

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

DPP-4 inhibitors and inhomogeneous static magnetic field, new
therapeutic potentialities for the treatment of neuropatic pain and
inflammation

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2014

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The Examination takes place at the Library of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, 19th of January 2015. at 11:00

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 19th of January 2015. at 13:00

1. INTRODUCTION AND AIM

Diabetes Mellitus, is a group of metabolic diseases resulted in hyperglycemia. According to the physicians advises the patients concentrate on keeping blood glucose levels in euglycemic level as much as it is possible, without causing hypoglycemia. Diabetes is due to either the β cells of the pancreas not producing enough insulin (type 1), or the periferial cells not responding properly to the insulin produced (type 2). In the year of 2013, an estimated 382 million people suffered from diabetes worldwide. The ratio of the type 2 diabetes is up to 90% of the cases with equal rates in both women and men. In 2012 and 2013 this metabolic disease resulted in 1.5 to 5.1 million deaths per year, making it the 8th leading cause of death.

Each types of diabetes increase the risk of long-term complications. Besides the retinophaty and kidney disfunction the most common complication is diabetic sensorimotor polyneuropathy, which occurs in 10-54% of patients with type 1 diabetes, while retinopathy occurs in 26.5% of patients and nephropathy in 32%. Similar rates exist in type 2 disease.

Painful diabetic neuropathy (PDN) is a common condition that will only increase as the diabetes epidemic grows. The PDN associated pain is usually described as “nipping pain,” “numbness,” or “increased pain sensation due to touch.” The intensity of the PDN induced pain is typically greater at the night hours. PDN usually develops in the lower legs and the feet; however, the hands are might also be involved. Neuropathy is most likely chronic and progressive. The pain in PDN greatly influences all areas of the patient's life, including mood, sleep, self-worth, independence, ability to work, and interpersonal relationships.

The objective of our research work was to evaluate two DPP-4 inhibitors in the analgetic and anti-inflammatory point of view that are used for the treatment of type 2 diabetes. Sitagliptin and vildagliptin were tested in *in vivo* models in mice. The anti-inflammatory action of these drugs were described previously in animal models and in human studies as well.

Inhomogeneous static magnetic field (SMF) is a non-pharmacologic alternative to treat neuropathy. SMF has known effects on living cells and organisms and the analgetic effect had already been proved by animal researches. The purpose of our investigation was to observe the analgetic and anti-inflammatory effects of inhomogeneous SMF on diabetic neuropathy in vivo model and the impact on blood glucose level and body weight of mice.

2. SCIENTIFIC BACKGROUND

Pancreas

The pancreas is a dual-function glandular organ in the digestive (exocrine function) and endocrine system (endocrine function). It is located in the abdominal cavity retroperitoneally. As a digestive organ, pancreas secretes pancreatic juice containing digestive enzymes that assist digestion and absorption of nutrients in the small intestine. Pancreas has a fundamental endocrine function as well, producing and secreting several important hormones into the bloodstream. The endocrine part of the pancreas is about 1-2% of the total weight of the gland and made up of approximately a million cell clusters called islets of Langerhans. Four main cell types exist in the islets: α cells secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin, and γ cells secrete pancreatic polypeptide. α and β cells are located peripheral in the islets, δ cells in the center and γ cells are detectable everywhere in the islets.

Insulin secretion regulators

Insulin is synthesized in the pancreas within the β -cells of the islets of Langerhans and releases rapidly from β -cells after glucose intake. This is the primary trigger mechanism for insulin release. Glucose enters into the β -cells through the glucose transporters, GLUT-2. The metabolic breakdown of glucose produces ATP, thus the physiological regulation of KATP channels and the consequent insulin release mechanism is pharmacologically exploitable. This is the point of intervention in the mechanism of insulin secretagogue oral antidiabetics. Beyond glucose, some amino acids like lysine, arginine and leucine also directly affects on β -cells and directly promotes insulin secretion. Although these do not act via GLUT-2 transporters, consequently they can be effective in case of GLUT-2 malfunction. Insulin secretion can be stimulated by parasympathetic release of acetylcholine as well.

Another regulating mechanism is the incretin effect. The first incretin hormone, glucose-dependent insulinotropic polypeptide (GIP) had been characterized in the 1970s. GIP is a 42 amino acid peptide produced predominantly in duodenal K cells in the proximal small intestine. GIP has also been localized to the central nervous system, where it may play a role in control of cell survival. The strongest stimulus for GIP secretion is nutrient intake, circulating level of GIP rise within minutes after food ingestion. Although GIP was shown to be a potent stimulator of glucose-dependent insulin secretion, removal of GIP from gut

extracts did not eliminate the incretin effect (insulin secretion, β -cell proliferation, inhibition of β -cell apoptosis), providing evidence for the existence of additional peptides with incretin-like activity. After the cloning and characterization of the proglucagon gene the second peptide with incretin activity was identified. Glucagon-like peptide-1 (GLP-1) is produced in enteroendocrine cells in the distal small bowel and colon. The plasma level of GLP-1 rise also rapidly after food intake which suggest that its secretion from the distal L-cells is promoted by neural and/or endocrine factors well before nutrients could act directly on enteroendocrin L-cells. Both GIP and GLP-1 contains alanine at position 2, accordingly they are excellent substrates of dipeptidyl peptidase-4 enzim that regulates the degradation of these hormones. Past the effect of DPP-4, both GIP and GLP-1 eliminates within minutes from the circulation after their secretion via kidneys as well.

Release of insulin is inhibited by somatostatin that is synthetized in δ -cells of pancreas. Somatostatin balances the blood glucose level fluctuation caused by the effects of insulin and glucagon. Release of insulin is also strongly inhibited by the stress hormone noradrenaline and adrenaline, which leads to increased blood glucose levels during stress acting on α -receptors of β -cells.

Medical therapy in type 2 diabetes

An appropriate diet and exercise are the basis of diabetic care. Physical exercise leads to a decrease in euglycemic markers such as HbA1c and improved insulin sensitivity. Diet must consist of nutrients with low glycemic index and low carbohydrate content. If lifestyle measures as diet and sport activities have not succeed in its object, anti-diabetic medication is required. People with type 2 diabetes usually do not need insulin therapy. It may be advised only if the measured blood glucose level can not be controlled by oral treatment.

Oral antidiabetics can be insulinotropic that may cause hyperglycemia as side effect because they promote endogenous insulin secretion and non-insulinotropic that enhance insulin sensitivity.

Alpha-glucosidase inhibitors as acarbose work by interfering with the absorption of carbohydrates in the intestines. These medicaments help to decrease blood glucose levels, but not as well as metformin or the sulfonylureas. Alpha-glucosidase inhibitors can be combined with other medicines if the first medicine was not effective. The main side effects of alpha-glucosidase inhibitors are flatulence, diarrhea, and abdominal pain; starting with a low dose may minimize these side effects. The medicine is usually taken three times per day with the

first bite of each meal. The common primarily advised tablet is metformin. Metformin is a biguanide medicine. It lowers blood glucose mainly by decreasing the amount of glucose that the patients' liver releases into the bloodstream. It also increases the sensitivity of the diabetic people body's cells to insulin. Metformin also showed in studies lower risk of other diabetic complications such as heart attack and stroke. Biguanide compounds are particularly useful in case of overweight patients, as it is less likely than some other glucose-lowering tablets to cause weight gain. Another advantage of metformin is that it generally does not cause hypoglycemia, which is a possible side effect of many other glucose-lowering tablets. The thiazolidinediones, also known as glitazones act by activating PPARs (peroxisome proliferator-activated receptors), a group of nuclear receptors, with greatest specificity for PPAR γ . Glitazones decrease plasma glucose, fat and insulin level, enhance glucose uptake in adipocytes and in the muscles without promoted insulin secretion. The only member on the market at the time is pioglitazone.

In less common cases sulfonylurea compounds are used in antidiabetic therapy. Sulfonylureas as gliclazide, glimepiride, and glipizide work by increasing the amount of insulin that pancreas produces. Sulfonylureas tend to be used if patient can not take metformin due to side effects or other reasons. Sulfonylurea could be taken in addition to other glucose-lowering tablets if one tablet does not control blood glucose level well enough on its own. There are many disadvantages and side effects in connection with sulfonylurea drugs. As sulfonylureas increase level of insulin, hypoglycemia is a possible and frequently dangerous complication. Weight gain is less serious but common side effect of the therapy. Meglitinides as nateglinide and repaglinide have a similar action to sulfonylureas. However, they are less commonly used. After taking a dose they quickly boost the insulin level, but the effect of each dose does not last long. Each dose must be taken shortly before main meals. Meglitinides are not generally used as a first-line treatment because they are more expensive than sulfonylureas however these drugs might be an option if the pateint has irregular agenda. As with sulfonylureas, possible side effects include weight gain and hypoglycaemia.

The applied chemicals in DPP-4 inhibitor group are in alphabetic order: alogliptin, anagliptin, gemigliptin, linagliptin, saxagliptin, sitagliptin, teneligliptin, vildagliptin. Dipeptidyl peptidase-4 (DPP4) is an enzyme which breaks down incretins. Incretins are hormones which are produced by the intestine in response to food. DPP-4 inhibitor medicines work by reducing blood glucose level by enhancing the effects of incretins as they prevent DPP4 from acting. DPP-4 circulates and is also present in the epithelial cells of the intestine, kidney, liver, lung, thymus, lymph node, spleen, prostate and in adipocytes, as well as on

activated lymphocytes and monocytes. Besides the incretin hormones, a number of bioactive peptides are potential substrates for DPP-4. These include neuropeptide Y, peptide YY, gastrin-releasing polypeptide, pituitary adenylate-cyclase-activating polypeptide (PACAP), insulin-like growth factor-1, substance P and various chemokines. DPP-4 is also known as the cell surface antigen CD26 and it can associate with the lymphocyte cell-surface molecules CD45 and adenosine deaminase (ADA) to have a co-stimulatory function in the immune response. Within incretin enhancers, sitagliptin and vildagliptin have been explored in the most detail. Both sitagliptin and vildagliptin are orally active and rapidly absorbed, 30 min after administration DPP-4 activity is already inhibited by almost 100%, and more than 80% inhibition lasts for more than 16 hours. Liver is not involved in the elimination but kidneys have much higher impact on it. FDA has therefore recommended that renal function is assessed prior to the start of sitagliptin treatment, and that in patients with moderate or severe renal insufficiency the dose of gliptin should be reduced. One of the gliptins may be advised in addition to metformin or a sulfonylurea, or even to both of these if HbA1c level is still high. Side-effects of these compounds are uncommon and are usually mild.

The incretin mimetic exenatide is a peptide, therefore can be used only by parenteral administration.

New guidelines mention that insulin can be used in case of no satisfactory response achieved with oral antidiabetic. This can save β -cells and rapidly adjust controlled metabolic conditions thus prevent complications.

Associated complications

Chronic elevation of blood glucose level leads to damage of blood vessels. In diabetes, the resulting problems are grouped under microvascular disease (diabetic retinopathy, nephropathy and neuropathy) and macrovascular disease (via atherosclerosis).

Diabetic retinopathy is a general term for all disorders of the retina caused by diabetes. It is the result of microvascular retinal changes. Hyperglycemia-induced intramural pericyte death and thickening of the basement membrane lead to incompetence of the vascular walls. These damages change the formation of the blood-retinal barrier and also make the retinal blood vessels become more permeable. New cases could be considerably reduced if the glycemic status was permanent and there was proper and vigilant treatment and monitoring of the eyes. Diabetic retinopathy is the leading cause of blindness for people aged 20 to 64 years in many countries. There are two major types of retinopathy: nonproliferative (with macular

edema) that is more common in patients with type 2 diabetes and proliferative that is usually associated with type 1 patients. As retinopathy often has no early warning signs the complication is often present at the diagnosis of type 2 diabetes.

Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is due to longstanding diabetes mellitus, and is a prime indication for dialysis in many developed countries. The evolution of the complication is highly influenced by the permanent increased blood glucose level and genetic factors. Throughout its early course, diabetic nephropathy has no symptoms. The first laboratory abnormality is the positive microalbuminuria test. The insufficient treatment leads to kidney failure.

Diabetic neuropathy has no morphologic signs. Abnormalities in specific examinations may be found at the time of diagnosis of diabetes due to its latent evolution. At a rough estimate about the third of patients with diabetes develop neuropathy both in patients with type 1 diabetes and in those with type 2 disease, so the condition is the most common and most costly complication. Painful diabetic neuropathy (PDN) symptoms exhibit a symmetrical “stocking and gloves” distribution and are often associated with nocturnal exacerbation. It can be presented from a mild pins and needle sensation to stabbing, burning, unremitting, or even unpleasant electric shock sensation. Other common manifestations of painful diabetic neuropathy are allodynia and hyperalgesia. The condition has relevant negative impact on physical and mental quality of life. After a profound questioning of the patient the disorder is assumable but specific examinations are available to confirm the suspicion. A widely used test is electroneurography test that is reliable in diagnostic and in follow up as well. The method of whole nerve biopsy (typically the sural nerve) is not commonly used neither as a diagnostic test because of the side effects nor in research studies because it can not be repeated in the same location. On the margin, the sural nerve biopsy is usually abnormal even when signs of diabetic neuropathy are minimal or absent. Unmyelinated fibers can be investigated by biopsy of the hairy skin too. The method of glabrous skin biopsy shows greater involvement of myelinated fibers in patients with type 1 diabetes than in those with type 2 disease with similar severity of neuropathy. The results highlight the fact that type 1 and type 2 diabetes are different diseases that probably have different mechanisms of nerve injury. As previous studies proved, in type 1 diabetes, the large effect of glucose control suggests that hyperglycemia is the primary driver of nerve injury, whereas in type 2 disease, the lack of effect implies that other factors beyond hyperglycemia are important.

At the moment there are no verified effective therapeutic possibilities to stabilize and turn back the destruction of myelinated fibers and axon degradation. The purpose of the therapy is to place emphasis on prevention and the treatment of symptoms. The proper glucose control is crucial in prevention of accompanied complications and progression. Current evidence supports the use of three major drug groups: antidepressants, antiepileptics and non specific analgetics including opioids. Within antiepileptic drugs, gabapentin and pregabalin show similar efficacy in treatment of painful diabetic neuropathy. In the group of antidepressants tricyclic antidepressants were showed beneficial in the treatment. However, they can have serious side effects. Newly introduced drugs are serotonin-norepinephrine reuptake inhibitor (SNRI) duloxetine and venlafaxine that are may be also useful for patients with painful neuropathy. Placebo controlled studies showed that opioid based drugs significantly improved pain in painful neuropathy but major drawbacks are tolerance and the risk of misuse. Possible causal therapy is α -lipoic-acid administration that can affect through its antioxidant and glutathione level decreasing properties. Another alternative for the treatment of some negative symptoms and signs is the hexose way inhibitor benfotiamine that is in a combination with vitamin B on the market. Topical treatment have had limited success in the therapy of painful neuropathy. Capsaicin cream is approved for topical relief of pain but many patients can not tolerate it because of the initial pain on application. Other non-pharmacologic treatments (transcutaneous electrical nerve stimulation, electromagnetic stimulation, spinal cord stimulation, low level laser treatment, massage, acupuncture) failed to demonstrate efficacy.

Inhomogeneous static magnetic field (SMF)

The most frequent use of SMF-exposure is represented in MRI (Magnetic Resonance Imaging) devices nowadays. Although MRI is used for diagnostic aim, animal studies proved that the strong homogeneous static magnetic field (3T) of MR has analgesic effect. Strong homogeneous or strongly inhomogeneous SMF-exposure can achieve well defined observable responses from cells and living subjects. Many previous studies targeted to evaluate the effects of SMF. A meta-analysis summarized 29 placebo and weaker SMF controlled studies. In their opinion there is no evidence for the analgesic effect consequently it can not be recommended as therapeutic alternative. A reflection summary debated with these conclusions on the basis of missing crucial SMF parameters. According to this review, SMF studies should be conducted only with known physical parameters of SMF (they suggest 10

parameters like the name of target tissue, the distance of the permanent magnet surface from the target, dosing regimen etc.) in the future.

To recognise the effects of SMF would be substantial for the therapeutical point of view and for the health and safety of the staff daily working with MRI as well.

Analgesic effect of SMF was convincingly demonstrated in previous studies in mice by László et al. SMF exposure could decrease the number of writhings after intraperitoneal injection of irritating agent by 54.07%. Further experiments investigated the duration of the effect; results showed that the antinociceptive effect of 10 minutes SMF exposure could be identified 30 minutes following the exposure. Additional studies aimed to explore the mechanism of action. Efficiency was evaluated in the writhing test in mice after different opioid antagonist pretreatment. These experiments resulted that SMF may act mostly by μ -opioid receptors, in a lesser extent by δ -opioid receptors but failed to affect the SMF-induced antinociception by κ -opioid receptors. Other studies confirmed the involvement of capsaicin sensitive fibers in the mechanism of action.

During the experimental work we used an inhomogeneous SMF generator device developed by László et al. which will be discussed in detail in the methodical chapters.

3. MATERIALS AND METHODS

Animals and ethics

Experiments were performed on 25–35 g CD1 male mice (Charles River, Gödöllő, Hungary), kept under standard conditions with standard rodent chow and water ad libitum. Animal procedures were approved by the local animal ethics committee and National Food Chain Safety Office Animal Health and Animal Welfare Directorate under the number 26/2007/DE MÁB in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII tv. 32§) and complied with the recommendations of the International Association for the Study of Pain and the Helsinki Declaration.

Sitagliptin and vildagliptin application

Mice were dosed with 1, 3 or 10 mg/kg sitagliptin or vildagliptin (Nanjing Ange Pharmaceuticals, Nanjing, Jiangsu, China) dissolved in saline by oral gavage (1 ml/100 g). Control groups were given the vehicle in the same amount and way. A single application was used in the case of one-day experiments, while daily application was used in the 21 day long experiments. Treatments and measurements were implemented 30 min after the oral gavage in every case.

Allyl-isothiocyanate (AITC)-induced inflammation model

Anesthesia was induced by thiopental (Trapanal, Sandoz, Basle, Switzerland) in an amount of 50 mg/kg intraperitoneally (i.p.), repeated as required. The inner and outer surface of the right ear was then smeared with 1% allyl-isothiocyanate (AITC) (Sigma-Aldrich, Budapest, Hungary) dissolved in paraffin oil, using a cotton-wool stick. This treatment was applied 30 min after the oral gavage (substances dissolved in saline or vehicle in the control group) and the procedure was repeated 45 min after the first application. Thus the oral administration of gliptins was performed firstly and the induction of inflammation was carried out secondly.

At the end of the experiment animals were sacrificed by cervical dislocation and ears were stored on -20 °C for the neutrophil accumulation assay.

Measurement of ear edema

Ear thickness was measured by a micrometer caliper (Oxford Precision, Leicester, England) with 0.1 mm accuracy before the AITC treatment, 15 min after the first AITC application, then by each hour during a 6 hour period after each AITC treatment. Gliptin treatment was performed 30 minutes before the commencement of ear edema induction.

Data were expressed in micrometers.

Measurement of neutrophil accumulation

Frozen ear samples were thawed at room temperature, chopped into small pieces, and homogenized in 0.05 M potassium phosphate buffer containing 0.5% HTAB (hexadecyltrimethylammonium bromide, Sigma-Aldrich, Budapest, Hungary), 1 ml buffer/ear. The homogenate was centrifuged at 11000 g at 4 °C for 10 min and 200 µl of the supernatant was placed into Eppendorf tubes. Myeloperoxidase activity was assayed by measuring the H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich, Budapest, Hungary). In its oxidized form, TMB has a blue color, which was measured spectrophotometrically at 620 nm. The reaction was performed in 96-well microtiter plates at room temperature. The reaction mixture consisted of 25 µl of the tissue sample, 25 µl of TMB (final concentration 0.16 mM) dissolved in dimethylsulfoxide (DMSO) and 200 µl H₂O₂ (final concentration 0.24 mM, Sigma-Aldrich, Budapest, Hungary) diluted in 0.08 M phosphate buffer pH 5.4. The optical density (OD) was measured at 5 min intervals for 30 min using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

Data was expressed in arbitrary units of absorbance.

Measurement of plasmaextravasation in the urinary bladder of mice

Mice were anaesthetized by i.p. administration of thiopental (50 mg/kg). A lateral tail vein was cannulated for intravenous administration. 1 or 3 mg of vildagliptin or sitagliptin was administered by oral gavage 30 minutes before the commencement of the capsaicin challenge. Evans blue (30 mg/kg) and 1 minute later capsaicin (1 mg/kg) was injected through the venous cannula. Each animal was sacrificed by transcardiac perfusion with 50 ml of 0.9%

w/v saline into the left cardiac ventricle 10 min after intravenous injection of Evans blue at 37 °C. The urinary bladder was then removed and weighed. Excised tissues were incubated in 1 ml of formamide for 48 h and Evans blue content was measured spectrophotometrically at 620 nm and expressed as µg/g wet mass of the tissue.

Induction of arthritis

Chronic arthritis of the right tibiotarsal joint of mice was induced by the subcutaneous injection of 0.1 ml of Freund's complete adjuvant (CFA, killed Mycobacteria suspended in paraffin oil, 1 mg/ml as provided by Sigma-Aldrich, Budapest, Hungary) into the plantar surface of the right hind paw and into the root of the tail. To enhance systemic effects, an additional injection into the tail was given on the following day. In order to minimize the suffering of mice, short-term general anesthesia was induced by 1% isoflurane (Abbott Laboratories, Budapest, Hungary) delivered in 1:2 oxygen/nitrous oxide mixture.

Measurement of mechano-nociceptive threshold

Touch sensitivity on the plantar surface was measured with von Frey filaments (Bioseb, Chaville, France) before the experiment, 3, 7, 10, 14, 17, and 21 days following the first CFA administration. The set of 20 monofilaments provided an approximate logarithmic scale of actual force and a linear scale of perceived intensity. Mice were placed into a Plexiglas cage with a pitted floor. Following animal acclimatization the operator placed the monofilament under the animal's paw and pressed against the surface till the animal indicated the pressure sensation by pulling back or shaking its paw, or the monofilament curved without any kind of reaction starting with 0.008 g and ranging up to 300 g.

Measurement of thermo-nociceptive threshold (Increasing hot plate test)

The plate (Supertech, Pécs, Hungary) in contact with the paws has been slowly warmed up from room temperature and the threshold temperature producing the first nocifensive behavior (e.g., pawlicking) was recorded. Since the temperature was increased gradually into the noxious range, stress associated with the testing procedure was minimized. The heated surface dimensions were 110 × 80 mm surrounded by 350 mm high transparent Plexiglaswalls. The commanding computer program was set to produce a 3 °C/min

temperature increase of the plate. When the hind paw licking or flinching was observed the threshold temperature was recorded. The measurement was terminated at the threshold level or when the plate temperature reached 50 °C to avoid tissue damage.

Data were expressed in °C.

Magnetic treatment

An inhomogeneous SMF was generated with an exposure system that was developed, validated and optimised for animal experiments by László et al. The device consisted of two ferrous matrices containing 10×10 mm (diameter×height) cylindrical neodymium iron boron (NdFeB) N50 grade magnets ($B_r=1.47$ T). The lateral periodicity of the inhomogeneous SMF was 10 mm. The individual magnets (supplied by ChenYang Technologies GmbH & Co. KG, Finsing, Germany) in both matrices were placed next to each other with alternating polarity. Magnets facing each other in the two matrices were oriented with opposite polarity. The matrices in the experiments were fixed in a holder in which the matrices were separated from each other in a distance of 50 mm. This distance could be changed between 50 and 90 mm. This arrangement allowed us to insert a 140×140×46 mm (length×width×height) Plexiglas animal cage with air holes into the exposure chamber. An air permeable opaque material covered the cage on four sides to make illumination circumstances similar in the exposure chamber and in the sham experiment. For SMF exposure, 2–3 animals were put into the Plexiglas cage at a time keeping in mind that mice are socially sensitive, then the cage with the animals was inserted into the exposure chamber for 30 min per day for a period of time animals were alive (minimum of six weeks). The treatment was whole body exposure, while animals were free to move in the cage. The magnetic exposure created with this magnetic exposure system did not cause any change in the behaviour of animals. Sham was provided by keeping 2-3 control animals (not exposed to SMF, sham) at a time in identical Plexiglas boxes for 30 min daily.

Induction of diabetes

Mice were rendered diabetic by a single dose of i.p. administered STZ provided by Tocris Bioscience in 100, 150, or 200 mg/kg body weight. STZ was prepared freshly by dissolving in 0.9% sterile physiological saline. Aside the 200 mg/kg dose, the two doses of STZ were set up for modelling the illness with reduced severity. All of the STZ-treated

animals were included irrespective of their blood glucose level, because the SMF exposure sequence was commenced one day after the STZ challenge.

Measurement of blood glucose level

Blood glucose level of STZ-treated (or placebo treated) and SMF-exposed (or sham) animals was measured by Accu-Check blood glucose monitor (Roche Hungary Ltd., Budaörs, Hungary) once a week. Blood samples were taken from the lateral tail vein.

Results were showed in mmol/l.

Experimental groups

Animals were randomly divided into the following experimental groups:

Group 1–4: No SMF exposure (sham).

Group 1: Animal number was 3. These animals did not get STZ at all. This was the non-treated group (no SMF, no STZ).

Group 2, 3 and 4. Animal number was identically 6. STZ was administered i.p. in the animals in an amount of 100, 150 or 200 mg/kg, respectively.

Group 5–8: 30 min/day inhomogeneous SMF exposure from day 0 until death.

Group 5: Animal number was 3. These animals did not get STZ at all. This was the SMF exposure without STZ-treatment group (SMF, no STZ) group.

Group 6, 7 and 8. Animal number was identically 6. STZ was administered i.p. in the animals in an amount of 100, 150 or 200 mg/kg, respectively.

Statistical considerations

Since baseline values of diverse groups in the gliptin experiments were significantly different in all measurements, a baseline correction was carried out on raw values. Baseline corrected values were regarded as primary outcome measures. Two-way ANOVA with replication was used for multiple group analysis with the time point of observation and the treatment option as factors for the complete duration of the experiments. Games-Howell tests were used as post hoc analysis for binary comparison of group averages. Significant differences at the 95% confidence interval were recognized, if $p < 0.05$.

Single-factor ANOVA (Analysis of Variance) was used to test significant differences among multiple data sets in SMF experiments. Statistical differences between two experimental groups were estimated by Dunnett's test. Unequal sized data sets were compared using the Bonferroni correction. Data series were considered significantly different, if their mean values differed with a probability (p) less than 0.05.

4. RESULTS

AITC-induced inflammation model

Both orally administered gliptins significantly decreased ear thickness in the complete time period compared to positive control (AITC only) in a dose-dependent manner. The maximum effect of AITC was measured at 2 hour post-challenge time in either case. For the complete examination period 10 mg sitagliptin as well as 1-10 mg vildagliptin was found to significantly decrease ear edema as compared to positive control ($p < 0.05$ to positive control).

Measurement of neutrophil accumulation

The evolved inflammation was shown by the high level of myeloperoxidase enzyme in the positive control group (AITC only) The model is suitable for measuring the extent of inflammation, since these data definitely diverge from the negative control group results. Sitagliptin treatment was found significantly effective in blocking the evolution of inflammation; every examined dose could reverse inflammation ($p < 0.005$ to positive control). (Blind samples were not included in the hypothesis testing.) The effect of vildagliptin treatment was similar to that of sitagliptin, although the dose of 3 mg/kg had only an insignificant impact.

Measurement of plasmaextravasation in the urinary bladder of mice

The capsaicin-induced plasma extravasation in urinary bladders of mice was inhibited by sitagliptin (1 mg $p = 0.025$ and 3 mg $p < 0.001$) and vildagliptin (both 1 mg and 3 mg $p < 0.001$) significantly. Difference in action was seen between the higher doses (3 mg/kg) of vilda- and sitagliptin. The lower dose of sitagliptin (1 mg/kg) produced the least significant inhibition compared to the control.

Touch sensitivity in CFA-induced arthritis

Results show that the mechano-nociceptive threshold of the untreated group was significantly higher than in the CFA treated (positive control) group during the whole 21 day experimental period. In the gliptin treated groups every threshold was significantly lower than

in the negative control group ($p < 0.001$ to negative control); consequently neither sita-, nor vildagliptin treatment was able to hinder the evolution of allodynia. Sitagliptin was significantly harmful in an amount of 3 and 10 mg/kg doses as compared to the positive control, meanwhile vildagliptin remained ineffective in all doses.

Increasing hot plate test in CFA-induced arthritis

Threshold temperature of the untreated group was significantly higher than in the CFA-treated group during the whole 21 day experimental period, following the first day. Every dose of either sitagliptin or vildagliptin significantly increased the threshold temperature, compared to the positive control group ($p < 0.05$ to positive control). Neither sitagliptin nor vildagliptin could inhibit inflammation; the threshold in all gliptin treated groups remained significantly lower than in the negative control groups ($p < 0.05$ to negative control).

Effect of inhomogeneous SMF exposure on the body weight of mice

Results suggested that there might be an effect of SMF treatment in the body weight of nondiabetic mice. In order to check this assumption, we introduced an additional set of experiments. We found no significant difference between body weight of SMF-treated and control mice in a 30-day long examination period. 30 min/day SMF exposure had no impact in the body weight of mice treated with 100 mg/kg STZ. The body weight in g averaged for the animals in the group treated with 150 mg/kg STZ without SMF exposure was significantly higher vs. group treated with STZ in an amount of 150 mg/kg but sham-exposed ($p < 0.0001$). Results were contrary in groups treated with 200 mg/kg STZ: body weight was lower in the SMF-exposed group ($p < 0.001$).

Note that diabetic mice died in the 6th week.

Effect of inhomogeneous SMF exposure on the blood glucose level of mice

For non-diabetic mice, there was no observable effect of SMF treatment. For diabetic mice, SMF treatment decreased average blood glucose level between 6.44 and 40.36%, by 22.83% on average ($p < 0.05$). In our experiments 100 mg/kg and 150 mg/kg STZ

administration resulted in latent diabetes, where no change was observed in blood glucose level compared to control.

Effect of inhomogeneous SMF exposure on peripheral nociception (increasing hot plate test)

For the diabetic mice (Group 8 and 4) the nociceptive temperature threshold was significantly higher without SMF treatment ($p < 0.01$), but the biological relevance is questionable (averages 44.22 and 43.158 °C, respectively). Due to the consistency of data and accordingly very small standard deviation (0.09 and 0.11), Group 6 and 2 were statistically slightly significantly different ($p = 0.043$), even though no biological difference could be attributed to this fact again (averages 43.54 and 43.268 °C, respectively).

5. DISCUSSION

Diabetes is a worldwide epidemic, and associated neuropathy is its most costly and disabling complication. In painful diabetic neuropathy, most commonly associated with the diabetic sensorimotor polyneuropathy subtype, patients may describe burning, electric, or stabbing pain. Allodynia (painful sensations to innocuous stimuli) and hyperalgesia (increased sensitivity to painful sensations) are other common manifestations of diabetic sensorimotor polyneuropathy. The aim of our experimental work was to evaluate the effects of DPP-4 inhibitors and inhomogeneous SMF against inflammation and pain.

According to our present data, we can conclude that sitagliptin and vildagliptin had a dose-dependent anti-inflammatory effect in *in vivo* mouse models. The applied methods were sensitive enough to detect the action of gliptins.

The anti-inflammatory action of these drugs were described in human studies and for exenatide. Sitagliptin significantly improves endothelial function and inflammatory state in patients with coronary artery disease and uncontrolled diabetes mellitus, forming a milestone in the way towards widening the spectrum of gliptins' indication. Both examined chemicals, sitagliptin and vildagliptin were able to decrease the AITC-induced inflammation in a dose-dependent manner however, sitagliptin had a higher impact. This effect cannot be explained by the regulatory role of GLP-1 on p38 MAPK, as it was described as an inducer; neither can it be attributed to the effect of gliptins on substance P metabolism. Moreover, the physiological role of GLP-1 is so dominant that its inhibition can still override the p38 MAPK-inducer property and the algogenic effect of elevated substance P. In our experiments, the anti-inflammatory action seems to be direct as demonstrated by the accumulated number of neutrophil cells (measured by myeloperoxidase enzyme activity) in the inflamed ear; this accumulation could be inhibited by the gliptin pre-treatment.

Similarly to the above mentioned tests, sitagliptin treatment had a higher impact in the compensation of the CFA-induced arthritis, where vildagliptin showed no effectiveness.

In case of measuring the high temperature sensitivity, both substances showed equal effectiveness. Our results lead to the conclusion that sitagliptin has a stronger influence on the evolution of inflammation; however, vildagliptin showed higher effectiveness in the inhibition of capsaicin-induced plasma extravasation in the urinary bladder. Although the investigated molecules have the same effectiveness in the treatment of type-2 diabetes, it seems that they do not act in the same way, in the immune response. Both substances represent promising options for the therapy of inflammatory disorders.

Several studies demonstrated that SMF exposure has no significant effect on body weight. In our experiments 150 and 200 mg/kg STZ led to opposite effects in the body weight, while no change was observed compared to control, when STZ was injected in a dose of 100 mg/kg. These results suggest that whole body SMF treatment is controversial and depends on the diabetic state. Daily SMF exposure was double-checked not to cause a significant body weight change between non-treated and SMF exposure without STZ-treatment groups. Neither have we experienced any difference in the food, nor in the water intake between exposed and unexposed animals.

Our experiments showed that 200 mg/kg STZ significantly elevated the blood glucose level by about four times. This level could be significantly reduced about 25% via daily SMF treatment. Summarising our observations, we may hypothesize that the effect of 30 min/day, whole body SMF exposure for several weeks can evoke an effect resembling IL-1 β receptor antagonist. Its effect may be equivalent to insulin intake reducing the malign influence of STZ, decreasing oxidative stress, lowering IL-1 β level, assisting insulin release, discouraging the recruitment of innate immune cells, and thus diminishing blood glucose.

An earlier investigation showed that in the course of diabetic neuropathy development STZ-treated mice show an allodynic phase in the first 3–4 weeks after STZ administration; then hypoaesthesia develops gradually. A similar effect may be seen in our present results; however, not for SMF exposed mice. This suggests that a homeostatic defence mechanism may have been activated when SMF was applied. The biphasic development of hyper- and hyposensitivity in temperature induced pain threshold is completely abolished by the daily SMF exposure. Following an initial decrease in the temperature threshold the weekly measured values were not significantly different from the control. This might imply that it was the spontaneous repair mechanism of the injured nerve indeed that was assisted by the SMF, and not the improvement of the diabetic state. The present results show that a 30 min/day whole body SMF treatment for several weeks does not significantly affect the peripheral thermonociceptive pain threshold measured by increasing-temperature hot plate assay in streptozotocin STZ-treated diabetic mice.

6. SUMMARY

1. The purpose of the experimental work was to evaluate two DPP-4 inhibitors, sitagliptin and vildagliptin in the analgetic and antiinflammatory point of view in vivo.
2. We investigated the effects of the inhomogeneous static magnetic field from the analgetic and antiinflammatory point of view in vivo.
3. According to our results substances had different impact but both gliptins showed analgetic and antiinflammatory effectiveness in a dose dependent manner. These properties had been verified in CFA-induced arthritis model by increasing hot plate test and von Frey test, and in mustard-oil-induced ear-inflammation via edema and via neutrophil accumulation as well. Sitagliptin was more potent in the mustard-oil-induced ear-inflammation model.
4. Both gliptins significantly inhibited capsaicin-induced plasmaextravasation in the urinary bladder in mice. In this measurement vildagliptin showed higher impact.
5. The different influence in various experiments suggests that they do not act in the same way.
6. Our results lead to the conclusion that both substances represent promising options for the therapy of inflammatory disorders.
7. The present study provides evidence that 30 min/day, whole body exposure to inhomogeneous SMF significantly diminishes plasma glucose level as compared to control in diabetic mice. Blood glucose level of mice not treated with STZ remained on the normal level with or without SMF exposure.
8. We did not experienced difference in body weight between SMF exposed and not exposed animals neither in healthy nor in diabetic status.
9. We did not experienced significant difference in peripheral nociception between exposed and sham animals neither in healthy nor in diabetic status.



Iktatószám: DEENKÉTK/352/2014.
Tételszám:
Tárgy: PhD Publikációs Lista

Candidate: Judit Ágnes Ujhelyi
Neptun ID: BZIK5C
MTMT ID: 10045973
Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation

1. László, F.J., **Szilvási J.**, Fényi, A., Szalai, A., Gyires, K., Pórszász, R.: Daily exposure to inhomogeneous static magnetic field significantly reduces blood glucose level in diabetic mice.
Int. J. Radiat. Biol. 87 (1), 36-45, 2011.
DOI: <http://dx.doi.org/10.3109/09553002.2010.518200>
IF:2.275
2. **Ujhelyi, J.**, Ujhelyi, Z., Szalai, A., László, F.J., Cayasso, M., Vecsernyés, M., Pórszász, R.: Analgesic and anti-inflammatory effectiveness of sitagliptin and vildagliptin in mice.
Regul. Pept. Epub ahead of print (2014)
DOI: <http://dx.doi.org/10.1016/j.regpep.2014.09.006>
IF:2.014 (2013)

Total IF of journals (all publications): 4,289

Total IF of journals (publications related to the dissertation): 4,289

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

29 October, 2014

