Herbal medicine of Kursi Wufarikun Ziyabit inhibits mitochondrial ATP production to activate AMPK in hepatocytes in therapy of type 2 diabetes

Mireguli Yasen ^{1#}, Lan Jiang ^{1#}, Mizhgona Sharofova ², Xuelei Xin ¹, Yusuf Nuraliev ³, Jianping Ye ^{4*}, and Haji Akber Aisa ^{1*}

¹ Key Laboratory of Chemistry of Plant Resources in Arid Regions, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Science, Urumqi, Xinjiang 830011, China.

² Research Center for Innovative Technologies, Academy of Sciences of Tajikistan, Dushanbe 734063, Tajikistan.

³ Institute of Avicenna's Medicine and Pharmacology, Academy of the Tajik Traditional Medicine, Dushanbe 734062, Tajikistan

⁴ Shanghai Diabetes Institute, Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai, China.

***Correspondence:** Haji Akber Aisa (haji@ms.xjb.ac.cn), Jianping Ye (yejianping@sjtu.edu.cn)

These authors contribute equally.

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Abstract

The herbal medicine of Kursi Wufarikun Ziyabit contains two herbs (*Geranium collinum* Steph.ex Willd and *Hypericum Scabrum* Lnn) in the formula of ethnomedicine. It is usually used in the treatment of diabetes mellitus with a significant therapeutic efficacy. However, the molecular mechanism remains unknown for the action of medicine. In this study, we investigated the mechanism with a focus on an extract (GC30) of the medicine in the regulation of energy metabolism in hepatocytes. GC30 was prepared from a crude extract of the medicine through elution of an AB-8 macro porous resin column loaded with the medicine in 30% ethanol. GC30 exhibited an activity in the inhibition of triglyceride (TG) accumulation in the mouse hepatocytes through a suppression of SREBP1c activity. FGF21 (fibroblast growth factor 21) expression was induced by GC30 in a dosage-dependent manner at concentrations of 25 - 100 µg/ml. The induction was observed in mRNA and protein of FGF21, which were peaked at 2 hours and lasted for 8 hours in the response to GC30 (100 µg/ml). The transcription of FGF21 gene was induced by GC30 for an increase in the FGF21 gene promoter activity. AMPK and PKA activities were induced by GC30 with an elevation in their phosphorylation status, which were associated with a reduction in ATP abundance and an increase in CREB phosphorylation in cells treated with GC30. Oxygen consumption of mitochondria was inhibited in the hepatocytes by GC30. These activities of GC30 were similar to those of diabetes medicines including metformin and berberine. The data suggest that GC30 inhibited ATP production in mitochondria to activate AMPK and PKA in the hepatocytes to induce FGF21 expression. This study suggests a novel activity of the herbal medicine in the regulation of glucose and lipid metabolism in the hepatocytes.

24 **Introduction**

25 Herbal medicines represent a resource of natural compounds in the control of blood glucose
 26 in the treatment of diabetes ^[1]. Folk medicines provide thousands of drug candidates with
 27 activities in the control of diabetes. In this case, a formula herbal medicine by the name of
 28 Kursi Wufarikun Ziyabit is an ethnomedicine from Avicenna's "Canon of Medicine", which
 29 has a long history in the treatment of diabetes mellitus. The formula contains two different
 30 herbs, *Geranium collinum* Steph.ex Willd and *Hypericum Scabrum* Lnn. They are common
 31 herbs in the countries of Central Asia. *Geranium collinum* is a perennial plant that belongs to
 32 the family of Geraniaceae, which grows in the tropical and subtropical areas with 810 species
 33 in 8 genera in the world. Decoction of the *Geranium collinum* roots has a record in the
 34 treatment of the gastric diseases, postpartum bleeding, and hemoptysis as an astringent. In
 35 China, Tajikistan and Mongolia, various types of geranium are used in the treatment of
 36 hepatic and gastrointestinal diseases in the folk medicines. The water extracts of *Geranium*
 37 *collinum* roots are also used in the treatment of inflammation-related diseases, such as
 38 rheumatism and gout. Aqueous extract of the aerial part of *Geranium collinum* (from the
 39 flowers and leaves) are widely used in the treatment of eye diseases in Tibetan and
 40 Mongolian traditional medicine. The alcohol extract of the aerial part of *Geranium collinum*
 41 has a potent anti-inflammatory effect in the treatment of pneumonia, catarrhal symptoms of
 42 stomach and intestines. *Geranium collinum* contains phenols (pyrogallol, catechol), saponins,
 43 tannins, carbohydrates (monosaccharides, sucrose, glucose, fructose, sorbose, rhamnose,
 44 arabinose, xylose, ribose, maltose, starch, hemicellulose), vitamin C, flavonoids (avikulyarin,
 45 quercetin, gvyaverin, kaempferol, etc.). The molecular basis of the Kursi Wufarikun Ziyabit

46 activity remains unknown in the treatment of diabetes mellitus. In this study, we addressed
47 this issue by testing the effect of Kursi Wufarikun Ziyabit on hepatocytes.

48

49 AMP-activated kinase (AMPK) is a molecular target in the control of blood glucose in the
50 type 2 diabetes ^[2]. AMPK is activated in the liver by the diabetic medicines, such as
51 metformin and berberine, to reduce the fasting blood glucose. The mechanism is related to
52 inhibition of gluconeogenesis in hepatocytes to decrease glucose production by the liver ^[3].
53 AMPK suppresses transcription-dependent expression of glucogenic genes by targeting the
54 transcription factors (FOXO1 and CREB, etc.) ^[2]. AMPK inhibits de novo lipogenesis
55 through a similar mechanism by targeting expression or phosphorylation of lipogenic proteins
56 (SREBP and ACC, etc.) ^[2]. A reduction in AMPK activity leads to production of glucose by
57 liver, which represents a mechanism for the hyperglycemia in type 2 diabetes. Activation of
58 AMPK is a molecular mechanism for the therapeutic activities of metformin and berberine in
59 the treatment of type 2 diabetes ^[4]. However, the activity of Kursi Wufarikun Ziyabit remains
60 unknown in the regulation of AMPK.

61

62 FGF21 is a fasting-induced hepatokine that promotes energy expenditure through an action in
63 multiple peripheral tissues including adipose tissue ^[5, 6]. Blood FGF21 is predominantly
64 secreted by the liver although other tissues including adipose tissue, pancreas, and skeletal
65 muscle also produce FGF21 ^[7, 8]. FGF21 induces beige cell functions through activation the
66 transcription factor PGC-1 ^[5]. The FGF21 action is mediated by a FGF receptor complex
67 containing β -klotho. Administration of recombinant FGF21 or transgenic overexpression of

68 FGF21 in mice provides a protection against diet-induced obesity and glucose disorders [9].

69 The activity of Kursi Wufarikun Ziyabit is unknown in the regulation of FGF21 expression.

70

71 In this study, we report that GC30 fraction of Kursi Wufarikun Ziyabit inhibited hepatic

72 steatosis and induced FGF21 expression in hepatocytes. The mechanism was associated with

73 an activation of AMPK through inhibition of ATP production in mitochondria. The study

74 provides a cellular and molecular mechanism for the pharmacological activities of Kursi

75 Wufarikun Ziyabit in the treatment of diabetes.

76 **Methods**

77 *Plant material*

78 The two herbs *Geranium collinum* Steph and *Hypericum scabrum* Lnn. were collected from

79 the Botanical Garden, Academy of Sciences of the Republic of Tajikistan (38.5357500 N,

80 68.7790500 E and 767 m above sea level, Dushanbe, Tajikistan) in June 2016. The plants

81 were identified by Qurbonov Mansur. A voucher specimen has been deposited in Xinjiang

82 Technical Institute of Physics and Chemistry, Urumqi, Chinese Academy of Sciences.

83 *Preparation of GC30 extract*

84 The medicine of Kursi Wufarikun Ziyabit was formed with *Geranium collinum* Steph (35g)

85 and *Hypericum scabrum* Lnn. (15g) in the formula of this study. The herbs were grinded into

86 powders individually and mixed at the ratio 2.3:1 as a powder mixture. The mixture was

87 extracted with 50% ethanol at 70 °C. The GC30 extract was obtained from a 50% ethanol

88 extract of the powder mixture following a purification process on an AB-8 macro porous

89 resin column, which was washed successively with distilled water (1BV), 30% ethanol (2BV),

90 and 70% ethanol (3BV). The eluents of 30% and 70% ethanol were concentrated under
91 reduced pressure and named as named GC30 and GC70, respectively. The compounds in
92 GC30 were determined as described in supplementary material.

93 *Cell culture and reagents*

94 The mouse hepatocyte cell line Hepa-1c1c7 (1c1c7 in this study) and HepG2 cells were
95 purchased from the American Type Culture Collection (ATCC) (Manassas, VA 20110) and
96 maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal
97 bovine serum at 37 °C in a 5% CO₂ incubator. Hepatic steatosis was established in HepG2
98 cells with a treatment by oleic acid (200μM) for 72 hr. The cells were treated with GC30 after
99 serum-starvation in DMEM medium containing 0.25% fatty acid-free bovine serum albumin
100 overnight. Berberine and metformin were used as positive controls in this study. Berberine
101 (BBR, 200275,), Metformin (Met, PHR1084, Sigma-Aldrich), Geraniin (GN, E-0742, Tauto
102 Biotech) and Ellagic acid (EA, E-0654, Tauto Biotech) were purchased from the
103 corresponding commercial sources.

104 *Western blot*

105 FGF21 Protein and AMPK phosphorylation were determined in Western blot as described
106 elsewhere ^[10]. The whole cell lysate was prepared in a lysis buffer with sonication as
107 described elsewhere ^[10]. Antibodies to FGF21 (ab171941) and actin (ab6276) were from
108 Abcam (Cambridge, MA). Antibodies to SREBP1c (sc-8984, Santa Cruz Biotech., USA),
109 pAMPKα (Thr172, #2531, Cell Signaling Biotech., USA) and AMPKα (sc-398861, Santa
110 Cruz Biotech., USA) were purchased from the commercial sources.

111 *mRNA expression*

FGF21 mRNA was determined in cells using qRT-PCR. Total RNA was prepared from the cells using TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA). *Fgf21* mRNA was determined in mouse 1c1c7 cells with the SYBR green primer (forward 5-CCTCTAGGTTTCTTTGCCAACAG-3; reverse 5-AAGCTGCAGGCCTCAGGAT-3) ^[11]. Target mRNA was normalized to ribosome 18S RNA in qRT-PCR. SYBR Green Master Mix (4309155, Applied Biosystems) was used in qRT-PCR with the ABI 7900 machine.

Lipids assay

Cells were lysed in PBS (1 g: 20 mL). The lipids were extracted from the lysate using a chloroform/methanol (2:1) mixture ^[12]. TG and glycerol were determined using the Serum Triglyceride Determination Kit (TR0100; Sigma-Aldrich). Oil red O staining was performed in HepG2 cells using a protocol described elsewhere ^[13].

Mitochondria and ATP assays

Mitochondrial activity was determined in response to GC30 treatment by monitoring the oxygen consumption rate (OCR) using a XF24 Analyzer (Seahorse Bioscience Inc., North Billerica, MA). OCR was determined in the cells following 1 h pretreatment by GC30 in the serum-free DMEM medium. The assay was conducted in the XF base medium (Cat # 102353-100, Agilent Seahorse XF) supplemented with glucose (25 mM), pyruvate (1 mM) and glutamine (4 mM) to fuel the cells. In the control, berberine (10 µM) was used as a positive control for mitochondrial inhibitor. Following chemicals were used at a final concentration in the assay to modulate the respiratory chain: oxidative phosphorylation uncoupler (Carbonyl cyanide 4-trifluoromethoxy phenylhydrazone, FCCP, 2 µM), the complex V inhibitor (Oligomycin, 1 µM), complex II and III inhibitor (Antimycin A, 0.5 µM)

and complex I inhibitor (Rotenone 0.5 μ M). ATP production by mitochondria was determined in two ways. The first was a luciferin-based ATP assay (ATP Determination Kit, Life technologies, Eugene, Oregon, USA) as described elsewhere ^[14] and the second was determined by the value of OCR change following GC30 treatment.

FGF21 gene reporter assay

A FGF21 luciferase reporter (0.2 mg DNA per well) was transfected into HepG2 cells in a 24-well plate (4 x 10⁵ cells per well) using Lipofectamine 2000 (Cat #11668027, ThermoFisher Scientific, Waltham, MA, USA). In the reporter vector, the luciferase gene was under control of a mouse FGF21 gene promoter (-1497/+5) as described elsewhere ^[15]. The reporter activity was determined using the dual luciferase substrate system (E1910; Promega, Madison, WI, USA), and normalized with the Renilla luciferase. Each experiment was repeated three times with consistent results.

Statistical analysis

In this study, the data were presented as the mean $\pm$ SEM. The experiments were repeated at least three times with consistent results. The representative immunoblotting is presented with quantification of three blots. Student's T test was used in the data analysis with significance at $p < 0.05$.

Results

Inhibition of TG accumulation in hepatocytes by GC30

To study the metabolic activity of herbal medicine of Kursi Wufarikun Ziyabit, GC30 was extracted from the powder of herb medicine and tested in hepatocytes. In the study, a hepatic

steatosis model was established in the mouse 1c1c7 hepatocyte cell line by treatment of the cells with oleic acid for 72 hr (OA, 200 μ M) (Fig. 1A). The TG abundance was quantified in the hepatocytes using the oil red O staining and a TG assay kit. In this model, treatment of the cells with GC30 during the induction of steatosis led to a significant reduction in the TG content, and the activity was comparable to the berberine activity (Fig. 1, A-C). Berberine was used as a positive control in the inhibition of TG accumulation^[16]. GC30 was used at 50 μ g/ml in this study. When GC30 was applied to the cells after establishment of hepatic steatosis, the GC30 activity was not observed in the reduction of TG content (Fig. 1D), suggesting that GC30 inhibits lipogenesis and does not induce lipolysis. BBR also failed to reduce TG in the established hepatic steatosis (Fig. 1D). These data suggest that GC30 inhibits TG accumulation in hepatocytes, and the mechanism is related to the inhibition of TG synthesis. The GC30 activity is comparable to that of BBR.

Inhibition of SREBP1c protein by GC30

In the hepatocytes, the transcription factor SREBP1c is one of the major driving forces in lipogenesis of hepatic steatosis. SREBP1c induces expression of multiple lipogenic enzymes through gene transcription in the induction of hepatic steatosis. SREBP1c protein was examined in the GC30-treated cells by Western blotting to understand the mechanism of lipogenesis inhibition. In a 24 hr study, SREBP1c was decreased in the cells by GC30 in a time-dependent manner (Fig. 2). A significant reduction was observed after 16 hr of treatment. In the control, BBR also reduced SREBP1c protein in the model (Fig. 2). The data suggest that GC30 inhibits SREBP1c activity in hepatocytes to suppress lipogenesis in the hepatic steatosis model.

Induction of FGF21 expression by GC30

FGF21 is a major endocrine factor from liver with an activity in the regulation of whole-body energy metabolism. FGF21 was examined in this study to determine GC30 activity in the regulation of endocrine function of hepatocytes. FGF21 protein was examined in the cell lysate by Western blotting. The protein was induced by GC30 in a dose-dependent manner and the highest activity was observed at dosages of 50 - 100 µg/ml (Fig. 3A). In the time-dependent study, FGF21 was induced by GC30 during 1 - 24 hr. The induction was peaked at 4 hr (Fig. 3B). These results suggest that GC30 may induce FGF21 expression in hepatocytes.

Induction of FGF21 gene promoter by GC30

FGF21 expression is regulated at the transcriptional level. mRNA and the gene promoter were examined to understand the mechanism of GC30 activity. An increase in mRNA was observed in FGF21 in response to GC30 treatment in dose- and time-dependent manners (Fig. 4, A and B). The gene promoter activity was examined using a luciferase reporter controlled by the FGF21 promoter DNA ^[15]. The reporter activity was induced by GC30 with 200% elevation over the control (Fig. 4C). In the positive control, the reporter activity was induced by Forskolin through activation of cAMP/PKA pathway (Fig. 4D). The GC30 activity was examined in the regulation of cAMP/PKA pathway with phosphorylation of the transcription factor CREB. An increase was observed in the phosphorylation in GC30-treated cells (Fig. 4E). The GC30 activity was similar to that of Forskolin (Fig. 4E). These results suggest that GC30 may induce FGF21 transcription in hepatocytes through activation of the cAMP/PKA signaling pathway.

Activation of AMPK and inhibition of ATP by GC30

AMPK is an energy sensor in cell whose activity is induced by a reduction of the intracellular ATP level. AMPK activity was examined to understand the mechanism of GC30 activity in the inhibition of hepatic steatosis. GC30 activity was examined in the regulation of AMPK activity by the phosphorylation status (pAMPK) in the cells. The pAMPK signal was induced in the cells treated by GC30 (Fig. 5A). BBR and metformin were used as the positive controls in the activation of AMPK (Fig. 5A). The GC30 activity in the activation of AMPK was not as strong as those of BBR or Metformin. In the study, GC30 was compared with another extract of Kursi Wufarikun Ziyabit (GC70), and other compounds including GN (Geraniin) and EA (Ellagic acid). The pAMPK signal was induced by all of them (Fig. 5A). The data suggest that GC30 activated AMPK in the hepatocytes.

Intracellular ATP was determined in the cells to understand the mechanism of AMPK activation by GC30. The ATP content was decrease by GC30, which was similar to those of other compounds including GC70, GN, EA, BBR and Metformin (Fig. 5B). These data suggest that GC30 may activate AMPK through reduction of ATP level in the cells.

Inhibition of mitochondrial ATP production by GC30

Mitochondria are the major source of ATP in hepatocytes. Mitochondrial function was examined to understand the ATP reduction in hepatocytes by GC30. The oxygen consumption rate (OCR) is an indicator of mitochondrial function as the mitochondrial activity is dependent on oxygen. OCR was measured in the GC30-treated cells and a significant reduction was observed in the basal and maximal (uncoupling) conditions (Fig. 6A). The ATP production is catalyzed by ATP synthase in mitochondria. Inhibition of the enzyme by oligomycin leads to a decrease in OCR. The difference of OCR at the basal and oligomycin-

treated conditions represents the ATP production capacity in mitochondria. Oxygen is also used in the heat production (uncoupling) by mitochondria. In response to the chemical uncoupler FCCP, OCR is induced for the maximal capacity of oxygen consumption in mitochondria. The difference between the basal and maximal OCR represents the spare capacity of mitochondria. ATP production and spare capacity were all decreased in the hepatocytes by GC30 (Fig. 6B). In the study, BBR was used as the positive control. The GC30 activity was similar to that of BBR in this assay (Fig. 6B). These data suggest that GC30 inhibited ATP production by mitochondria in the hepatocytes.

Compound profile of GC30

GC30 was analyzed with liquid chromatograph-mass spectrometry to determine the compound profile. Twenty compounds were identified in GC30 according to the major peaks in the fingerprints of GC30. Those include Quercetin, Quercetin derivative and others. Quercetin provides a basis for certain activities of GC30 in the regulation of ATP production.

Discussion & Conclusions

Our data demonstrates that the GC30 extract of herbal medicine of Kursi Wufarikun Ziyabit is able to inhibit hepatic steatosis and induce FGF21 expression in hepatocytes. The medicine has been used for hundreds of years in the treatment of diabetes and liver diseases in forks medicines of the countries of Central Asia. However, the cellular and molecular mechanisms were not known for the therapeutic activities. To address this issue, we prepared the GC30 extract of Kursi Wufarikun Ziyabit, and tested its activity in the hepatocyte model. GC30 was found to have activities in the inhibition of development of hepatic steatosis and induction of FGF21 expression in the hepatocyte model. The data suggest that liver is a potential target of

the herbal medicine in the treatment of diabetes and liver disease. The mechanism is related to improvement of hepatocyte function by inhibition of steatosis and induction of the endocrine activity. These observations provide a cellular mechanism for the therapeutic activities of the herbal medicine.

Our data suggests that in hepatocytes, GC30 activated the AMPK and cAMP/PKA signaling pathways to generate the pharmacological activities. The AMPK pathway provides a mechanism for inhibition of hepatic steatosis. In cells, AMPK is activated by lack of fuel supply in the stress conditions. AMPK activation acts to restore the fuel supply in cells through an impact in multiple metabolic pathways ^[2]. Inhibition of the de novo lipogenic pathway by AMPK shifts energy metabolism from anabolism to catabolism, which includes induction of utilization of glucose and fatty acids in the production of ATP. AMPK activation by GC30 represents a molecular mechanism for the GC30 activity in the inhibition of hepatic steatosis. The cAMP/PKA pathway stimulates fuel mobilization and utilization in ATP production through stimulation of glycose and fatty acid oxidation in mitochondria. It also induces FGF21 expression in hepatocytes. Those activities support another molecular mechanism of the GC30 activity in the inhibition of hepatic steatosis. Although the cAMP/PKA pathway induces gluconeogenesis in hepatocytes, the activity may be offset by the activation of AMPK in the GC30-treated cells. AMPK is known to inhibit gluconeogenesis in the liver. Activation of the two pathways (AMPK and cAMP/PKA) provides two molecular mechanisms for the activities of GC30 in the regulation of hepatocyte metabolism.

266

267 GC30 exhibited an activity in the suppression of ATP production in mitochondria, which was
 268 similar to those of BBR and metformin. BBR and metformin are widely used in the control of
 269 blood glucose in the diabetic patients worldwide. They share an activity in the activation of
 270 the AMPK signaling pathway by inhibition of ATP production in mitochondria ^[17]. In current
 271 study, GC30 was found to reduce the intracellular level of ATP by suppression of
 272 mitochondrial oxygen consumption, which provides a mechanism for the activation of
 273 AMPK and cAMP/PKA pathways. Activation of the two pathways is likely a compensatory
 274 response to the ATP depletion in favor of ATP production. A similar reaction was reported in
 275 our previous study of BBR, which inhibited ATP production by mitochondria with a
 276 reduction in oxygen consumption ^[18]. Interestingly, such an activity was also reported for
 277 metformin by other group ^[19]. Consistently, ATP enhances de novo lipogenesis and
 278 gluconeogenesis in liver in the pathogenesis of insulin resistance ^[20, 21]. Inhibition of ATP
 279 production by an uncoupler (2,4 dinitrophenol, DNP) prevented insulin resistance in dietary
 280 obese rats ^[21]. Elevation of intracellular ATP promotes insulin resistance and inflammation in
 281 previous studies by us ^[14, 22]. Suppression of ATP production by mitochondria provides a
 282 cellular basis for the GC30 activities in the activation of AMPK in hepatocytes. The
 283 molecular target of GC30 remains to be identified in mitochondria.

284

285 The compound profile was examined in GC30 using the liquid chromatography-mass
 286 spectrometry (LC-MS) technology. Twenty compounds were identified in GC30, in which
 287 quercetin and its derivatives were found. Quercetin has an activity in the inhibition of

mitochondrial function to decrease ATP production ^[23], which may contribute to the GC30 activity in the regulation of AMPK. Quercetin inhibits lipogenesis in adipocytes ^[24], which is consistent with the GC30 activity in the inhibition of lipogenesis in hepatocytes.

In summary, we investigated the activity of herbal medicine of Kursi Wufarikun Ziyabit in the regulation of hepatocyte metabolism in this study. The study was conducted with the GC30 extract in hepatocytes. GC30 inhibited the development of hepatic steatosis and induced expression of FGF21 through a transcriptional mechanism in hepatocytes. The activities were associated with activation of the AMPK and cAMP/PKA pathways. GC30 inhibited ATP production in mitochondria to activate the two signaling pathways through ATP depletion. The GC30 activities are similar to those of BBR and metformin. Quercetin was identified in GC30 together with other 19 compounds to support the mechanism of GC30 activities. The study suggests that the herbal medicine may generate the therapeutic activities by targeting liver. However, this possibility remains to be tested in vivo.

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Author contribution: MY, LJ, XX, MS and YN conducted the experiments and analyzed the data. JY and HAA had the concept of the study, designed the experiments. JY wrote the

310 manuscript. HAA is the guarantor of this work and has full access to all the data in the study
311 and takes responsibility for the integrity of the data and accuracy of the data analysis. All
312 authors reviewed the results and approved the final version of the manuscript. There is NO
313 conflict of interest to disclose.
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LEGENDS

Fig. 1 Inhibition of TG accumulation by GC30 in HepG2 cells. HepG2 cells were treated with oleic acid (200 μ M) for 72 hours to induce TG accumulation in the hepatic steatosis model. GC30 was used at 50 μ g/ml in this study. A. TG detection with oil red O staining. Lipid droplets are indicated by the red color in the HepG2 cell cytoplasm following oil red O staining. GC30 was applied to the cells together with the oleic acid during induction of TG accumulation. B. TG quantification by OD of color change. OD was determined in the extract of hepatocytes for oil red O. C. TG quantification by the TG assay kit. TG was extracted and quantified with the TG assay kit. D. GC30 treatment of established steatosis. In the bar figure, the results are mean $\pm$ SE (n=3). * $p < 0.05$; # $p < 0.05$ over the control.

Fig. 2 Inhibition of SREBP1c protein expression in hepatocytes by GC30. SREBP1c protein was examined in HepG2 hepatocytes after GC30 (100 μ g/ml) or BBR (10 μ M) treatment by Western blot. The bar figure represents mean $\pm$ SE (n=3). *** $p < 0.001$ over the control.

Fig. 3 Induction of FGF21 expression in hepatocytes by GC30. FGF21 expression was examined in 1c1c7 hepatocytes after GC30 treatment. A. FGF21 protein expression. The whole cell lysate was used in the Western blot for FGF21 protein. The dose-dependent study was done with GC30 treatment for 4 hr. B. FGF21 protein in a time-course study. GC30 (100 μ g/ml) was used to treat the cells for 24 hr. The bar figure represents mean $\pm$ SE (n=3). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ over the control.

Fig. 4 Activation of FGF21 gene transcription by GC30. A. Induction of FGF21 mRNA by GC30 in the dose-dependent study. The FGF21 mRNA was determined in 1c1c7 cells

following a 2 hr treatment with different dosages of GC30. mRNA was determined using qRT-PCR. B. Induction of FGF21 mRNA by GC30 in the time-dependent study. The FGF21 mRNA was determined in 1c1c7 cells with GC30 (100 µg/ml) treatment over 24 hr. C. Induction of the FGF21 luciferase reporter by GC30. The FGF21 reporter was transiently transfected into 293 cells, and the cells were treated with GC30 (50 µg/ml) for 16 hr. D. Induction of the FGF21 gene promoter activity by Forskolin. The reporter activity was determined after Forskolin (10 µM) treatment of the transfected cells. E. Induction of CREB phosphorylation by GC30. The phosphorylation was determined in whole cell lysate by Western blotting after treatment of the cells with GC30 (50 µg/ml) and Forskolin (10 µM). In the bar figure, the results are mean ± SE (n=3). * p < 0.05; ** p < 0.01 ; *** p < 0.001 over the control.

Fig. 5 Induction of AMPK phosphorylation and inhibition of ATP by GC30. A. Induction of AMPK phosphorylation by GC30. The HepG2 cells were treated with GC30. The phosphorylation of AMPK was examined in the whole cell lysate at 60 mins after exposure to GC30 (50 µg/ml), GC70 (50 µg/ml), GN (10 µM), and EA (10 µM) by Western blotting. BBR (10 µM) and Met (10 µM) was in the positive controls as AMPK activators. B. Inhibition of ATP by GC30. ATP was determined in the whole cell lysate after GC30 treatment of HepG2 cells. The fold change of ATP is presented in the unit of µM/g protein. In the bar figure, the results are mean ± SE (n=3). * p < 0.05; ** p < 0.01 over the control.

Fig. 6 Inhibition of mitochondrial ATP production by GC30. A. Dynamic change of oxygen consumption rate (OCR) of mitochondria in response to GC30. OCR was determined in the HepG2 cells following GC30 (50 µg/ml) for 1 hr. In the positive control, BBR (10 µM)

360 was used. OCR was determined using the XF24 Analyzer over 110 minutes. B. ATP
 361 production. The value was a result of OCR difference before and after oligomycin treatment.
 362 C. Spare capacity of mitochondria. This was a result of OCR difference between the basal
 363 and maximal conditions. The results are mean $\pm$ SE (n=3). ** p < 0.01; ++ p < 0.01 relative to
 364 the control.

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Fig. 1 Inhibition of TG accumulation in hepatocytes by GC30

A. Oil red O staining

B. Quantification of oil red O

C. TG in cell lysate

D.

20X

Fig. 2 Inhibition of SREBP in hepatocytes by GC30

Fig. 3 Induction of FGF21 expression by GC30 in 1c1c7 cells

A. GC30 dose-dependent effect

B. GC30 time-dependent effect

Fig. 4 Induction of FGF21 transcription by GC30

Fig. 5 Induction of AMPK phosphorylation and inhibition of ATP by GC30

Fig. 6 Inhibition of mitochondrial ATP production by GC30

A. Oxygen consumption

B. ATP production

C. Spare capacity