Form 3

Report for Joint/Usage Research Program for Endocrine/Metabolism (Fiscal Year 2019)

Date: 2020/04/12

To Director of Institute for Molecular and Cellular Regulation, Gunma University

Principal Applicant	
Institution	International Center for Chemical and Biological Sciences (ICCBS), University of Karachi
Position	Associate Professor
Name	Dr. M. Hafizur Rahman

We report on the results of joint research in fiscal 2019 as below.

(**Program No. 18013**)

1. Research Title	**Exploring the Insulin Secretory Mechanisms of Hymecromone and Eupatorin *In vitro* and *In vivo***
2. Purpose and Significance of the research project	***Purpose***: The present study aims to identify potent and safer insulin secretagogues, particularly from natural sources. Recently, we identified two compounds, namely Hymecromone and Eupatorin; however, their mechanism(s) remain unknown. In this collaborative project, we sought to investigate these two lead compounds for further in-depth insulin secretory mechanisms in mice pancreatic islets and MIN6 cells. ***Significance of the research project***: Impaired insulin secretion is a key feature in the pathophysiology of non-obese type 2 diabetes, mostly found in Asia. Classical insulin secreting agents, such as sulfonylurea, stimulate insulin secretion irrespective of glucose concentrations which is the main cause for hypoglycemia as well as oxidative stress and excessive burden to exhausted islets in diabetic patients. Therefore, identification of new insulin secretagogues which could minimize the risk of hypoglycemia is of great interests. In this context, this collaboration is pursued to identify novel candidate compound that does not overload islets, protects β-cells and has significant glucose-dependent insulinotropic effects. The discovery of novel insulin secretagogue(s), particularly from natural sources with drug targets other than sulfonylureas may prove a better alternative insulin secretagogue to work in glucose-stimulated manner. The initial data of the project revealed few potential findings that show window of opportunity to address the unmet needs in current diabetes research. Furthermore, the project established a bridge between scientifically advanced country like Japan and scientifically lagging country like Pakistan. In summary, the project underlines important role in developing an anti-diabetic drug seed. Additionally, this will encourage young researchers to strengthen their research capacity in exploring different anti-diabetic drugs in future.
3. Period of The Program	April 1, 2019 ～ March 31, 2020
4. Project Members	

Name	Age	Gender	Institution/Department	Position	Role
(Principal Applicant) Dr. M. Hafizur Rahman	46	M	International Center for Chemical and Biological Sciences/ Dr. Panjwani Center for Molecular Medicine and Drug Research	Associate Professor	PI of the project, supervised and monitored all aspects of the project including Project conception to Data curations and analysis, result compilation and report preparation.
(Research Collaborators) Israr Khan	31	M	International Center for Chemical and Biological Sciences/ Dr. Panjwani Center for Molecular Medicine and Drug Research	Graduate student	Performed experiments under the supervision of PI.
Fariha Naz	27	F	International Center for Chemical and Biological Sciences/ Dr. Panjwani Center for Molecular Medicine and Drug Research	Graduate student	Performed experiments under the supervision of PI.

※If additional space is required, attach a separate sheet.

5. Collaborative Researcher of IMCR	Name of the Laboratory	Molecular Endocrinology and Metabolism	Name	Professor Tetsuro Izumi, MD, PhD

6. Research Plans

Eupatorin and Hymecromone were tested to explore their insulin secretory mechanisms in BALB/c and NMRI mice islets. Freshly isolated mice islets were incubated in different glucose concentrations in the presence of Hymecromone/Eupatorin with or without agonist/antagonist of K-ATP channel/Ca^{2+} channel/cAMP/PKA/Epac2 and secreted insulin was measured by ELISA kit. Research plans are following:

i) Toxicity studies of Eupatorin and Hymecromone in MIN6 cells.

ii) Insulin secretion activity by Eupatorin and Hymecromone, and their optimum dose selection for mechanistic study.

iii) Explore the role of Eupatorin and Hymecromone on K-ATP channel, Ca^{2+} channel, cAMP-PKA, cAMP-Epac2, PLC-PKC, and MEK-$ERK_{1/2}$ insulin secretory pathways.

iv) Roles of Eupatorin and Hymecromone on insulin secretion kinetics.

v) Data analysis, result compilation and report preparation.

Research plans i-iii has been performed by our research group. Research plan iv will be performed soon by Dr. Tetsuro Izumi research group at Institute for Molecular and Cellular Regulation (IMCR), Gunma University, Japan.

7. Research results:

We established a collaborative research project on Hymecromone (HCM) and Eupatorin (EPT) based on their roles in insulin secretion. Going forward, HCM and EPT were investigated for further in-depth insulinotropic mechanism(s) in mice islets and MIN6 cells, respectively. The insulin and cAMP contents were measured using ELISA kits. K^+-channel currents were recorded in MIN6 cells with the whole-cell patch-clamp technique. The *in vitro* findings were further evaluated by *in silico* docking studies. HCM and EPT were found non-toxic upto 400 μM in MIN6 cells. The dose-response data revealed that HCM showed optimum activity at 200 μM and EPT at 50 μM, respectively. Therefore, these doses were used for mechanistic studies.

Hymecromone (200 μM) only potentiated stimulated insulin secretion at 16.7 mM glucose and 25 mM glucose concentration in mice islets and MIN6 cells, respectively. At 16.7 mM glucose, HCM showed better insulin secretory activity than tolbutamide alone and an additive effect in tolbutamide-induced insulin secretion suggest that the insulin secretory mechanism of HCM is different from sulfonylurea. This was further justified when islets were depolarized with 25 mM KCl, HCM-mediated insulin secretion was enhanced even in the presence of diazoxide indicate that depolarization of islets facilitated Ca^{2+} influx to augment intracellular Ca^{2+}. Furthermore, verapamil, an L-type Ca^{2+} channel blocker, showed significant inhibition on HCM-induced insulin secretion. Subsequently, additive effect in IBMX- (a phosphodiesterase inhibitor) and forskolin (adenylate cyclase activator)-mediated insulin secretion was observed. The increased cAMP content was observed following HCM treatment. We used inhibitor for PKA (H-89) and Epac2 (MAY0132) alone and in combination. We found that at 16.7 mM glucose concentration, the HCM-induced insulin secretion was significantly inhibited in the presence of each H-89, MAY0132 and in combination, respectively. In case of PKC (calphostin-C) and $ERK_{1/2}$ (U-0126) inhibitors, HCM-mediated insulin secretion was not significantly inhibited.

For Eupatorin, we found that EPT (50 μM) potentiated insulin secretion at 16.7 mM glucose concentration and little to none effect at 3 mM glucose concentration. Comparing with tolbutamide, EPT showed higher stimulated insulin secretion at 16.7 mM and 25 mM glucose concentration in mice islets and MIN6 cells, respectively. Furthermore, EPT showed additive effects in tolbutamide-induced insulin secretion suggest that EPT works on downstream signaling pathways. In the presence of verapamil, an L-type Ca^{2+} channel blocker, EPT-induced insulin secretion was significantly inhibited at 16.7 mM glucose concentration. We observed that EPT exhibited additive effect in IBMX- and/or forskolin-induced insulin secretion, respectively. Furthermore, increased cAMP content was observed following EPT treatment. Additionally, H-89, a PKA inhibitor, and MAY0132, an Epac2 inhibitor showed significant inhibitory effect on EPT-induced insulin secretion at 16.7 mM glucose suggest that the effect of insulin secretion *per se* cAMP-dependent.

Taken all these data together, we conclude that Hymecromone and Eupatorin potentiate insulin secretion predominantly through cAMP signaling cascade, distal to the K-ATP channel coupled with stimulatory glucose; however, the conclusive target(s) is(are) still to be explored.

8. Publications and/or Presentations resulting from Joint Research Program with IMCR.

①Please describe a list of publications in which the name of the collaborative researcher of IMCR appears and send one paper reprints of each publication to IMCR.

Publication in progress

②Please describe a list of publications which include the description that the research is supported by Joint Research Program with IMCR and send one copy of each publication to IMCR.

Publication in progress

③Enter the name of the conference, the date of the conference, and the title of the presentation of the conference.(up to 3 cases)

Presentations resulting from Joint Research Program.

- Regarding the research achievements of Joint/Usage Research Project 18013, we have presented our Project data in several international scientific meetings. Following are the details.

[1]. An abstract of potential findings on Hymecromone in insulin secretion has been presented in 62nd Annual Meeting of Japan Diabetes Society held during May 23 – 25, 2019, Sendai, Japan. In this abstract, 3 authors from Dr. Izumi group have been included. Below is the title of the abstract and author's name.

Abstract title: Hymecromone potentiates glucose-stimulated insulin secretion through cAMP-PKA signaling pathway

Authors: Hafizur Rahman, M. Israr Khan, Abdul Hameed, Huma Aslam Bhatti, Muneeb Ali, Zaheer Ul-Haq, Faisal Khan, Ghulam Abbas, **Hao Wang**, **Kohichi Matsunaga**, **Tetsuro Izumi**

[2]. Eupatorin and Hymecromone data were also presented in the 7th International Symposium-cum-Training Course on Molecular Medicine and Drug Research, held in Karachi, Pakistan, November 4-7, 2019. In this abstract, 4 authors from Dr. Izumi group have been included. Below is the title of the abstract and author's name.

Abstract title: Exploring the Insulin Secretory Mechanisms of Hymecromone and Eupatorin in Mice Islets

Authors: Rahman M. Hafizur, Abdul Hameed, M. Israr Khan, Kiran Maryam, Muneeb Ali, Zaheer Ul-Haq, **Hao Wang**, **Miaomiao Zhao**, **Kohichi Matsunaga**, **Tetsuro Izumi**, Achyut Adhikari, Huma Aslam Bhatti

④Implementation status of information exchange with faculty members in charge of joint research.

We sometimes communicated with Professor Izumi by e-mail for academic discussion. Now, Dr. Abdul who previously worked at my lab is working at Professor Izumi's lab as a post-doctor.