THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of cardioprotective effect of the drug candidate BGP-15 in a preclinical in vivo and human ex vivo model

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DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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1. Introduction

The metabolic syndrome is a cluster of symptoms, such as high blood pressure, hyperglycemia, and abdominal obesity. It increases morbidity of type 2 diabetes mellitus and cardiovascular diseases. Metabolic syndrome was first defined by the WHO in 1998, but in following years multiple other criteria systems appeared, to further increase the accuracy of the definition. The latest of these systems is the Harmonization (2009). The prevalence of the disease, and its distribution in different sex and age groups is highly dependent on which criteria system we base our study on, but overall, it shows an increasing tendency.

Main risk factors include abdominal obesity, insulin resistance, low HDL level, and high blood pressure. There are studies that focus on the genetic determination, for example the 11-β-hydroxisteroid dehydrogenase, or the endothelial nitrogen oxide synthase genes, and the role they play in the development of the disease. Without a doubt, in its pathogenesis insulin resistance plays a key role, and is often paired with increased amount of abdominal visceral fat tissue. In this state the body tries to keep up the normoglycemic level, through increasing the number of β-cells, and/or the increased capacity of insulin secretion. If the increased β-cell function and/or mass stays functional for an extended period, the manifestation of the type 2 diabetes is shifted in time, which leads to a hyperinsulinemia. With the increased level of insulin resistance β-cells get exhausted, they lose their functionality. Insulin takes part in regulating the glucose and lipid levels of the body, in its presence lipogenesis intensifies, while lipolysis declines. The insulin resistance of the fat-tissue is important regarding the pathophysiology of the metabolic syndrome. Through its development the FFA (free fatty acid) mobilization from the triglyceride stored in the fat tissue is sped up (lipolysis).The fat-tissue, through excessive releasement of proinflammatory cytokines, is also connected to the pathophysiology of the metabolic syndrome.

The increased risk of cardiovascular diseases developed by patients with insulin resistance is well known. First, the hypertension is characteristic, which is cumulatively caused by the activity of the renin-angiotensin system, the salt-reabsorption effect of the insulin, and the increased activity of the sympathetic nervous system. The second dangerous cardiovascular threat is the development of diabetic cardiomyopathy, which leads to diastolic dysfunction, heart muscle hypertrophy, and in advanced cases, the deterioration of systolic functions. In addition to type 2 diabetes and cardiovascular diseases, metabolic syndrome may be the root cause of numerous other illnesses.

As for therapy, first action should be a lifestyle change, most importantly a correct diet, and regular physical activity. These methods could be sufficient in the early stages of the syndrome, at later stages application of various drugs might be necessary, mostly antidiabetics, antihypertensive and antihyperlipidemic agents. The available drugs have limits and often severe side effects. Development of new treatment methods should be explored.

During our research, we focused on the proven non-toxic, Hungarian developed drug candidate BGP-15 (O-(3-piperidino-2-hydroxy-1-propyl) nicotinic acid amidoxime), an agent similar to proplanolol in structure, which in indication of diabetes is already passed the II. Phase of clinical trials. It has a protective effect in various area, mainly cardiac disease, severe muscle dystrophy, or polycystic ovary syndrome, and there were studies about its skin-protective effect in case of acute light damage. It's mechanism of action are diverse, and despite the numerous studies its effect on the cardiac mechanisms is still unclear.

2. Aims

The aim of our experimental work, which is the basis of the present dissertation, was to investigate the cardiovascular effects of BGP-15 and to understand the underlying mechanisms as accurately as possible. Due to its structural similarity to propranolol, it came to our mind, that the drug candidate may have a β-blocking effect. In our first experimental design, we compared the effect of this two molecules on inotropy using human right atrial trabecule sample. Based on our previous studies, PKG axis activating effect of BGP-15 also came to the fore, which was studied using hypercholesterolemic New Zealand rabbits as animal model.

3. Material and Methods

3.1. Examination of isolated trabecula

3.1.1. Tissue sample

The study was designed in accordance with the principles of the Declaration of Helsinki, and it was approved by the Medical Research Council with ethical approval reference number ETT TUKEB 39762-3/2016/EKU. Patients admitted to the Department of Cardiology and Cardiac Surgery (University of Debrecen, Debrecen, Hungary) for an open-heart surgery (mostly to carry out coronary bypass or valve prosthesis implantation), were asked to participate in this investigation. Written informed consent was obtained from each participant prior to inclusion.
After dissection, the atrial sample was placed into ice-cold, oxygenated Krebs solution. From the sample, one trabecula carnea was isolated (diameter: 0.7–1 mm; length: 3–6 mm) and mounted at 10 mN resting tension in a 10 mL vertical organ chamber (Experimetria TSZ-04, Experimetria Kft, Budapest, Hungary) containing Krebs solution, oxygenated with 95% O2 and 5% CO2 (35.5 ◦C; pH = 7.4). The trabecula was paced by a platinum electrode (1 Hz, 2 ms, 7–10 V) by means of a programmable stimulator (Experimetria ST-02, Experimetria Kft, Budapest, Hungary) and power amplifier (Experimetria PST-02, Experimetria Kft, Budapest, Hungary). The contractile force was characterized by the amplitude of the isometric twitches.

3.1.2. Protocols

Two protocols were carried out, which early steps were the same. The atrial samples were equilibrated in Krebs solution for 45 min. Three, consecutive cumulative adenosine concentration-response (E/c) curve was generated, each followed by a wash-out period.
Protocol I.- Indirect effect: atria received a cumulative ISO E/c curve (from 1 nmol/L to 100 μmol/L) and half maximal effective concentration (EC50) was determinated and after a wash out period administered. After stabilization of the contractile force, a cumulative BGP-15 E/c curve was generated. After a 50-min wash-out, samples were stimulated with the EC50 of ISO again, and then a cumulative propranolol E/c curve was constructed.
Protocol II – Direct effect: in the Direct group, atria were subjected to a cumulative E/c curve with BGP-15 or propranolol with no previous isoproterenol administration.

3.1.3. Data analysis

Evaluating the curves, the contractile force was interpreted as the distance (amplitude) of the lower and upper envelope curves of the densely registered individual contractions.

Negative inotropic effects were seen in case of adenosine, BGP-15, and propranolol. The initial contractile force was defined as the state before the administration of the lowest concentration. The lowest contractile force to given concentration was used to calculate the effect, which was defined as the percentage decrease in the initial contractile force:

Positive inotropic effect was observed after administration of isoproterenol. Also, the initial contractile force was the state before the administration of the lowest concentration, and the highest contractile force after administration was used to calculate the effect of given concentration. The effect in this case was defined as the percentage increase in the initial contractile force.

3.2. Examination of BGP-15 treated rabbits

3.2.1. Animal model

All experimental protocols were approved by the local Ethics Committee of University of Debrecen (permission: 25/2013DEMÁB and 10/2018). The animals received humane care in accordance with the "Principles of Laboratory Animal Care" by EU Directive 2010/63/EU. Male New Zealand white rabbits (2700-3200 g) were used for the studies (Charles River Laboratories Inc., Wilmington, MA, USA). Rabbits were kept under a 12-12 h light-dark cycle, and 2 weeks of adaptation period was provided before the start point of the study. Hypercholesterolemia, thus atherosclerotic cardiovascular disease was generated in rabbits using "atherogenic" diet for 4 months. Atherogenic chow contained 1% additive cholesterol and 5% saturated fat.

3.2.2. Protocol

Protocol I.: For acute studies, hypercholesterolemic (HC, n=10, at the 16. week of atherogenic treatment) and age-matched healthy Control (n=6, on standard rabbit chow) rabbits were used. HC animals were fed with atherogenic chow (1 % additive cholesterol and 5 % saturated fat) for 16 weeks, before baseline data acquisition. Echocardiography was performed on each animal under light ketamine-xylazine (30/3 mg/kg i.m.)anesthesia at week 16, which was followed by the administration of one single bolus of BGP-15-solution (10 mg/kg in saline, i.v.), through the marginal ear vein. A 20-minute-long period was provided for drug

distribution, after standard echocardiographic imaging was performed again on each animal, under the influence of BGP-15. Statistical analyses were performed on 4 subgroups, as the follows: (I) Control rabbits at baseline conditions (Control Pre), (II) the same Control animals under the influence of BGP-15 (Control Post BGP-15), (III) HC animals at baseline (HC Pre) and (IV) HC animals after BGP-15 i.v. bolus administration (HC Post BGP-15).

Protocol II.: For the long-term studies, another population of rabbits were used. Healthy Control rabbits (n=10) received standard rodent chow, while the diseased group hypercholesterolemic, HC, n=10) and the BGP-15-treated group (HC+BGP-15, n=10) received atherogenic chow (described above) for 4 months. BGP-15 was administered orally to the animals in the HC+BGP-15 group (10 mg/kg, in saline), every day. Control and HC animals were left untreated. At the endpoint of the study, echocardiography was carried out, followed by blood sample collection from the marginal ear vein. On the next day, animals were sacrificed under deep anesthesia, thoracotomy was performed, and the excised thoracic aorta was subjected to ex vivo vascular studies. Heart was carefully placed into ice-cold Ca^{2+} -free Krebs buffer, and tissue samples was immediately frozen in liquid nitrogen and stored for further molecular biological analyses and isolated cardiomyocyte experiments. Tissue wet/dry weights (lung, kidney) were measured and the tibial length was determined (n=5). Heart and aortic root samples from 3 rabbits/group were stored in 4% formalin solution for histological stains.

3.2.3. Echocardiography

Transthoracic echocardiography was performed under ketamine-xylazine anesthesia (30/3 mg/kg, i.m.). The chest hair of rabbits was removed, and data acquisition was performed in accordance with the recommendations of the American Society of Echocardiography. A Vivid E9 sonograph (GE Healthcare, New York, NY, USA) equipped with a sector 12S-D probe was used, and the echocardiographic dataset was recorded from parasternal long-and short axis, and as well as from apical 3- and 4 chamber views. Ejection fraction (EF), fractional shortening (FS), myocardial wall thickness in systole and diastole, left atrial (LA) size, aortic (Ao) diameter, mitral- and tricuspid annular plane systolic excursion (MAPSE, TAPSE) was determined from M-mode recordings. Heart rate (HR), stroke volume (SV), cardiac output (CO) and left ventricle mass was calculated. Pulsed wave Doppler (PW) and tissue Doppler (TDI) echocardiography was also carried out, where transmitral E and A wave velocities, E/A ratio and E wave deceleration time (DecT) was measured. Wall motion was defined by TDI e’ and a’ waves and e’/a’ ratios, measured at the mitral and septal annulus, and the E/e’ ratio was calculated. Ejection time (ET), isovolumic contraction and relaxation time (IVCT, IVRT,

respectively) was determined, and Tei-index (Myocardial Performance Index, MPI) was calculated as IVRT+IVCT/ET. At the level of the aortic valve, left ventricle outflow tract (LVOT) velocities (V, maximal and mean) and pressure gradients (PG, maximal and mean) were recorded. Speckle tracking method was performed offline on recordings obtained from apical long axis (APLAX) and 4 chamber views by using EchoPAC PC software (ver. 112, GE Healthcare, New York, NY, USA) Q-analysis/2DStrain measurement option. Endocardial wall (region of interest, ROI) was manually traced. Systolic global longitudinal strain (GLS), strain rate (SR) during the isovolumic relaxation period (SR IVR) was determined, and E/SRIVR ratio was calculated.

3.2.4. Serum parameters and morphometry

Blood was collected from the marginal ear vein after 12-h fasting, at the endpoint of the long-term study protocol. Serum parameters were determined on the Roche Cobas Integrated platform (Roche Diagnostics GmbH, Mannheim, Germany). Body weight of the animals was measured, then rabbits were sacrificed by thoracotomy under deep anaesthesia, and organ samples from heart, lung, liver and kidney were excised (n=5/group). Tissue samples were weighed using a milligram scale. Whole heart and then left ventricle was precisely isolated from 5 rabbits, weighed and normalized to the length of the tibia. Left ventricle samples were then placed into 4 % formalin solution and stored for histological staining. Kidney, lung and liver samples were kept overnight at 60°C, after wet/dry tissue ratios were determined. Left ventricle samples (from another 5 animals) were immediately excised after thoracotomy, placed into Ca 2+ -free Krebs buffer, then were rapidly frozen in liquid nitrogen and stored at -80°C for molecular biological analyses and force measurement of isolated myocytes. Thoracic aorta was excised, washed, and the distal section was subjected to ex vivo vascular assays, whereas the proximal part was stored in 4% formalin solution for histological staining.

3.2.5. Histology

Left ventricle samples harvested from the long-term study group animals were subjected to Masson's Trichrome staining to visualize collagen fibers. Tissue samples stored in 4% formalin solution were embedded into paraffin, and 5 µm thick sections were made. After deparaffinization, Masson's trichrome staining was carried out based on the protocol provided by the manufacturer (Sigma-Aldrich Co., St. Louis, MO, USA). On the stained slides, cytoplasm and muscle fibers appear in red, whereas collagen fibers display blue-purple coloration. Area of the fibrosis was measured using the manual area-tracking function of the

Scion Image for Windows software (ver. 4.0.2., Scion Corporation, USA), and was expressed as the percentage of the total field-of-view (FOV) area (n=3/groups, 3-5 sections/animal). Paraffin-embedded sections of thoracic aorta at 5 μm thickness were subjected to Movat pentachrome staining to visualize atherosclerotic plaques (n=3/groups, 3-5 sections/animal). Staining protocol provided by the manufacturer (Abcam Plc., Cambridge, UK) was followed, and the stained slides were analyzed using a light microscope (40x or 100x magnification). Thickness of the intima and media was measured using the length measuring function of Scion Image for Windows software (ver. 4.0.2., Scion Corporation, USA), and intima/media ratios were then calculated.

3.2.6. Vascular assays

After sacrificing the rabbits in the long-term study group, the distal part of the thoracic aorta was excised, and 2 mm-wide rings were cut off. The isolated aortic rings were mounted using a wire instrument in an organ bath system containing Krebs solution oxygenated with 95% O_2 and 5% CO2 (36 C; pH = 7.4), at 10 mN resting tension. The isometric contractile force was measured by a transducer (SD-01; Experimetria Ltd., Hungary) connected to workstation with SPEL Advances Isosys software (SOFT-02; MDE GmbH, Heidelberg, Germany). On the rings, three concentration-response (E/c) curves were constructed (separated by wash-out periods) with norepinephrine (NE), acetylcholine (Ach) and adenosine-5'-triphosphate (ATP). Before the Ach and ATP E/c curves, the aortic rings were pre-contracted at the half-maximal effective concentration (EC50) of NE determined from the NE E/c curve. Responses of aortic rings obtained from the same animal were averaged (n=4 per animal). The effect of NE was defined as an increase of the contractile force in addition to the resting tension (10 mN). The effect of Ach and ATP was defined as a percentage change in the initial tension of the ring.

3.2.7. Western blot

300 mg deep-frozen myocardial tissue samples were used for each protein isolation. The powdered tissueswere homogenized in a buffer containing 25 mM Tris-HCl, pH = 8, 25 mM NaCl, 4 mM Na-orthovanadate, 10 mM NaF, 10 mM Na-pyrophosphate, 10 nM okadaic acid, 0.5 mM EDTA,1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein concentration was measured by the QuantiPro™ BCA Assay Kit (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany). Samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then were transferred onto a nitrocellulose membrane via electro-blotting (at 40 mA for 120 min). After blocking with 3% BSA,

membranes were incubated overnight at 4°C with primary antibodies, as the follows: anti-sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a), anti-heat-shock protein72 (hsp72), anti-vasodilator-stimulated phosphoprotein (VASP), anti-phopsho(Ser239)-vasodilator-stimulated phosphoprotein clone 16C2 (p-VASP), anti-phosphodiesterase 9a (PDE9a), anti-phosphodiesterase 5a (PDE5a), anti-phospholamban (PLB), anti-phospho(Ser16)-phospholamban (p-PLB), and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, as a housekeeping protein), obtained from Sigma-Aldrich (Sigma-Aldrich-Merck KGaA and Abcam (Abcam Plc., Cambridge, UK). Antibodies were used in dilutions recommended by the manufacturer. Visualization of protein bands were made by horseradish peroxidase-conjugated secondary antibodies and Enhanced Chemiluminescense reagent (WesternBright™, ECL, Advansta Inc., Menlo Park, CA, USA). Detection and data analysis were carried out by a C-Digit® blot scanner equipped with Image Studio Digits ver.5.2. software (LI-COR Inc., Lincoln, NE, USA). Data was averaged from 3 independent experiment (n = 6 samples/group), and protein expressions are shown as the percentage of pixel density relative to those of the Control group, which was considered 100 %.

3.2.8. Determination of myocardial cGMP-content

Quantification of cGMP in left ventricle myocardial samples of the rabbits were carried out by a direct competitive immunoassay (Abcam Plc., Cambridge, UK). After preparing standard absorbance-cGMP concentration curve, samples of animals (n=4/group, each measured in duplicates) were homogenized in 0.1M HCl. Measurement protocol recommended by the manufacturer was strictly followed. The amount of horseradish-peroxidase (HRP)-conjugated cGMP, bound to the specific G-protein-covered 96-well plate was measured by determining optical density (OD) at 450 nm, using a plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany). The amount of cGMP in myocardial samples was calculated using the equation described by the manufacturer, and data was expressed as pmol/mg tissue in each group.

3.2.9. Titin assays

Analysis of titin isoform composition and titin N2-Bus phosphorylation assays were carried out. Frozen myocardial samples were homogenized in a relaxing buffer and were centrifuged. Lanes were loaded with equal amounts of protein and titin isoforms were separated by agarose-strengthened 1.8% SDS-PAGE. Total protein was stained by Sypro Ruby (Invitrogen, Carlsbad, CA, USA) and were analyzed densitometrically to determine titin N2BA/N2B isoform ratios.

Proteins were then transferred onto a PVDF-membrane to determine titin N2B bus phosphorylation. Signal intensity was analyzed after bands were visualized using a Fuji-LAS 4000 imaging system. The ratio of phospho/total titin was normalized to those of the Control group (=100 %).

3.2.10. In vitro PDE inhibitor screening assay

The ability of BGP-15 (substance, dissolved in distilled water) to inhibit the phosphodiesterase-1 enzyme (PDE1) was determined using a specific colorimetric assay (Abcam Plc., Cambridge, UK). The basis of the assay is the cleavage of cGMP by PDE1 to 5'-GMP, which is further cleaved into GMP and phosphate by the enzyme 5'-nucleotidase, and the phosphate is quantified using Malachite Green reagent. After preparing the standard curve (absorbance - 5'-GMP concentration), wells of a 96-well microplate were loaded with assay buffer, 0.5 mM cGMP substrate, 5'-nucleotidase enzyme (5 kU/ml), PDE1 enzyme (4 mU/µL), and the test compound (BGP-15). BGP-15 was added in concentrations of 40, 100, 200 and 500 µM to "test" wells, respectively. Blank wells contained only the assay buffer (for background subtraction), further, "positive control" wells were loaded with 40 µM isobutyl-1-methylxanthine (IBMX, IC_{50} for PDE1 = 25 µM). "Control" wells contained the reaction mixture without any drug. After incubation at 37°C for 50 mins, a modified Malachite Green assay reagent was added to each well, the reaction ended, and the green color was able to develop for 20 mins. Absorbance was measured using a Varioskan LUX spectrometer (ThermoFisher Scientific Inc., Waltham, MA, USA) at 620 nm, and the amounts of the generated 5'-GMP were calculated. PDE1 activity in "test" wells were normalized to "Control" PDE1 activity, which was considered 100 %. Data was analyzed using one-way ANOVA with Tukey post-test.

3.3. Statistical analysis

In the first study design, normality of data was checked using Shapiro–Wilk normality test. Two data sets, if passed the normality test, were compared with unpaired t test (without or with Welch's correction, depending on homogeneity or heterogeneity of variances, respectively). For non-Gaussian data, Mann–Whitney U test was used. To compare more than two data sets, one-way ANOVA followed by Tukey post-testing was applied (after verifying the Gaussian data distribution). The linear relationship of two data sets was analyzed with Pearson or

Spearman correlation in the case of Gaussian or non-Gaussian distribution, respectively. For the sake of illustration, linear regression was also performed. To assess the relationship between two binomial data sets, Fisher's test was used. Difference of means (or medians) was considered significant at $p < 0.05$. Statistical analysis was performed with GraphPad Prism 8.4.2 for Windows (GraphPad Software Inc., La Jolla, CA, USA), while other calculations were made by means of Microsoft Excel 2016 (Microsoft Co., Redmond, WA, USA).

In the second study design, data is presented as the mean value of the group ± standard error of the mean (SEM).

At Protocol I, baseline echocardiographic parameters were compared with the same parameters recorded 20 mins after BGP-15 administration, both in healthy Control and hypercholesterolemic (HC) rabbits. As this procedure required the determination how a response is affected by two factors (BGP-15-treatment and atherogenic diet), two-way repeated measure ANOVA (2-way-RM-ANOVA) was performed on longitudinal data across two timepoints, and interaction of the two factors was also calculated. In Protocol II, to compare endpoint parameters of 3 groups, Gaussian distribution was estimated by Shapiro-Wilk normality test. Statistical analysis then was performed using one-way analysis of variance (ANOVA) followed by Tukey post-test (when normality test was passed), or Kruskal-Wallis test followed by Dunn's post-test (when normality test was not passed). Analyses were carried out using GraphPad Prism software for Windows, version 8.4.2. (GraphPad Software Inc., La Jolla, CA, USA). Probability values (p) less than 0.05 were considered significantly different.

4. Results

4.1. Examination of isolated trabecula

4.1.1. Contractile Force of the Right Atrial Samples

The contractile force, measured at the end of the wash-out period after the third adenosine E/c curve, was used to characterize the naïve contractility of the samples. The averaged contractile force did not differ significantly between atria that were (n = 16) and were not (n = 10) stimulated with ISO in a later phase of the investigation. In the case of atria never treated with ISO (Direct group), there was no significant difference between contractility of its two subgroups, the BGP-15 subgroup (n = 6) and Propranolol subgroup (n = 4). Within atrial samples subjected to ISO, according to their response to ISO, four types were identified: samples that gave a negative inotropic response (n = 2), weak positive inotropic response (n = 5), strong positive inotropic response (n = 7) and extremely strong positive inotropic response (n = 2). Hereinafter, the atria producing weak positive inotropic response (Weak subgroup) and strong positive inotropic response (Strong subgroup) were pooled as the Indirect group (n = 12). (Overall, the Indirect group consisted of atrial samples showing positive inotropic response to ISO that was greater than 0% and was not greater than 100%. The reason for this data pooling is that these two subgroups contain the samples free from any paradoxical or irregular behavior.) The contractile force of samples in the Weak subgroup was greater than that in the Strong subgroup, although this difference did not reach the level of statistical significance. In summary, the naïve contractility of samples in the present study was similar (apart from the two samples giving an extremely strong positive inotropic response to ISO.

4.1.2. Response to Adenosine

Responsiveness of the atrial samples to adenosine was evaluated based on the third adenosine E/c curve. Regarding the adenosine-evoked direct negative inotropic effect, there was no significant difference between the Direct and Indirect groups (names of which refer to the absence or presence of subsequent ISO stimulations, respectively). Also, within the two groups, the different subgroups (also distinguished based on subsequent events) did not differ significantly from each other. Thus, the susceptibility of samples to adenosine was similar.

4.1.3. Response to Isoproterenol

ISO elicited a positive inotropic effect from the atrial samples (except for two samples). The positive inotropic response was categorized to be weak, if its maximum did not reach 50% (i.e.,

an extra 50% in addition to the initial value), strong, if the maximum was between 50–100%, and extremely strong, if the maximum was above 100%. As mentioned above, samples giving negative inotropic and extremely strong positive inotropic responses were excluded from the further analysis. The positive inotropic effect of ISO was significantly higher in the Strong subgroup than in the Weak subgroup at most ISO concentrations. Both main features of ISO's effect, the maximal response and the potency (characterized by pEC50 being the negative common logarithm of the ISO concentration producing half-maximal response),
showed a statistically non-significant negative correlation with the initial contractile force (Pearson r= 0.41, p=0.19; Spearman r=0.32, p=0.31, respectively).

4.1.4. Response to BGP-15 and Propranolol

In the isolated, paced, human right atrial myocardium, both BGP-15 and propranolol evoked a strong, concentration-dependent, negative inotropic effect (regarding both the direct and indirect ones). Beyond the indubitable similarities (most importantly, the practically equal maximal responses), the effect of BGP-15 and propranolol showed some differences. The main difference between effects of BGP-15 and propranolol is that BGP-15 possessed a considerably smaller potency as compared to propranolol, in terms of both the direct and indirect negative inotropic effects. In addition, BGP-15 seems to have a somewhat stronger direct effect, whereas propranolol appears to exert a stronger indirect action, especially at upper medium concentrations. Furthermore, BGP-15 evoked a substantially greater indirect negative inotropic effect on samples with strong responsiveness to ISO (at medium and upper medium concentrations). In contrast, propranolol did not discriminate significantly between samples with different susceptibility to ISO (although, similarly to BGP-15, it tended to elicit a somewhat greater indirect negative inotropic effect on samples that gave a stronger response to ISO). It should be noted here that subgroups of the Direct group are the most suitable for comparing effects of BGP-15 and propranolol, as experimental conditions for these two negative inotropic agents were the same in these subgroups. ISO was very potent in three atrial samples. Since EC50 estimation for these three samples was less reliable than for the others, furthermore, since the estimated EC50 values were used to stimulate the samples in the Indirect group, the influence of these samples on the BGP-15 and propranolol E/c curve data was tested. Therefore, the evaluation of the BGP-15 and propranolol E/c curves in the Indirect group was repeated with the exclusion of the three "overpotent" samples. This exclusion did not alter the major findings of the evaluation presented so far, leading only two changes worth to mention: differences of BGP-15 E/c curves between the Direct and Indirect groups became statistically

non-significant; and only one propranolol E/c curve remained in the Weak subgroup that hindered the statistical analysis. Thus, the uncertainty in the estimation of the three EC50 values did not meaningfully modify results of the present investigation.

To explore the influence of diabetes mellitus on the effect of BGP-15, another evaluation was also performed via dichotomizing the adenosine, ISO, and BGP-15 E/c data of the Indirect group, according to the presence or absence of diabetes mellitus in the history of patients. The results were astonishingly similar to those obtained by comparing the Weak and Strong subgroups of the Indirect group. Thus, ISO and BGP-15 exerted a significantly greater inotropic effect on the diabetic samples than on the non-diabetic ones. The reason for this might be a big overlap of the Strong subgroup with the data set containing the diabetic samples of the Indirect group. Of 12 samples in the Indirect group, 7 gave a strong response to ISO. Of these 7 samples, 4 were derived from diabetic patients. In contrast, of 5 samples producing weak response to ISO, none was obtained from a diabetic patient. Starting from these findings, it was concluded that diabetes mellitus was a major (if not the greatest) factor to increase ISO sensitivity of the atrial samples. Unfortunately, the limited sample (patient) number did not allow us to evaluate whether type 1 or type 2 diabetes mellitus (or both ones) is (are) responsible for the above-mentioned phenomenon. Nevertheless, occurrence of the different types of diabetes mellitus in our database shows the dominance of the type 2: 7/9/22 (type 2 diabetes mellitus/all diabetes mellitus/all patients, for the pooled Direct and Indirect groups), and 3/4/12 (the same solely for the Indirect group).

4.1.5. Associations between Patient Data and Sample Features

During the recruitment period, 30 patients were included (7 females and 23 males) ranging from 29 to 78 years (59.5 ± 12.4). From the 30 samples obtained, 26 proved to be technically sound. Of them,10 samples, derived from patients (3 females and 7 males) ranging from 40 to 70 years (58.7 ± 9), were randomized into the Direct group, while 16 samples, obtained from patients (2 females and 14 males) ranging from 29 to 76 years (57.9 ± 13.5), were sorted into the set receiving ISO. Of this latter set, 12 patients (1 female and 11 males) from 29 to 76 years (58.7 ± 15.4), who provided atrial samples giving “unextreme” response to ISO (i.e., the effect of ISO was neither negative nor extremely strong), formed the Indirect group. The relationship of the occurrence of certain conditions (yes or no) with the naïve contractility (small or large), and with the response to ISO (weak or strong) were assessed. All patients suffered from several diseases and took numerous drugs. From these factors, NO donors and trimetazidine appeared to beneficially influence the contractile force of the atrial samples. Furthermore, diabetes

mellitus and hypertension seemed to increase, while proton-pump inhibitors tended to decrease the response to ISO. From these associations, none proved to be statistically significant.

4.2. Examination of BGP-15 treated rabbits

4.2.1. Diastolic function

Echocardiographic effects of single-dose intravenous BGP-15 was evaluated using HC rabbits at the 16 th week of atherogenic diet and age-matched healthy Control animals (standard rabbit chow). Baseline (drug-free) echocardiographic data of rabbits from both groups was compared to data obtained under the influence of BGP-15 (i.v. bolus), using 2-way repeated measure (RM) ANOVA. (Adjusted p values were also calculated by Sidak multiple-comparison test. In single-dose echocardiographic studies, Control and HC animals differed in baseline echocardiographic values, as it was expected. E/A and e'/a' ratios were decreased in HC animals after 16 weeks of atherogenic treatment. E/e' ratio and Tei- index was increased in HC rabbits, but Ejection Fraction (EF) only slightly decreased, in accordance with the echocardiographic data obtained from the long-term study group population (Protocol II) and as well with our previous findings in a similar rabbit model. Ejection fraction was unaltered in both study groups after BGP-15-injection. Drug administration significantly decreased heart rate ($p=0.0142$, 2-way RM-ANOVA). BGP-15- treatment significantly increased E/A ratio, but only in HC rabbits ($p=0.0008$ in HC pre vs. HC Post BGP-15, Sidak MC). As transmitral E/A ratio may be affected by heart rate, diastolic function was also evaluated by more standardized parameters. Tissue Doppler (TDI) e' wave velocity and e'/a' ratios were significantly increased after BGP-15 administration in both groups, but the changes were more prominent in HC animals ($p=0.0004$ for TDI e'; and $p=0.0012$ for e'/a'; HC Pre vs. HC Post BGP-15; Sidak MC). The E/e' ratio (indicative for LV filling pressure) decreased after BGP-15 bolus administration in both groups ($p=0.042$, 2- way RM-ANOVA), but the change again was more distinct in HC animals ($p=0.003$, HC Pre vs. HC Post-BGP-15, Sidak). Tei-index decreased significantly in HC animals under the influence of the drug ($p=0.0028$, Sidak MC). As echocardiographic parameters of HC animals were highly affected by the BGP-15 injection, but parameters of Control rabbits remained nearly unchanged, during the following chronic studies, healthy Control rabbits were not treated with BGP-15. In long-term studies (Protocol II, 10 mg/kg BGP-15 per os, for 16 weeks), echocardiographic changes showed similarities with those of single-dose studies. After 16 weeks of treatment, left atrial enlargement (defined as the ratio of left atrial diameter and aortic root diameter, LA/Ao) was shown in HC group compared to healthy

Control (p<0.0001, Control vs. HC), which was counteracted by the 16-week-long BGP-15 treatment (p=0.0478 HC+BGP-15 vs. HC). Wall thickness in systole and diastole, as well MAPSE and TAPSE values were unchanged among groups. More prominent changes were found in diastolic function, as E/A and tissue e'/a' ratios were significantly decreased in the HC group (p<0.0001 and p=0.0013 vs. Control, respectively), and Deceleration Time (DecT) was lengthened (p=0.0008), while values of BGP-15-treated rabbits were similar to those of Controls, thus improved compared to HC (with p values 0.0993, 0.0002 and 0.0125 vs. HC, respectively). E/e' ratio (indicative for LV filling pressure) dramatically increased in HC group (p<0.0001 vs. Control) and significantly decreased in HC-BGP-15 (p=0.0048 vs. HC). The same pattern was observed in isovolumic relaxation time (IVRT: p<0.0001 HC vs. Control; p=0.0003, HC+BGP-15 vs. HC) and Tei-index (p<0.0001, HC vs. Control; p<0.0001, HC+BGP-15 vs. HC) In conclusion, both single-dose and chronic BGP-15 treatment significantly improved echocardiographic parameters, particularly those of indicative for diastolic function, compared to data obtained from rabbits only received atherogenic diet (HC).

4.2.2. Serum parameters and morphometry

As it was expected, severe hypercholesterolemia and dyslipidemia developed in HC rabbits after 16 weeks of atherogenic diet (Protocol II). Total cholesterol, LDL, HDL, ApoA and ApoB levels significantly increased in both HC and HC+BGP-15 groups in comparison to the Control. BGP-15-treatment failed to significantly improve serum lipid parameters, liver enzymes or CK and CK-MB levels. Levels of serum osteocalcin (a marker that has recently been proposed to negatively correlate with atherosclerosis and coronary heart disease), decreased in both HC and HC+BGP-15 samples compared to Control.

4.2.3. Endothelium-dependent vasorelaxation

The response to NE of aortic rings isolated from rabbits (Protocol II) differed significantly between the Control and HC groups at 10 and 100 nmol/L NE concentrations. The moderate decrease in the susceptibility to NE produced by the atherogenic diet was not affected by the BGP-15 treatment. In all the treatment groups, Ach elicited first (≤ 1 μmol/L) an arterial relaxation, and at higher concentrations (> 1 μmol/L), it caused a gradually evolving arterial contraction. Atherogenic diet significantly attenuated the relaxing action of Ach (Control vs. HC). BGP-15-treatment did not affect the endothelial dysfunction observed in response to the atherogenic diet. ATP caused an arterial relaxation in all the 3 treatment groups. This effect was significantly stronger in the Control group than in both the HC and HC+BGP-15 groups. In

agreement with the results obtained with Ach, BGP-15 did not alter the influence of the atherogenic diet.

Movat pentachrome staining revealed dramatic atherosclerotic plaque coverage in the intimal surface of HC and HC+BGP-15 groups, while aortic samples of the Control group were free from lesions, as it was expected. Accordingly, mean intima/media ratio significantly increased in the HC group vs. Control, and slightly decreased in the HC+BGP-15 group compared to HC. Despite the decreased intima/media ratio, aortic root cross-sections of the HC+BGP-15 group still showed severe atherosclerotic plaque coverage, thus, in accordance with the results obtained from the ex vivo vascular studies and blood parameters, we conclude that BGP-15 failed to improve vascular status in this particular rabbit model.

4.2.4. cGMP level

Myocardial cGMP levels were determined by a specific assay in left ventricle tissue samples of animals in the Control, HC and HC+BGP-15 groups (n=4 animals/group). Cyclic GMP levels of Control and HC animals did not differ significantly (10.49±1.584 vs. 12.94±2.301 pmol/mg of tissue, Control and HC, respectively), while cGMP levels in BGP-15-treated animals significantly increased (33.89±5.271 pmol/mg of tissue), in comparison to those of HC samples (p=0.0052)

4.2.5. PDE1 in vitro

A phosphodiesterase activity assay was performed to assess the ability of BGP-15 to inhibit the enzyme. Without inhibition (Control), 20 mU PDE enzyme generated 2 nmoles of 5'GMP from 200 μM cGMP at 37 °C in 60 minutes. A non-specific PDE inhibitor, IBMX (3-isobutyl-1-methylxanthine; 40 μM) significantly decreased PDE activity to 67% (vs. Control). BGP-15 dose-dependently inhibited PDE activity comparably to IBMX, which was significant (compared to Control) in concentrations of 100 μM and 200 μM

4.2.6. PKG pathway

Western blot analyses revealed the deterioration of the Protein Kinase G (PKG) pathway in the left ventricle myocardium of HC animals. Chronic BGP-15-treatment has been found to alter the expression levels of key mediators in the cGMP-PKG axis. PKG protein expression was significantly elevated in both HC and HC+BGP-15 groups compared to Control (p=0.0228). When normalized to the Control levels (100%), cGMP/PKG ratio was significantly lower in the HC group compared to Control (n=4, p=0.0052), but was elevated in HC+BGP-15 group

compared to HC (p=0.0117). The ratio of p-(Ser239)-VASP/VASP (indicative for PKG enzyme activity) followed an exactly similar pattern (p=0.0471, Control vs. HC), and was restored in the BGP-15-treated group (p=0.0173,HC vs. HC+BGP-15). Although the total level of the SERCA-inhibitor phospholamban significantly decreased in the HC+BGP-15 groups compared to HC (p=0.0284), the expression of SERCA, or the PLB/SERCA ratio did not show marked differences between the groups. In contrast, the phosphorylation of phospholamban at Ser16 (relative to the total PLB) significantly increased in the HC+BGP-15 group in comparison to HC (p=0.0274). Expression of PDE9A was elevated in both HC and HC+BGP-15 groups compared to the Control (p=0.0283 and p=0.0035, respectively). Contrarily, the expression of the other cardiac cGMP-specific phosphodiesterase, PDE5A, was elevated in HC group (p=0.0056 vs. Control), but decreased in the HC+BGP-15 group compared to HC (p=0.025). The expression of hsp72 did not show marked differences among groups (data not shown). For all proteins of interest, bands were first normalized to GAPDH as a housekeeping protein, and signal intensities are shown as a percentage of Control (which was considered 100%).

5. Discussion

To the best of our knowledge, the first study is the first dealing with the inotropic action of BGP-15, and compared it to that of propranolol, the prototypical non-cardioselective β-adrenergic antagonist with additional membrane stabilizing activity (when used at high concentrations). The relevance of this comparison stems from the fact that BGP-15 and propranolol have a significant structural similarity, so BGP-15 may exert β-blocker activity. In our model using isolated, paced, human atrial trabeculae carneae, contractility can be reliably examined, without the disturbing influence of the chronotropic effect. In the present study, we observed that BGP-15, at low concentrations (≤10 μmol/L), exerted a negligible negative inotropic effect (including its direct and indirect types), while it elicited robust negative inotropy at higher concentrations (≥1 mmol/L). In adult patients, BGP-15 was found to exert an insulin-sensitizing effect at 2–3 mg/kg/day doses that may produce BGP-15 concentrations in micromolar order of magnitude in the body. Taking this observation together with that BGP-15 is ineffective as a negative inotropic agent until 10 μmol/L, it can be concluded that beneficial metabolic effects of BGP-15 will not be accompanied by a significant decrease in contractility in the intermediate range (between 10 μmol/L and 1 mmol/L), the indirect negative inotropic effect of BGP-15 significantly depended on the atrial responsiveness to ISO: only samples exhibiting strong positive inotropic response to ISO produced a considerable negative inotropic response to BGP-15 his appears to be a beneficial property in favor of BGP-15, because it may reduce the cardiac function stimulated by an increased sympathetic tone to a greater extent than the resting activity of the heart. This phenomenon shows some abstract similarities to the negative chronotropic effect of local anesthetics, i.e., they can effectively slow the heart in tachycardia but not in bradycardia (exerting a so-called use-dependent effect). In addition, we investigated the relationship between functional properties of the atrial samples (contractile force, response to ISO) and data of the patients (gender, underlying health conditions, medications used). It should be noted that the patients had multiple diseases and took several drugs, rendering this investigation difficult. Nevertheless, the strong response to ISO seems to be associated with hypertension and diabetes mellitus. Regarding this latter association (i.e., the enhanced ISO sensitivity with the diabetic condition), it should be noted that most of the strong ISO responder samples were obtained from diabetic patients (predominantly suffering from type 2 diabetes mellitus). Thus, it is reasonable to conclude that the response to ISO has an inverse relationship with the “fitness” of the atrial myocardium. This conclusion is further supported by the fact that all pathological

conditions, investigated in the current study, tended to increase the response to ISO. Conversely, when contrasted the behavior of atrial samples obtained from diabetic vs. non-diabetic patients, the results showed convincing similarity to the outcome of the comparison of samples givingst rong vs. weak response to ISO. Thus, BGP-15 exerted a significantly stronger indirect negative inotropic effect on the diabetic atrial samples than on the non-diabetic ones. This finding corresponds with the results of others, who found a bidirectional relationship between insulin resistance and enhanced β-adrenergic stimulation in the heart. This relationship may stem from a mutual counter-regulation between signaling pathways of insulin and β-adrenergic agonists. This mechanism may underly the ability of β-blockers to ameliorate insulin resistance in the heart.

In the second study, we found that both acute and chronic administration of the nicotinic-acid derivate BGP-15 improves cardiac function of rabbits suffering atherosclerotic cardiovascular disease. BGP-15 application preserved diastolic parameters of the heart, restored PKG activity, increased the phosphorylation of the myofilament protein titin, that is responsible for diastolic tension. Most importantly, we found that these effects were independent of vascular status, serum lipid levels and atherosclerosis, which factors failed to improve after the treatment, in contrast to the restored cardiac function. According to our echocardiographic studies presented here, we were able to demonstrate that both single-dose (i.v. bolus) and long-term (oral) administration of BGP-15 may improve the diastolic performance of the heart. Tissue e'/a' ratios (being relatively independent of heart rate) were significantly increased after BGP-15 injection, suggesting that BGP-15 directly affects myocardial relaxation. Single dose BGP-15-treatment did not alter ejection fraction in Control or HC rabbits, however EF was significantly elevated in BGP-15-treated rabbits of the long-term study group in comparison to values of the HC animals. One can speculate that this may be the result of the persistently improved diastolic performance and lower heart rates. It has recently been proposed that the fundamental molecular basis of diastolic dysfunction is the deterioration of the PKG signaling pathway in the myocardial tissue. Considering that our in vivo results demonstrated significant improvement in diastolic function as a result of BGP-15-treatment, key mediators of the cGMP-PKG cascade were investigated.

A main result of this present study is that long-term BGP-15-treatment elevated cGMP levels, hence PKG activity (indicated by normalized p-VASP/VASP and cGMP/PKG ratios) in the diseased myocardium. Cyclic GMP levels are regulated by a family of phosphodiesterase enzymes (PDs), of which PDE5a and PDE9a isoforms (expressed in the heart) are highly selective to cGMP over cAMP. Correspondingly, we were able to detect an increase in PDE9a

expression in left ventricle samples of HC rabbits, which was unaltered by the chronic BGP-15 treatment. Moreover, we found increased expression of PDE5a in the HC myocardium, while PDE5a levels decreased significantly in the HC+BGP-15 group. Among substrates of PKG, the expression of phospholamban (PLB), a key regulator of cardiac relaxation was investigated by Western blot. Phospholamban is a small regulatory protein inhibiting the SERCA pump, thus it contributes to prolonged relaxation of myocytes. When PLB is phosphorylated by PKG or PKA at its Ser16 (or at Thr17 by PKA), its inhibitory effect on the SERCA pump is relieved. Total PLB expression decreased in the samples of BGP-15-treated animals, moreover the ratio of Ser16 PLB (to total PLB) increased. These findings correlate to our in vivo data, as we were able to demonstrate that the prolonged IVRT normalized in the BGP-15-treated animals.

Besides calcium homeostasis abnormalities, the two other mechanism contributing to diastolic dysfunction are alterations in the regulation of myofilament proteins; and changes in the extracellular matrix (ECM). Here, we performed Masson's trichrome staining which demonstrated fibrotic remodeling in the hearts of the HC group. In line with the above, at the molecular level we were able to demonstrate that BGP-15-treatment induces favorable changes in phosphorylation state and isoform composition of titin. We found that cardiac titin N2BA/N2B expression ratio significantly shifted towards the stiff N2B form, moreover, titin N2-Bus phosphorylation tended to decrease in the samples of HC animals.

In conclusion, both single-dose intravenous and chronic oral administration of BGP-15 improved diastolic dysfunction in a rabbit model of atherosclerosis and cardiac dysfunction, independent of vascular status. Decreased PDE5A expression with long-term treatment and in vitro inhibition of PDE1A and recovery of N2-Bus phosphorylation in titin isoforms have been demonstrated. Based on our results, the full scanning of the mechanism of action of BGP-15, a potential cardiological drug candidate with several protective effects, requires further investigation. In our research, we have demonstrated a beneficial effect in reducing diastolic dysfunction and a stronger effect in case of diabetic patients, suggesting that BGP-15 may become a deficient pharmacon for metabolic syndrome as well as diabetic heart failure.

6. Summary

The metabolic syndrome is a serious, potentially life-threatening disease, treating it could be a lifelong process. The currently available therapies are limited, both in active agents, and overall effectiveness. During our research, we focused on BGP 15, a drug candidate already past the second clinical trial phase, which has numerous, already proven beneficial effects. In our first experimental setup, we verified BGP 15's negative ionotropic effect on human trabecules we acquired from open heart surgeries. This effect is highly dependent on the previous isoproterenol precontraction (indirect group), it was only independent in the case of higher concentration. Our significant achievement is that the negative ionotropic effect of BGP-15 showed connection to the existing diabetical state. The sample donors suffering from diabetes mellitus showed a significantly stronger positive ionotropic reaction to isoproterenol, and BGP-15 had a stronger negative ionotropic effect on these samples. In the second experimental setup, we used a hypercholesterolemic rabbit model to study the short (single i.v. bolus) and long term (p.o. for 16 weeks) effect of BGP-15 on the cardiac functions. Based on our experiences, BGP 15 improved diastolic functions, while having no effect on the vascular NO release. We validated BGP-15's cardiac effects with functional testing, and to understand the processes in the background, we conducted molecular biological tests. Based on the results, BGP-15 restored the titin isoforms, and increased the ratio of Ser16 phospholambane. It reduced the total phospholambane and increased cGMP level, which through PKG pathway plays an important role in the pathophysiology of heart failure. We proved the blocking effect of BGP-15 on the PEDA5A in vivo and on the PDE1 in vitro. Our studies prove multiple diastolic parameter of the heart failure could be improved by BGP-15, and these effects are expressed stronger on a diabetic heart. Given the seriousness of the metabolic syndrome as a cluster of metabolic abnormalities and the limitations of the available therapies, focusing on BGP-15 in future studies is something worth considering.

7. List of publications

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List of publications related to the dissertation

1. Priksz, D., **Lampé, N.**, Kovács, Á., Herwig, M., Bombicz, M., Varga, B., Wilisicz, T., Szilvássy, J., Pósa, A., Kiss, R., Gesztelyi, R., Ráduly, A. P., Szekeres, R., Sieme, M., Papp, Z., Tóth, A., Hamdani, N., Szilvássy, Z., Juhász, B.: Nicotinic-acid derivative BGP-15 improves diastolic function in a rabbit model of atherosclerotic cardiomyopathy.
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   J. Clin. Med. 9 (5), 1-18, 2020.
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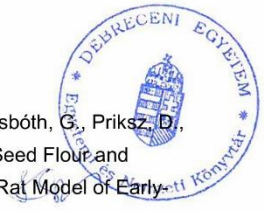

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