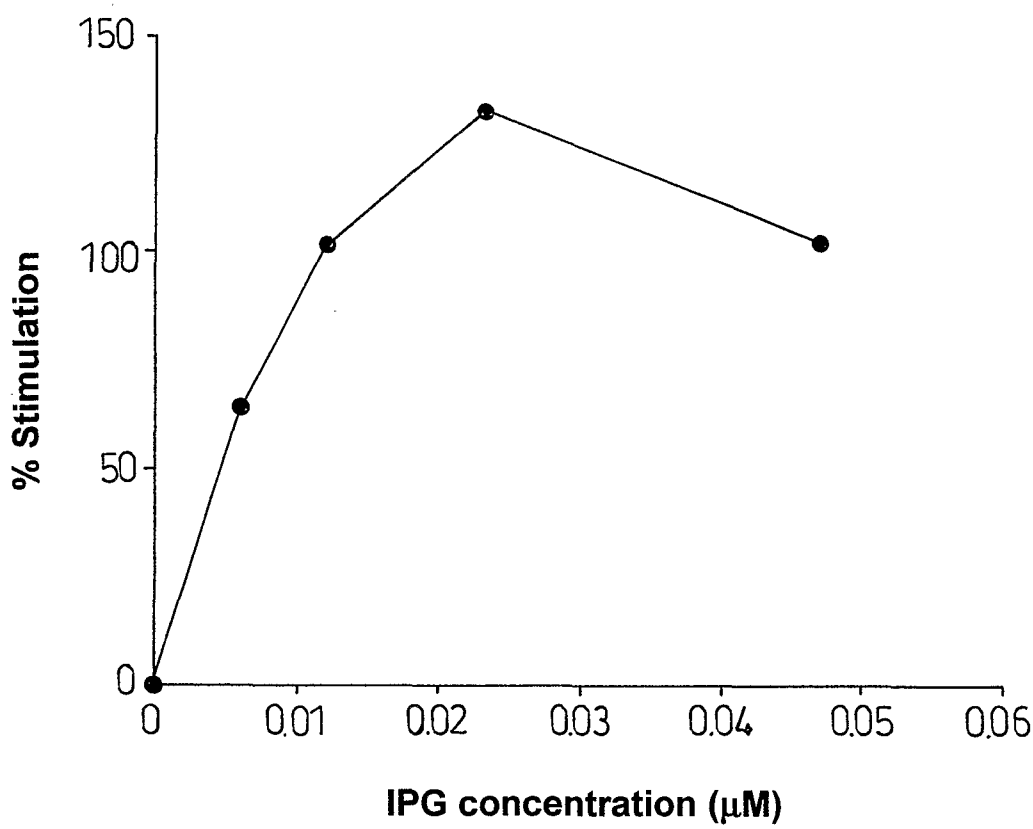
150
100
50
0
% Stimulation
0
0.01
0.02
0.03
0.04
0.05
0.06
IPG concentration (μM)

Fig. 16

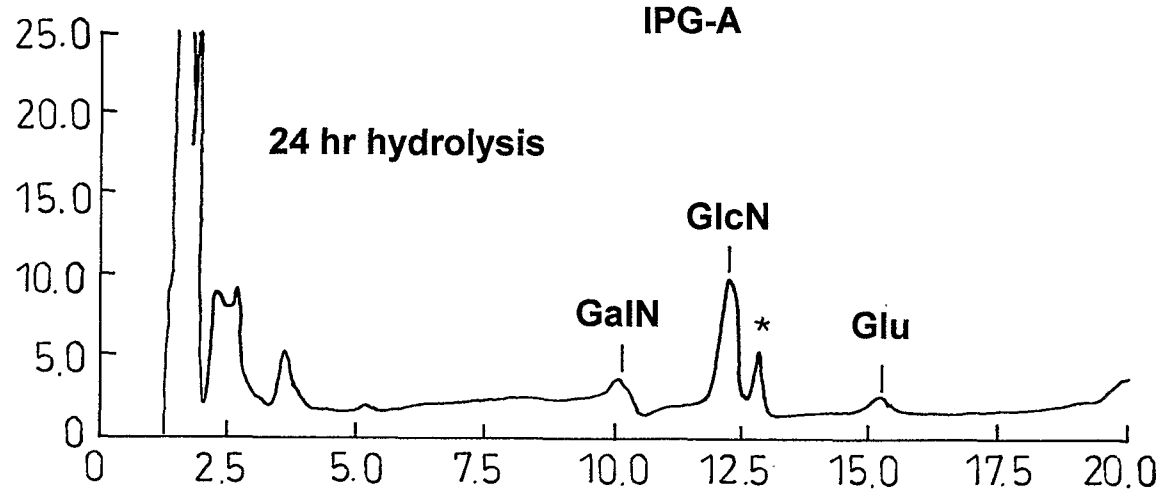

Fig. 17A

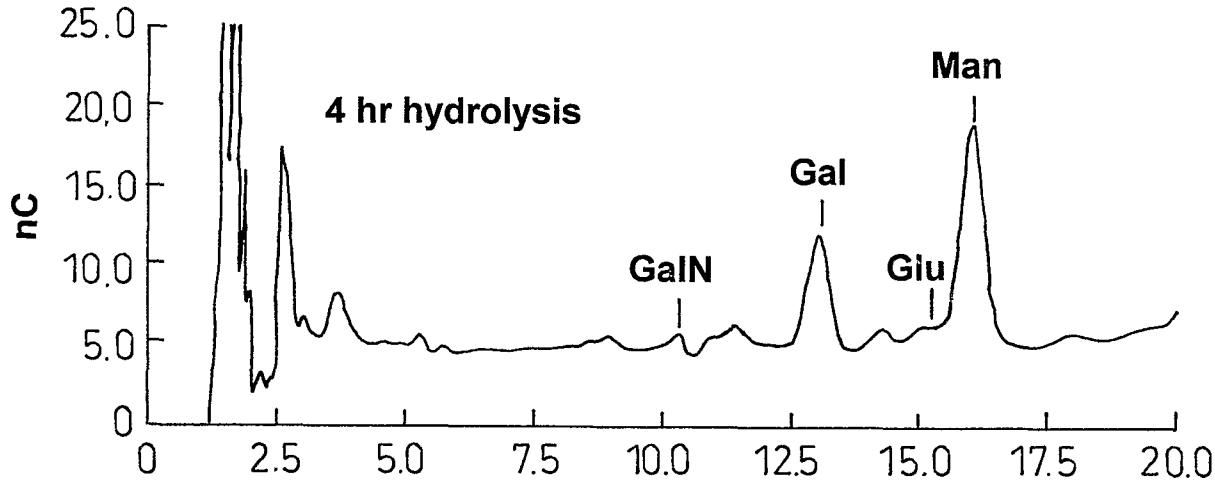

Fig. 17B

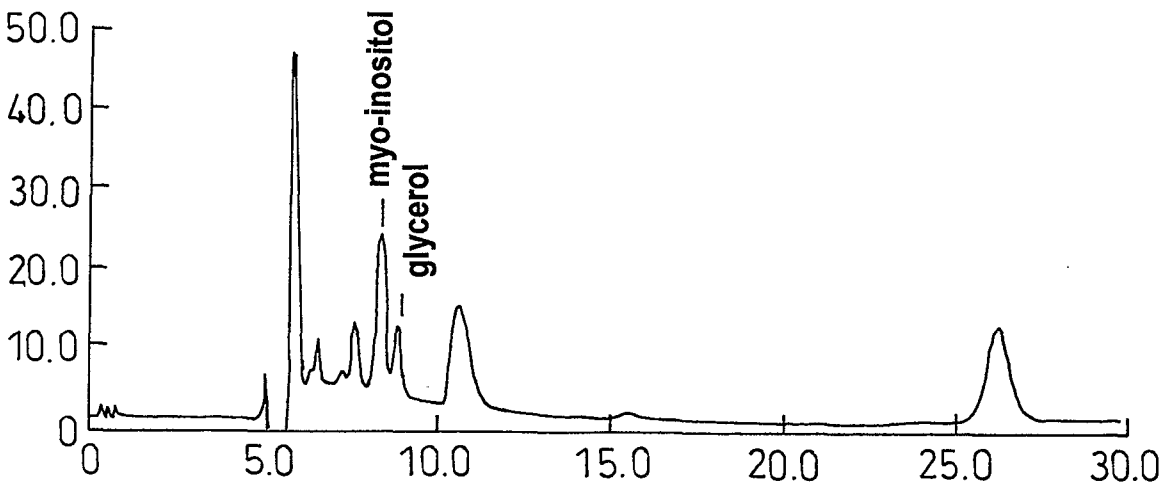

Fig. 17C

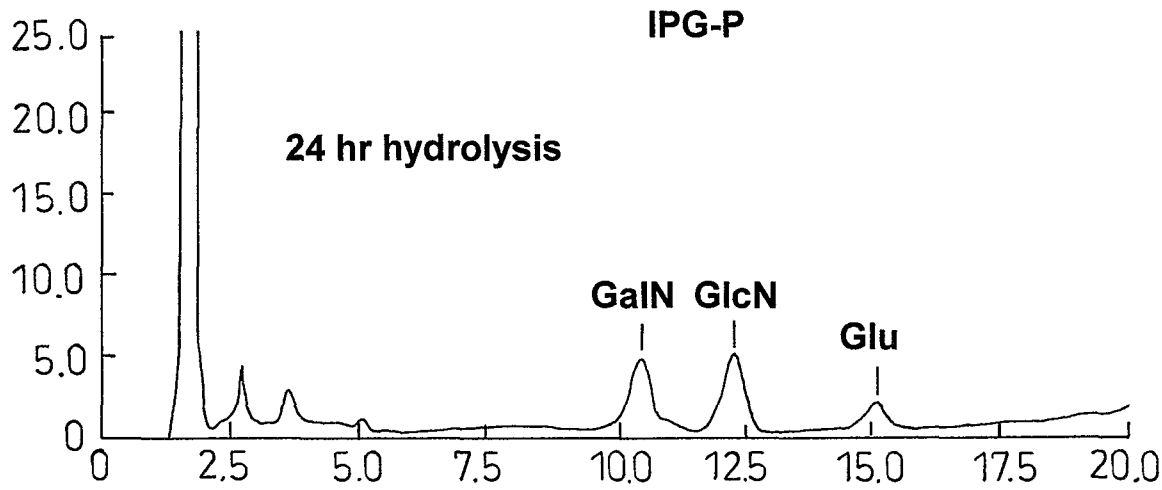

Fig. 18A

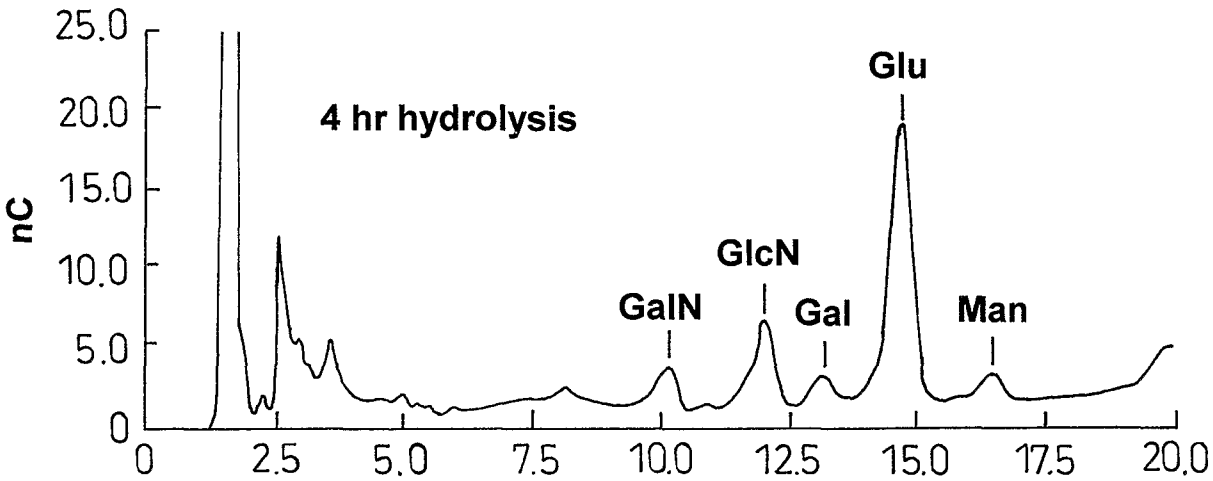

Fig. 18B

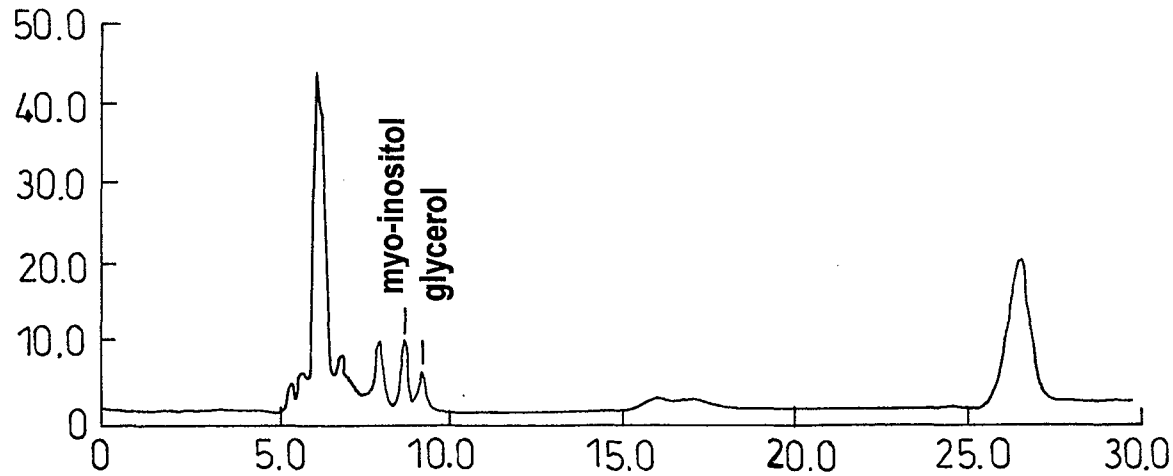

Fig. 18C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/02763

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07G3/00 C07H1/08 A61K31/70 A61P3/00

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 C07G C07H A61K A61P

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, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 24406 A (INST MEDICAL W & E HALL ;HANSEN DIANA (AU); SCHOFIELD LOUIS (AU)) 4 May 2000 (2000-05-04) the whole document claim 74 ---	1-16
X	GIGG R ET AL: "SYNTHESIS OF GLYCOSYLPHOSPHATIDYLINOSITOL ANCHORS" GLYCOPEPTIDES AND RELATED COMPOUNDS, DEKKER, NEW YORK,, US, 1997, pages 327-392, XP000897779 page 369 -page 380 --- -/--	1-12

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

[X] 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)

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2001	25/09/2001

Name and mailing address of the ISA	Authorized officer
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	de Nooy, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/02763

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 6 004 938 A (FRICK WENDELIN ET AL) 21 December 1999 (1999-12-21) example 3 -----	13-16

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-12 (in part)

The initial phase of the search revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved that it is impossible to determine which parts of the claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, it appears impossible to execute a meaningful search and/or to issue a complete search report over the whole breadth of the above mentioned claims. Consequently, the search has been directed to the use of the claimed compounds in the preparation of a medicament for the treatment of a condition ameliorated by administration of a PGM second messenger.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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PCT/GB 01/02763

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