Title: TREATMENT OF METABOLIC DISEASES

Abstract: The present invention relates to the combined use of an inhibitor of the purine bio synthetic pathway, and a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis in the treatment of a metabolic disease.
Treatment of metabolic diseases

The present invention relates to the use of a combination of an inhibitor of the purine biosynthetic pathway and a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis for the treatment and/or prevention of a metabolic disease, a pharmaceutical composition comprising the same, a method for producing the pharmaceutical composition as well as methods for treating and/or preventing a metabolic disease.

There is a continuous need for therapeutics for the treatment of metabolic diseases such as diabetes, obesity, dyslipidemia or the metabolic syndrome (MoHer, 2001, New drug targets for type 2 diabetes and the metabolic syndrome. Nature 414, 821-827).

Type 2 insulin-resistant diabetes amounts to 90-95% of all diabetes cases. This heterogenous disorder afflicts approximately 6% of the adult population in Western societies. It is desirable to control hyperglycaemia in patients afflicted by type 2 diabetes in order to attenuate the development of chronic complications such as retinopathy and nephropathy. Current treatments mainly intend to reduce the hyperglycaemia itself.

Treatment with insulin is a mainstay in diabetes treatment but can lead to hypoglycaemia and weight gain.

Sulphonylureas and related insulin secretagogues increase insulin release from pancreatic islet cells. Adverse effects comprise hypoglycaemia and weight gain. Many patients who respond initially become refractory to treatment.

Metformin reduces hepatic glucose production but causes gastrointestinal disturbances and can lead to lactic acidosis.

The newer peroxisome proliferators-activated receptor gamma (PPARγ) agonists (thiazolidinedones) enhance insulin action but are associated with side effects such as weight gain, oedema, and anaemia.

Thus new therapeutic approaches with fewer side effects are needed. Preferably, these treatments address the underlying metabolic mechanisms for example of obesity and lipid metabolism.
One treatment of metabolic diseases suggested in the art includes the application of AICA-riboside (AICAR), a natural endogenous metabolite. In cells AICA-riboside is phosphorylated to AICA-ribotide (ZMP, active metabolite of AICA-riboside) which mimics the effect of AMP and can allosterically activate the enzyme AMP-activated kinase (AMPK), a key regulator of metabolism.

AMP-activated kinase (AMPK) serves as a sensor of ATP availability in the cell. Cellular stress depletes ATP and increases AMP which can activate AMPK, which in turn switches on catabolic pathways and switches off ATP-consuming processes. In the liver AMPK activation leads to a reduction of lipogenesis and an increase in fatty acid oxidation. In addition, AMPK increases muscle fatty acid oxidation. It is thus plausible that an increase in AMPK activity has a positive impact on the treatment of diabetes. Experiments with obese Zucker rats showed that administration of AICAR enhanced glucose transport in the muscle and suppressed hepatic glucose production. AMPK is also activated by metformin, the most widely used drug for reducing blood glucose levels in type 2 diabetic patients (Ruderman and Prentki, 2004. AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. Nature Reviews Drug Discovery 3, 340-351).

Although the administration of AICA-riboside (5-aminomidazole-4-carboxamide ribonucleoside; AICAR) or AICA-ribotide (5-aminimidazole-4-carboxamide ribonucleotide, ZMP) has been suggested for the treatment of a variety of metabolic diseases, a major drawback of AICAR is its low oral bioavailability due to rapid metabolism. Poor bioavailability was shown (<5%) when administered orally in solution (Dixon et al., 1991. AICA-riboside: Safety, tolerance, and pharmacokinetics of a novel adenosine-regulating agent. J. Clin. Pharmacol. 31, 342-347). Furthermore, AICAR is metabolized to uric acid through normal purine pathways (Dixon et al., 1993. Acadesine (AICA-riboside): Disposition and metabolism of an adenosine-regulating agent. J. Clin. Pharmacol. 33, 955-958).

It was reported that AICAR can exhibit anti-inflammatory properties, presumably by activating AMP-activated kinase (AMPK), a kinase known to be activated by AICAR (Giri et al., 2004, 5-Aminimidazole-4-carboxamide-1-β-4-ribofuranoside inhibits proinflammatory reponse in glial cells: a possible role of AMP-activated kinase. The Journal of Neuroscience 24(2), 479-487). The application of AICAR inhibited the lipopolysaccharide-(LPS)-induced expression of proinflammatory cytokines in several cell types and in an animal model of inflammation. In the animal experiments rats were intraperitoneally (i.p) injected with AICAR before LPS treatment. The authors suggest that AMPK may be exploited as a target for anti-inflammatory drugs such as AICAR. However
they point out that AICAR has a high clearance and is poorly bioavailable with oral
administration.

WO 93/03734 suggests to use an AMP-mimetic such as AICAR as single agent as anti-
cholestermic/anti-hyperlipemic treatment.

Ruderman and colleagues propose to administer an AMPK-activator (e.g. AICAR) orally
or subcutaneously to treat obesity (WO 01/93873) and conditions associated with insulin
resistance (WO 02/09726).

Winder suggests to use an AMPK-activator, specifically AICAR, for the treatment of type
2 diabetes and insulin resistance (WO 01/97816) and obesity or paralysed muscle (WO
01/93874) whereby AICAR as a single drug is injected subcutaneously or administered
intracellularly.

WO 90/09163 describes AICA riboside prodrugs in orally bioavailable form for lowering
blood glucose levels.

WO 89/00854 suggests the use of various purine nucleosides including AICAR or
ribavarin alone or in combination with various other agents including methotrexate or
succinylaminoimidazole carboxamide riboside for enhancing extracellular adenosine e.g.
in arthritis. Several administration modes are suggested including intravenous, oral, rectal
or topical administration. The examples are directed to subcutaneous or intravenous
administration.

The present invention relates to the use of a combination of

a) an inhibitor of the purine biosynthetic pathway, and
b) a compound susceptible to enzymatic degradation/metabolism by an enzyme
involved in purine biosynthesis

for the preparation of a medicament for oral administration for the treatment and/or
prevention of a metabolic disease, preferably selected from the group consisting of
diabetes, obesity, dyslipidemia or metabolic syndrome

Especially, the present invention relates to the use of a combination of
a) an inhibitor selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156 579-02-1), and sulfasalazine, and
b) the compound AICA riboside

for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome.

In a third aspect, the invention relates to the use of a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, preferably AICA riboside, for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome, wherein said compound is administered simultaneously, separately or sequentially with an inhibitor of the purine biosynthetic pathway, preferably methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156 579-02-1), and sulfasalazine.

In a fourth aspect, the invention relates to the use of an inhibitor of the purine biosynthetic pathway, preferably methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156 579-02-1), and sulfasalazine, for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome, wherein said inhibitor is administered simultaneously, separately or sequentially with a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, preferably AICA riboside.

Consequently, the invention focuses on the oral administration of two agents for the treatment of metabolic diseases.

According to the present invention, the term "treating" or "treatment" refers to all actions where an amelioration of the respective disease is obtained. Especially, these terms also refer to conditions where the outbreak of the respective disease is prevented.

Metabolic disease can be defined as a state of metabolic dysregulation e.g. insulin resistance, hyperinsulinaemia, obesity, preferably central obesity, diabetes, preferably type 2 diabetes, but also type 1 diabetes, dyslipidaemia, metabolic syndrome, hypertension, and premature atherosclerosis, as well as a predisposition for these disorders. In the literature

Insulin resistance is a state in which insulin at a physiological concentration does not exert its usual biological effect. In some instances, the effect of insulin on certain processes (for example glycogen synthesis) can be impaired, whereas its effect on others (for example diacylglycerol synthesis) can be normal or even enhanced.

In the present invention the first agent is an inhibitor of the purine biosynthetic pathway. In the de novo purine biosynthetic pathway, the purine ring is synthesized in mammals utilizing amino acids as carbon and nitrogen donors and CO$_2$ as carbon donor. The de novo pathway for purine nucleotide synthesis consists of metabolic steps and leads to inosine 5’-monophosphate (IMP). IMP is the common precursor for AMP and GMP synthesis. AMP and GMP are then converted into ATP and GTP, respectively.

The committed step in the de novo synthesis of purine nucleotides is the formation of 5-phosphoribosylamine from 5-phosphoribosyl-1-pyrophosphate (PRPP) and glutamine. The first five steps of the pathway lead to the formation of 5-aminimidazole ribonucleotide. The essence of these five reactions is (1) displacement of pyrophosphate by the side chain amino group of glutamine (glutamine PRPP amidotransferase), (2) addition of glycine (GAR synthetase), (3) formylation by N10-formyltetrahydrofolate (GAR transformylase), (4) transfer of a nitrogen atom from glutamine (FGAM synthetase), and (5) dehydration and ring closure (AIR synthetase). The second phase of the pathway transforms 5-aminimidazole ribonucleotide into inosinate. The essence of these steps is (6) carboxylation (AIR carboxylase), (7) addition of aspartate (SAICAR synthetase), (8) elimination of fumarate (adenylosuccinate lyase), (9) formylation by N10-formyltetrahydrofolate (AICAR transformylase), and (10) dehydration and ring closure (IMP cyclohydrolase). The vertebrate enzymes catalysing steps 9 and 10 are present on a single polypeptide chain (ATIC, see below) (Chapter 29 "Biosynthesis of Nucleotides", pp. 739-762, in Biochemistry by L. Stryer, fourth edition, 1995, W.H. Freeman and Company, New York).

In addition to the de novo synthesis of purine rings, cells have the capability to recycle ("salvage") purine bases and nucleosides through so-called salvage pathways. These pathways utilize the preformed bases or nucleosides by contrast to the new synthesis in the de novo pathway. Throughout this invention, the purine biosynthetic pathway refers to the de novo pathway.
Preferably, the inhibitor is selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate (a methotrexate metabolite), MX-68 (CAS RN 156 579-02-1), and sulfasalazine. MX-68 is a methotrexate derivative (Matsuoka et al., 1997, J. Med. Chem. 40(1): 105-111).

Methotrexate and its major metabolite 7-hydroxy-methotrexate are taken up by cells and polyglutamated. Methotrexate polyglutamates have been shown to be even more active than the parent drug as inhibitors of a variety of folate-dependent enzymes, for example AICAR transformylase (Allegra et al., 1985, Proc. Natl. Acad. Sci. USA 82, 4881-4885).

Most preferred is methotrexate. Methotrexate has been extensively tested for clinical applications and methods for formulating and administering Methotrexate are abundantly available to those skilled in art.

Alternatively, the following inhibitors may be used:


The second agent is a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis.

Many pharmaceutically effective compounds are metabolically processed, some being activated while others are inactivated or their pharmacokinetic characteristics such as, for example, efficacy, specificity, half life, clearance rate, side effects, and/or drug targeting are modified. Most often metabolic modification, in particular degradation, is undesired. Extensive efforts have been undertaken to control metabolic degradation ranging from chemical modification of the compounds (e.g. prodrugs), the modification of the pharmaceutical formulation or the mode of administration, as well as the use of enzyme inhibitors.
Generally speaking, the term "degradation" relates to the modification of compounds that results in a partial or complete loss of the biological activity of the unmodified compound by e.g. reduction in molecular size (e.g. cleavage), addition of moieties (e.g. sugar moieties) that increase body clearance, reduction or oxidation of functional groups, etc.

The term "metabolism" of a pharmaceutically effective compound in the context of the present invention with respect to intermediates (metabolites) relates to the enzymatic conversion into the next (downstream) intermediate of the pathway or, in case of reversible reactions, into the previous (upstream) intermediate of the pathway.

In the context of this invention, "compounds" can be metabolic intermediates of biosynthetic pathways such as the de novo purine synthesis pathway: 5-Phosphoribosyl-l- pyrophosphate (PRPP), 5-phosphoribosylamine (PRA), 5-phosphoribosylglycinamide (GAR), 5-phosphoribosylformylglycinamide (FGAR), 5-phosphoribosylformylglycinamidine (FGAM), 5-Phosphoribosyl-5-aminoimidazole (AIR), 5-Phosphoribosyl-5-aminoimidazole-4-carboxylic acid (CAIR), 5-Phosphoribosyl-5-aminoimidazole-4-N-succinocarboxamide (SAICAR), 5-Phosphoribosyl-5-aminoimidazole-4-carboxamide (AICA-ribotide).

Preferably, the compound is AICA-riboside (5-aminoimidazole-4-carboxamide ribonucleoside; AICAR). However, throughout the invention, instead of AICA riboside, 5-Phosphoribosyl-5-aminoimidazole-4-carboxamide (AICA-ribotide; ZMP) can also be used.

Throughout the invention, the compounds can not only be synthetic drugs but also natural metabolites (e.g. intermediates in biosynthesis or biodegradation pathways).

In a preferred embodiment, said enzyme is AICAR transformylase.

AICARFT (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; EC 2.1.2.3; synonym AICAR transformylase) and IMPCHase (IMP cyclohydrolase; EC 3.5.4.10) catalyze the penultimate and final steps, respectively, of the de novo purine biosynthetic pathway. Rayl et al. cloned a cDNA for human AICARFT/IMPCHase from a hepatoma cDNA library (Rayl et al., 1996, J. Biol. Chem. 271: 2225-2233).

Both enzymatic activities are present in the same protein, designated ATIC, in all species of prokaryotes and eukaryotes studied. The human ATIC cDNA encodes a deduced 591-amino acid protein that is 81% identical to the chicken sequence. Rayl et al. (1996) created truncation mutants of the cDNA and measured their enzymatic properties. In this way they
were able to localize the AICARFT activity within the amino-terminal 223 amino acids and the IMPCHase activity to the carboxyl-terminal 406 residues.

Throughout the invention, the inhibitor a) and the compound b) are for simultaneous, separate and/or sequential administration.

Depending one the overall mode of administration or merely from a standpoint of convenience, the coadministered drugs may be administered at different times in different formulations and in different locations.

Throughout the invention, the terms "combined" or "in combination" or "combination" as used herein in the context of an inhibitor of the purine biosynthetic pathway (e.g. methotrexate) being combined with at least one compound susceptible to enzymatic degradation/metabolism (e.g. AICA-riboside or AICA-ribotide) relate to the functional combination of the inhibitor (Methotrexate) as an AICAR-transformylase inhibitor together with at least one therapeutic or prophylactic compound.

"Combined" or "in combination" or "combination" should be understood as a functional coadministration only, wherein some or all compounds may be administrated separately, in different formulations, different modes of administration (subcutaneous, intravenous, intravenous, oral, etc.), and different times of administration. The mode of administration, the formulation, the time(s) of administration, and other pharmacologically related measures for each compound separately or both compounds together depend on the properties of inhibitor (methotrexate) and those desired compound(s) that are to be coadministered.

The compound and/or the inhibitor are administered orally. This means that the substances are formulated such that they are suitable for oral administration. The skilled person is aware of such methods of formulation (see e.g. Remington's Pharmaceutical Sciences by E.W. Martin, 1975, 15th edition; Mack Publishing Co., pp. 1405-1412 and pp. 1461-1487). In general, oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, or magnesium carbonate.

In a further preferred embodiment, the coadministration of the inhibitor, preferably methotrexate and at least one pharmacologically effective compound is performed in one formulation, and/or in the same mode and/or at the same time of administration.
Generally speaking, for combinational therapy, Methotrexate and a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis can be administered together at the same time of administration and in the same formulation, and at the same location, e.g. orally.

In a further preferred embodiment, the inhibitor a) is administered before the compound b). In this case, it is also envisaged that the administration of the inhibitor a) may be continued during the administration of the compound b).

The skilled person would be able to determine the amounts to be administered based on his professional experience. Preferably, the inhibitor, preferably methotrexate, is administered in an amount of less than 10mg/day, preferably less than 1mg/day, and said compound, preferably AICA riboside, is administered in an amount of less than 500mg/day, preferably less than 100mg/day.

If the inhibitor is Sulfasalazine, higher doses, possibly up to 3 g/day, are preferred.

In a preferred embodiment of the invention, further folic acid is used, preferably at a dose of less than 5mg/day. Preferably, folic acid is also administered orally.

The administration of folic acid is expected to decrease Methotrexate-induced side effects due to inhibition of other folate-dependent enzymes than AICAR transformylase. Folic acid is widely used together with Methotrexate for the treatment of rheumatoid arthritis. Folic acid had been extensively tested for clinical applications and methods for formulating and administering folic acid are abundantly available to those skilled in art (Whittle and Hughes, 2004. Folate supplementation and methotrexate treatment in rheumatoid arthritis: a review. Rheumatology 43 (3), 267-271).

The invention further relates to a pharmaceutical composition comprising either together or in separate dosage forms

a) an inhibitor of the purine biosynthetic pathway, and
b) a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, and
c) an excipient suitable for oral administration.

With respect to the pharmaceutical composition of the invention, all embodiments defined above for the uses of the invention also apply.
The invention further provides a method for producing the pharmaceutical composition of
the invention, wherein the inhibitor a), the compound b) and the excipient c) are
formulated to a pharmaceutical composition. With respect to formulation and amounts, the
embodiments as defined above for the uses of the invention also apply to this method of
the invention.

The invention further provides a method of treating and/or preventing of a metabolic
disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or
metabolic syndrome in a mammal, which method comprises orally administering to the
mammal an effective amount of

a) an inhibitor of the purine biosynthetic pathway, and
b) a compound susceptible to enzymatic degradation/metabolism by an enzyme
   involved in purine biosynthesis.

Furthermore, the invention relates to a method of treating and/or preventing of a metabolic
disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or
metabolic syndrome in a mammal, which method comprises orally administering to the
mammal an effective amount of

a) an inhibitor selected from the group consisting of methotrexate, amethopterin, 7-
   hydroxy-methotrexate, MX-68 (CAS RN 156 579-02-1), and sulfasalazine, and
b) the compound AICA riboside.

With respect to these methods of the invention, the same applies as for the above defined
uses of the invention.

Throughout the invention, the administration of two compounds in a therapeutic effective
amount includes that one or each of the compounds is administered in a subtherapeutic
amount, i.e. that the amount of each compound on its own is not sufficient to provide a
therapeutic effect, but that the combination of the compounds results in the desired
therapeutic effect. However, it is also included that each of the compounds on its own is
administered in a therapeutically effective amount.

The invention is further illustrated by the following examples, which are not intended to be
limiting for the scope of the present invention. Inter alia, the examples demonstrate the
beneficial effect of treatment with Methotrexate on the bioavailability of orally administered AICAR (see Example 6).

Since Methotrexate is the prototype of an inhibitor of the purine biosynthetic pathway, and since AICAR is known to be a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, it is believed that the findings for both agents can be generalized. This generalization does not only include the generalization from Methotrexate to amethopterin, 7-hydroxy-methotrexate (a methotrexate metabolite), MX-68 (CAS RN 156 579-02-1), and sulfasalazine, but it is also believed that these findings can be extended to each of said inhibitors and to each of said compounds.

Examples

Example 1: Pharmacokinetics

**Determination of the enhancement of the oral bioavailability of AICAR by Methotrexate**

Groups of 5 rats are dosed with a single oral dose of methotrexate at a dose of 2.5 mg/kg by oral gavage each day for 5 days. 2 hours after the last dose of methotrexate, AICAR at a dose of 100mg/Kg is given by oral gavage. Urinary excretion of AICAR is measured according to the method of Baggott and colleagues (Arthritis & Rheumatism Volume 41, Issue 8, Pages 1407 - 1410, 1998). Control rats are dosed with an equivalent volume of saline and urinary excretion of AICAR similarly measured. Enhancement of oral bioavailability is recorded as the difference between urinary excretion of AICAR in treated and control rats.

**Determination of the enhancement of the oral bioavailability of AICAR by Sulfasalazine**

Groups of 5 rats are dosed with a single oral dose of sulfasalazine at a dose of 100 mg/kg by oral gavage each day for 5 days. 2 hours after the last dose of sulfasalazine AICAR at a dose of 100mg/Kg is given by oral gavage. Urinary excretion of AICAR is measured according to the method of Baggott and colleagues (Arthritis & Rheumatism Volume 41, Issue 8, Pages 1407 - 1410, 1998). Control rats are dosed with an equivalent volume of saline and urinary excretion of AICAR similarly measured. Enhancement of oral bioavailability is recorded as the difference between urinary excretion of AICAR in treated and control rats.
Example 2: Determination of IC50 values of test compounds

An AICAR transformylase enzyme assay was described previously (Xu et al., 2004, J. Biol. Chem. 279, 50555-50565). An AICAR transformylase inhibition assay to determine IC50 values of test compounds can be performed in a solution containing 25 nM ATIC enzyme, 50 µM AICAR, and 8.5 µM N\textsubscript{10}-formyltetrahydrofolate (1Of-ThF).

Example 3: Treatment of Zucker diabetic fatty rats with a combination of Methotrexate and AICAR.

The Zucker diabetic fatty rat (ZDF) is a rodent model of obesity-associated non-insulin dependent diabetes mellitus (NIDDM) that closely resembles the human disorder. This rat has a mutant, functionally deficient leptin receptor and a genetic defect that predisposes the animal to diabetes, as it becomes obese and lipid accumulates in the pancreatic β-cell.

Male ZDF rats (ZDF/Gmi-fa/fa) and their heterozygous (ZDF/Gmi+/fa) lean littermates are commercially available from Charles River Laboratories (Wilmington, MA) at five weeks of age. Typically, ZDF rats are fed a diet containing 16.7% fat (e.g. Purina 5008), on which they can develop overt diabetes at 6 to 8 weeks of age. To initiate the intervention before the ZDF rats developed diabetes, they are fed a lower-fat laboratory chow (e.g. Altromin 1324, 10.1% fat).

The intervention study can be carried out as described by PoId and colleagues (PoId et al. 2005, Diabetes 54, 928-934). The following groups are studied (n=12 animals per group): ZDF AICAR treated group (AICAR-group), ZDF Methotrexate treated group (MTX group), ZDF AICAR+Methotrexate treated group (AICAR-MTX group), ZDF untreated control group (untreated group), and lean untreated control group (lean group). Methotrexate (2.5 mg/kg) and AICAR (100 mg/kg) are administered orally once daily throughout the treatment period. The two control groups are left untreated. The study is initiated when the rats are 5 weeks old and lasts for 8 weeks, until 13 weeks of age. Fasting plasma glucose and insulin levels as well as body weight and food and water consumption are measured weekly.

Analytical procedures. Plasma glucose and insulin are determined using plasma obtained by tail-vein bleeding from rats fasted overnight (10 hours). Plasma glucose concentrations
are determined using a Beckman glucose analyser (Beckman Instruments, Palo Alto, CA, USA). Insulin levels are determined using an ultrasensitive rat insulin enzyme-linked immunosorbent assay kit (DRG Diagnostics, Marburg, Germany).

Example 4: Determination of the effect of a combination of AICAR and Methotrexate on GLUT1 and GLUT4 mRNA expression in human primary muscle cell cultures

Defects in whole-body glucose uptake are closely linked to impaired insulin-stimulated glucose transport activity. The glucose transporter 4 (GLUT4), the major glucose transporter expressed in skeletal muscle, plays a characteristic role in regulating glucose transport under insulin-stimulated conditions. Therefore, pharmacological treatment that increases glucose transporter expression has a positive impact on glucose homeostasis.

The mRNA expression levels of GLUT1 and GLUT4 are measured following a published protocol (Al-Khalili et al., 2005, Diabetologia 48, 1173-1 179).

Cell culture. Human muscle biopsies, obtained from individuals without metabolic disorders (rectus abdominis, approximately 1 to 3g), are collected in cold Phosphate Buffered Saline (PBS). Myoblasts are grown in growth medium (5.5 mmol/l glucose Ham’s FIOmedium with 20% fetal calf serum). When differentiation is started, growth medium is replaced by differentiation medium (5.5 mmol/l glucose, DMEM, 4% fetal calf serum for 2 days, thereafter 2% fetal calf serum). Differentiation of myoblasts to myotubes is started with 8-day treatment with AICAR (200 µmol/l), methotrexate (1 µmol/l), and insulin (400 pmol/l) and solvent control. The final concentration of DMSO is adjusted to 0.1% for each group. On the day of the assay, the myotubes are washed free of reagents and incubated with serum-free DMEM for 6 hours and used for RNA analysis.

Real-time PCR analysis of mRNA expression. Cell cultures are washed three times with RNase-free PBS and harvested directly for RNA extraction (RNAeasy Mini Kit; Qiagen, Crawley, UK). The RNA is DNase-treated before reverse transcription (RQI RNase-free DNase; Promega, Sothampton, UK). cDNA is prepared from 1 µg of RNA samples using the TaqMan reverse transcription reagent (Applied Biosystems, Foster City, CA, USA). The quantification of PCR products is analysed by real-time PCR (TaqMan) using a standard curve method (User Bulletin 2, ABI PRISM 7900 Sequence Detection System). The sequences of the oligonucleotide primers and probes for the PCR reaction are designed using published data (PubMed), or acquired by assays-on-demand (Applied Biosystems). All data are analysed by using the values of the 18S gene levels as a baseline.
Oligonucleotide primer and probe sequences for the human GLUT1 and human GLUT4 are listed in Al-Khalili et al., 2005, Diabetologia 48, 1173-1 179.

Example 5: Determination of the effect of a combination of AICAR and Methotrexate on GLUT4 protein expression in rat muscles

This experiment is designed to show that administration of a combination of AICAR and Methotrexate leads to an increase of the glucose transporter 4 (GLUT4) protein in the muscles of an animal model (Holmes et al., 1999, Journal of Applied Physiology 87, 1990-1995).

Treatment of rats. Male Sprague-Dawley rats (Saco, Wilmington, MA, USA) are given food and water ad libitum. The following groups are studied (n=7 animals per group): AICAR treated group (AICAR-group), Methotrexate treated group (MTX group), AICAR+Methotrexate treated group (AICAR-MTX group), and untreated control group. Methotrexate (2.5 mg/kg) and AICAR (100 mg/kg) are administered orally once daily for 5 days in succession. The control group is left untreated. Rats are anesthetized by intraperitoneal injection of pentobarbital sodium and the epitrochlearis and gastrocnemius/plantaris muscles are collected and rapidly frozen at liquid nitrogen temperature.

Analytical methods. For GLUT4 protein measurement the muscle tissue is ground to powder under liquid nitrogen. A homogenate (1:9 dilution) is prepared in HEPES buffer containing protease inhibitors as described in Holmes et al., 1999. Proteins of these homogenates are separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot analysis. GLUT4 protein is detected with GLUT4 polyclonal antibody RalGFT (Biogenesis, Sandown, NH) and a peroxidase-conjugated donkey anti-rabbit IgG and a chemiluminescence detection system (Amersham Life Sciences). The intensity of the GLUT4 signals is determined via densitometric analysis and values of treated groups are compared to the non-treated group.

Example 6: Effect of Methotrexate treatment on the oral bioavailability of AICAR

The data of this experiment demonstrate the beneficial effect of treatment with Methotrexate on the bioavailability of orally administered AICAR. Rats treated with Methotrexate and AICAR (group 1) showed higher AICAR plasma concentrations
compared to control groups treated with Methotrexate alone (group 2), AICAR alone (group 3) or saline controls (group 4).

**Study protocol: Treatment of animals**

**Group 1**
5 rats (Lewis) were treated with methotrexate (Calbiochem, San Diego, CA, USA; catalogue number 454125) for 5 days at 1mg/Kg (1ml of a solution in normal saline - 0.9%) intraperitoneal (ip). Two hours after the last dose of methotrexate, AICAR (AICA-Riboside, Calbiochem, San Diego, CA, USA; catalogue number 123040, 100 mg/Kg) was dosed as an oral gavage in normal saline (1 ml). Blood samples were taken 4 hours (hr) post dose and plasma separated and frozen at -20°C until analysis.

**Group 2**
5 rats (Lewis) were treated with methotrexate for 5 days at 1mg/Kg ip (1ml of a solution in normal saline - 0.9%). Two hours after the last dose of methotrexate, normal saline was dosed as an oral gavage (1 ml). Blood samples were taken 4hr post dose and plasma separated and frozen at -20°C until analysis.

**Group 3**
5 rats (Lewis) were treated ip with 1ml of normal saline for 5 days. Two hours after the last dose of saline, AICAR (100 mg/Kg) was dosed as an oral gavage in normal saline (1 ml). Blood samples were taken 4hr post dose and plasma separated and frozen at -20°C until analysis.

**Group 4**
5 rats (Lewis) were treated ip with 1ml of normal saline for 5 days. Two hours after the last dose of saline, normal saline (1 ml) was dosed as an oral gavage. Blood samples were taken 4hr post dose and plasma separated and frozen at -20°C until analysis.

**Sample Preparation**

Plasma samples and standards (0.5ml) were each transferred to an Amicon Centrifree Micropartitioning Cartridge (No. 4104; Amicon Div., W.R. Grace & Co., Danvers, MA 01923) which has a molecular weight (m.w.) cut off at approximately 30,000. The ultrafiltration devices were centrifuged at 2000 x g for 30 minutes using a fixed angle centrifuge rotor. The clear, almost colourless ultrafiltrated samples were then transferred to a Waters WISP 712 Autosampler for chromatography.
Sample Analysis

Reverse-phase HPLC was performed at room temperature using a Beckman Ultrasphere ODS column (5 μm; 250 x 4.6 mm) with a mobile phase of 1.5% methanol in 10mM ammonium phosphate (pH3) at a flow rate of 1.0ml/min. AICAR has a retention time of 6 minutes. Detection was at 270nm using a Waters model 484 UV detector and a Hewlett-Packard Model 3390A integrator.

Results

Compound levels of AICAR in plasma (ng/ml; nd = not detected)

Table 1

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Claims

1. Use of a combination of
   a) an inhibitor of the purine biosynthetic pathway, and
   b) a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis
   for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome.

2. The use of claim 1, wherein said enzyme is AICAR transformylase.

3. The use of claim 2, wherein said compound is AICA riboside.

4. The use of any one of claims 1 or 2, wherein said inhibitor is selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine.

5. The use of claim 4, wherein said inhibitor is methotrexate.

6. Use of a combination of
   a) an inhibitor selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine, and
   b) the compound AICA riboside
   for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome.

7. The use of any of claims 1 to 7, wherein the inhibitor a) and the compound b) are for simultaneous, separate and/or sequential administration.
8. The use of any of claims 1 to 7, wherein where the inhibitor, preferably methotrexate, is administered in an amount of less than 10mg/day, preferably less than 1mg/day, and said compound, preferably AICA riboside, is administered in an amount of less than 500mg/day, preferably less than 100mg/day.

9. The use of any of claims 1 to 8, further including the use of folic acid, preferably at a dose of less than 5mg/day.

10. Use of a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, preferably AICA riboside, for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome, wherein said compound is administered simultaneously, separately or sequentially with an inhibitor of the purine biosynthetic pathway, preferably methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine.

11. Use of an inhibitor of the purine biosynthetic pathway, preferably methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine, for the preparation of a medicament for oral administration for the treatment and/or prevention of a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome, wherein said inhibitor is administered simultaneously, separately or sequentially with a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, preferably AICA riboside.

12. The use of any of claims 10 or 11, with the features of any of claims 2, 5, 7, 8 and 9.

13. A pharmaceutical composition comprising either together or in separate dosage forms
   a) an inhibitor of the purine biosynthetic pathway, and
   b) a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis, and
14. The pharmaceutical composition of claim 13, wherein said enzyme is AICAR transformylase.

15. The pharmaceutical composition of any of claims 13 or 14, wherein said inhibitor is selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine.

16. The pharmaceutical composition of any of claims 13 to 15, wherein said compound is AICA riboside.

17. The pharmaceutical composition of any of claims 13 to 16, further comprising folic acid.

18. A method for producing the pharmaceutical composition of any one of claims 13 to 17, wherein the inhibitor a), the compound b) and the excipient c) are formulated to a pharmaceutical composition.

19. A method of treating and/or preventing a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome in a mammal, which method comprises orally administering to the mammal an effective amount of

a) an inhibitor of the purine biosynthetic pathway, and

b) a compound susceptible to enzymatic degradation/metabolism by an enzyme involved in purine biosynthesis.

20. The method of claim 19, with the features as defined in claims 1 to 6.

21. A method of treating and/or preventing a metabolic disease, preferably selected from the group consisting of diabetes, obesity, dyslipidemia or metabolic syndrome in a mammal, which method comprises orally administering to the mammal an effective amount of
a) an inhibitor selected from the group consisting of methotrexate, amethopterin, 7-hydroxy-methotrexate, MX-68 (CAS RN 156579-02-1), and sulfasalazine, and

b) the compound AICA riboside.

22. The method of any of claims 19 to 21, with the features as defined in any of claims 7 to 9.