DOI 10.1007/s11094-019-02043-7
Pharmaceutical Chemistry Journal, Vol. 53, No. 7, October, 2019 (Russian Original Vol. 53, No. 7, August, 2019)

MOLECULAR BIOLOGICAL PROBLEMS OF DRUG DESIGN AND MECHANISM OF DRUG ACTION

A LOW MOLECULAR WEIGHT NERVE GROWTH FACTOR (NGF) MIMETIC GIVEN PER OS INCREASES THE SURVIVAL OF PANCREATIC β-CELLS IN A STREPTOZOTOCIN MODEL OF DIABETES

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Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 53, No. 7, pp. 3 – 6, July, 2019.

Original article submitted January 20, 2018.

We present here results of studies of the antidiabetic activity of compound GK-2 (*bis*-(N-monosuccinyl-glutamyl-L-lysine) hexamethylenediamide), an NGF mimetic, in a model of streptozotocin-induced type 2 diabetes mellitus in Wistar rats. Two-week prophylactic courses of oral GK-2 did not decrease blood glucose levels in healthy animals but reduces the severity of hyperglycemia and eliminated the insulin resistance effect induced by streptozotocin. Morphological analysis of the pancreas of the animals using monoclonal antibodies to insulin showed that while streptozotocin decreased the number of insulin-producing cells in the pancreas, GK-2 produced a statistically significant reduction in this harmful effect and promoted recovery of pancreatic islet size. A strong correlation was found between the extent of the cytoprotective action as indicated by morphometric measures and the strength of the hypoglycemic effect.

Keywords: diabetes mellitus, GK-2, NGF, streptozotocin, insulin resistance.

Researchers in the Department of Drug Chemistry, V. V. Zakusov Science Research Institute of Pharmacology have used the β-fold in loop 4 of NGF to construct a dipeptide mimetic with the working code GK-2 [1] [Russian Federation patent No. 2410392, 2010; US patent 9,683,014 B2,2017; CN patent 102365294 B,2016,19].

GK-2 has marked neuroprotective activity in in vitro experiments on immortalized and primary cell cultures and in vivo in various models of Alzheimer's disease, as well as in focal and global cerebral ischemia and hemorrhagic stroke [2].

In accordance with the previously proposed view that the physiological and pathological processes occurring in neurons and pancreatic β-cells are similar [3], there is value in identifying the antidiabetic activity of substances with neuroprotective actions.

Previous studies in rats [4, 5] and mice [6] have shown that the antihyperglycemic activity of GK-2 is apparent in a model of type 2 diabetes mellitus (DM2) induced by streptozotocin (STZ). The aim of the present work was to study the effect of GK-2 in conditions of prophylactic oral administration with assessment of measures of insulin resistance (IR) and morphometric analysis of the state of the insulin-producing apparatus of the pancreas.

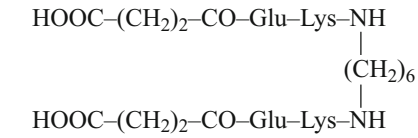

Fig. 1. Formula of NGH loop 4 mimetic GK-2.

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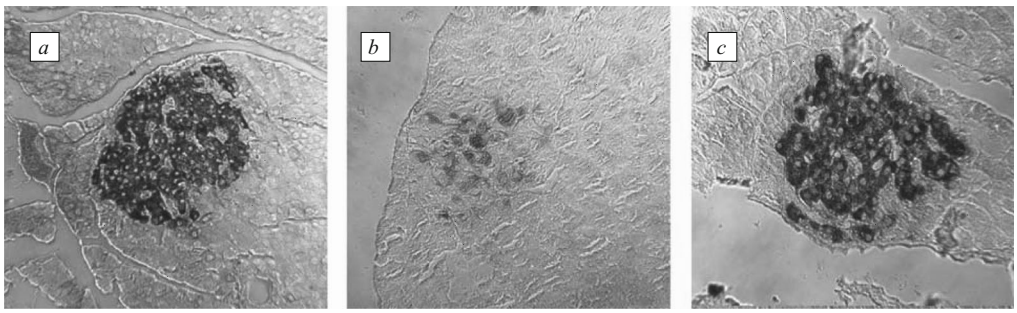

Fig. 2. Pancreatic islets from animals of different groups: *a*) passive control; *b*) active control; *c*) rats given prophylactic p.o. GK-2. Magnification × 1600.

EXPERIMENTAL PHARMACOLOGICAL SECTION

Experiments were performed using adult male Wistar rats ($n = 40$) with starting body weight 250 – 270 g obtained from Stolbovaya. Animals had free access to feed (except for the 16 h preceding administration of STZ) and drinking water. Animals were kept in accordance with standard SP 2.2.1.3218-14 of August 29, 2014, No. 51. The experiments were approved by the Biomedical Ethics Committee of the V. V. Zakusov Science Research Institute of Pharmacology (protocol No. 6 of April 16, 2018).

The diabetogenic toxin was STZ (Sigma, USA). Dipeptide GK-2 (*bis*-(N-monosuccinyl-glutamyl-L-lysine) hexamethylenediamide) ($T_m = 120 – 128°C$; $[\alpha]_D^{25} = -44.0°$ (c, 0.1, H_2O)) (Fig. 1) was synthesized at the Department of Chemistry, V. V. Zakusov Science Research Institute of Pharmacology, Russian Academy of Medical Sciences, using a method as described previously [7].

Experimental design. DM2 was modeled by single i.p. injections of freshly prepared STZ solution in cold citrate buffer pH 4.5 (45 mg/kg). This dose was selected to model DM2 on the basis of a previously demonstrated 48% reduction in the blood insulin level and survival of 30% of viable β-cells in the pancreas [8].

Rats were randomized to three groups: 1) rats of the passive control group ($n = 12$) received physiological saline (PS) 2 ml/kg orally for 14 days followed by a single i.p. dose of citrate buffer and, for the last seven days, p.o. PS; 2) the active control group ($n = 14$) received p.o. PS (2 ml/kg) for 14 days, followed by STZ, and, on the last seven days, p.o. PS; 3) rats of the experimental group ($n = 14$) received GK-2 dissolved in PS p.o. (5 mg/kg) for the 14 days preceding administration of STZ and the last seven days during development of DM2.

Blood (tail vein) glucose was assayed using a One Touch Ultra kit (USA) at two time points: at the end of the 14 days of prophylactic administration (to identify any possible hypoglycemic effect of GK-2 in healthy animals) and seven days after administration of STZ to assess the hypoglycemic activity of GK-2 and IR.

Insulin tolerance test. Access to food was restricted 18 h before the test, with continuing free access to water. All animals received s.c. insulin at a dose of 0.4 IU/kg. Blood samples were collected from the tail vein immediately before (0) and 45 and 90 min after administration of insulin. Animals were sacrificed by decapitation after this test.

TABLE 1. Levels of Glycemia in the Insulin Tolerance Test

No	Group	Glucose level, mM ($M \pm m$)		
		0 min (baseline)	45 min	90 min
1	Passive control	4.3 ± 0.1	2.0 ± 0.1 (– 53.5 %)	3.3 ± 0.2 (– 23.3 %)
2	Active control	13.5 ± 2.2*	10.9 ± 1.7* (– 19.3 %)	10.0 ± 1.9* (– 25.9 %)
3	p.o. GK-2	6.6 ± 0.5#	3.0 ± 0.2# (– 54.5 %)	3.0 ± 0.2# (– 54.5 %)

* Significant differences between the passive control and active control groups ($p < 0.05$).
# Significant differences between the experimental group and the active control group ($p < 0.05$).

TABLE 2. Morphometric Parameters of Pancreatic Islets in Male Wistar Rats

No	Group	Total area of β-cells		Mean islet area, μm²
		absolute, μm²	% of section	
1	Passive control	238149 ± 21554	22.2 ± 2.6	19292 ± 2813
2	Active control	77244 ± 16899*	5.9 ± 1.6*	7256 ± 2012*
3	GK-2	183831 ± 12906#	16.1 ± 1.0#	13755 ± 2050#

* Significant differences between the passive control and active control groups ($p < 0.05$).
# Significant differences between the experimental group and the active control group ($p < 0.05$).

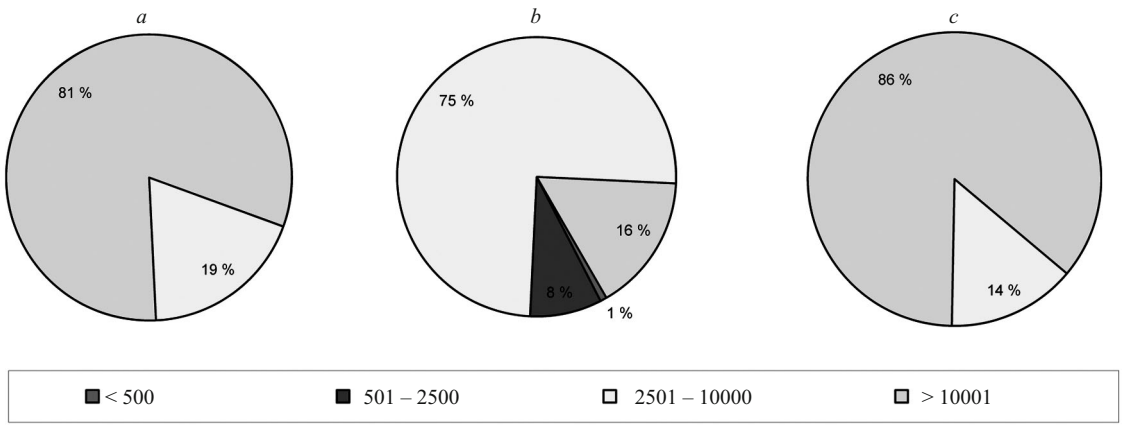

Fig. 3. Proportions of β-cell islets of different sizes, μm^2: *a*) passive controls; *b*) active controls; *c*) rats given prophylactic p.o. GK-2.

Immunohistochemical analysis of pancreatic islets. Rat pancreases were fixed in 10% neutral formalin (pH 7.4) (Sigma, USA), dehydrated, and embedded in paraffin blocks. Sections of thickness 5 μm were prepared using a microtome (Jung RM2035 Microtome, Germany). β-cells were identified using primary (guinea-pig anti-insulin 1:500, Abcam, UK) and secondary (anti-guinea pig 1:500, Abcam, UK) monoclonal antibodies labeled with peroxidase. Phosphate buffer PBS (Sigma, USA) was used as the medium for washing and applying antibodies. Detection was with a DAB Vector Peroxidase reagent kit (USA).

Microscopic analysis. Morphometric studies were run using an Aristoplan microscope (Leitz, Germany) with a DCM-800 digital camera (Mikromed, Russia), a personal computer, and ScopePhoto software, at a magnification of x1600. The number and density of β-cells were determined in absolute units, along with the ratio of β-cell area to the total area of microscope preparations in the field of vision. The resulting photomicrographs were processed in ImageJ v. 1.52 to determine the mean staining intensity of islets, which correlates with β-cell insulin content.

Published data on the heterogeneity of the reactions of pancreatic islets of different sizes to the harmful effects of STZ [9] led us to carry out a differential analysis of islets by area with determination of the percentages of islets of each size range (<500 μm^2, 501 – 2500 μm^2, 2501 – 10,000 μm^2, >10,001 μm^2) among the total number.

Results were analyzed statistically in Biostat. The nature of the distribution of the data was assessed using the Shapiro-Wilks test. As data distributions were normal, the statistical significance of between-group differences was identified by ANOVA. Arithmetic means M were computed along with standard errors of arithmetic means SEM. Differences between mean values were regarded as significant at $p < 0.05$. Interactions between quantitative parameters were assessed by Spearman rank correlation.

RESULTS AND DISCUSSION

Administration of GK-2 to healthy animals for 14 days did not induce any change in the blood glucose level, which was 5.4 ± 0.3 mM in the passive control group, 5.6 ± 0.2 mM in the active control group, and 5.4 ± 0.2 mM after administration of GK-2, which is evidence that GK-2 had no intrinsic hypoglycemic effect.

The insulin tolerance test was run on experimental day 21 (Table 1). Glucose levels in healthy (passive control) rats decreased by 53.5% in response to administration of insulin and recovered quickly. In the active control group, the response to insulin was weak (mean 19.4% decrease), i.e., the IR effect appeared. Prophylactic administration of GK-2 prevented the development of IR, such that the insulin response wasimilar to that in healthy animals (54.5% decrease in glycemia).

Results from morphometric analysis of the pancreas (Table 2) provided evidence of decreases in the absolute and relative numbers of β-cells in the group of untreated animals (active controls). Islets in untreated animals with DM2 were characterized by decreased numbers of insulinocytes, changes in cell shape, and dystrophic changes (Fig. 2*b*). Prophylactic p.o. administration of GK-2 to rats led to significant increases in the absolute and relative numbers of β-cells and islet area, along with recovery of the morphological characteristics of β-cells (Fig. 2*c*). Morphometry data showed good correlation with measures of glycemia: the correlation coefficient between the glucose level and the total area of pancreatic β-cells was 0.863 and that between the glucocorticoid level and the percentage content of β-cells on sections was 0.899.

Quantitative assessment of the staining intensity of insulin-producing β-cells was performed by evaluating the photomicrograph density using ImageJ v. 1.45. Images from the passive and active control groups had values of $169,617 \pm 21,697$ and $101,402 \pm 5,581$ respectively (the dif-

ference between the active and passive control groups was significant, $p < 0.05$). In the group of rats with prophylactic administration of GK-2, this measure increased to 132,133 ± 1,653 units (the difference between the active control group and the experimental group was significant, $p < 0.05$). Thus, analysis of islet staining intensity confirmed the protective effect of GK-2 in relation to insulin-producing pancreatic cells. These results are consistent with data from measurements of islet size. These studies showed that while the group of healthy animals was dominated by large islets (the proportion of islets of greater than 10,001 μm^2 was 81%), untreated animals with DM2 were dominated by small islets (Fig. 3). Prophylactic p.o. GK-2 completely restored the ratio of islets of different sizes to the level seen in the passive control group. Feng, who studied the effects of the CD117 factor on β-cell proliferation in islets of different sizes [9], took the view that the increase in the relative number of large islets is evidence of weakening of the process of apoptosis.

Overall, the data obtained here provide evidence that GK-2 does not have hypoglycemic activity in healthy animals but is able to reduce the level of glycemia in rats with DM2 and to eliminate the insulin resistance typical of DM2. Data from immunohistochemical analysis of the pancreas of the animals provided evidence that GK-2 has cytoprotective actions in relation to insulin-producing pancreatic β-cells. Strong correlations were found between morphometric parameters and the blood glucose level. It is important to emphasize that GK-2 has the effects described above when given p.o., which is the most appropriate for the prophylaxis of the manifestations of DM2. In this regard, dimeric NGF mimetic GK-2 differs fundamentally from peptides of more complex structure, for example, glucagon-like peptide GLP-1 agonists, which have antidiabetic activity only when given s.c. or i.v. [10].

Thus, as follows from the data obtained here, effective neuroprotective compound GK-2 can also protect pancreatic β-cells. The search for antidiabetic substances among orally active neurotrophin mimetics with cytoprotective actions is consistent with current views on the suitability of developing substances preventing the progressive death of β-cells during the development of DM2 [11].

This study was supported under state contract for 2019 – 2020, Theme No. 0521-2019-0003 “Seeking pharmacological means for selective activation of the signal transduction pathways of tyrosine kinase receptors as the basis for creating drugs without the side effects of native neurotrophins.”

REFERENCES

1. T. A. Gudasheva, T. A. Antipova, and S. B. Seredenin, *Dokl. Akad. Nauk*, **434**(4), 549 – 552 (2010).
2. S. B. Seredenin and T. A. Gudasheva, *Zh. Nevrol. Psikhiat. im. S. S. Korsakova,* No. 6, 63 – 70 (2015).
3. R. U. Ostrovskaya, S. V. Ivanov, and I. V. Ozerova, *Éksperim. Klin. Farmakol.*, **80**(9), 20 – 28 (2017).
4. P. Yu. Povarnina, T. A. Gudasheva, O. N. Vorontsova, et al., *Byul. Éksperim. Biol. Med.*, **151**(6), 634 – 637 (2011).
5. S. V. Ivanov, R. U. Ostrovskaya, I. V. Ozerova, and N. N. Zolotov, *Proceedings of the V Russian Congress of Pharmacologists, “Scientific Basis of the Search for and Creation of New Drugs”* [in Russian], Yaroslavl (2018), p. 96.
6. R. U. Ostrovskaya, S. S. Yagubova, T. A. Gudasheva, and S. B. Seredenin, *Acta Naturae*, **9**(33), 94 – 102 (2017).
7. N. M. Sazonova, A. V. Tarasyuk, D. V. Kurilov, et al., *Khim.-Farm. Zh.*, **49**(7), 10 – 19 (2015); *Pharm. Chem. J.*, **49**(7), 439 – 448 (2015).
8. R. U. Ostrovskaya, N. N. Zolotov, I. V. Ozerova, et al., *Bul. Exp. Biol. Med.*, **157**(3), 344 – 349 (2014).
9. Z. C. Feng, L. Donnelly, J. Li, et al., *Lab. Invest.*, **92**(4), 543 – 555 (2012).
10. A. J. Scheen, *Expert Opin. Biol. Ther.*, **17**(4), 485 – 496 (2017).
11. I. Song, C. Muller, J. Louw, and L. Bouwens, *Cur. Drug Targets*, **16**(5), 516 – 524 (2015).