Form 3

Report for Joint/Usage Research Program for Endocrine/Metabolism (Fiscal Year 2018)

Date: 2019/04/24

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 2018 as below.

(**Program No. 18013**)

1. Research Title	**Roles of Eupatorin and Hymecromone as Potent Insulin Secretagogues and their Mechanisms**
2. Purpose and Significance of the research project	***Purpose***: The present study was designed with aims to identify more efficacious insulin secretagogues from natural sources and to explore their possible mechanisms. Among hundreds of tested compounds, two compounds, namely Hymecromone and Eupatorin, showed significant insulinotropic effects in mice pancreatic islets in our preliminary studies. In this collaborative project, we investigated these two potential compounds in-depth and explored their insulin secretory mechanisms. ***Significance of the research project***: Insulin secretory defect is a major anomaly in the pathophysiology of Asian non-obese type 2 diabetic subjects. Classical insulin secreting agents, such as sulfonlyureas, stimulate insulin secretion irrespective of glucose concentrations which is the main reason for hypoglycemia as well as oxidative stress and excessive burden to exhaust islets in diabetic patients. Therefore, there is a need for a collective approach to develop new insulin secretagogue(s) with more pharmacological efficacy and minimize the sulfonylurea-associated risks. In this regard, this collaboration is of great essence to identify novel candidate compound that has glucose-dependent insulinotropic effects. The discovery of novel insulin secretagogue(s) as a drug candidate from natural sources with glucose-stimulated insulin secretion and drug targets other than sulfonylureas prove a better alternative insulin secretagogue. The main areas we are focusing on particularly distinct advantages in terms of benefit to risk ratio, low risk of drug-induced hypoglycemia compared to synthetic marketed drugs. The data of the project revealed some interesting and novel findings that will provide a future opportunity to address the unmet needs in current diabetes research. Furthermore, the project established a bridge between scientifically more advanced country like Japan and scientifically lagging country like Pakistan. In summary, the project will play an important role for diabetes drug development. Additionally, this will encourage young researchers significantly to strengthen their research capacity in developing different anti-diabetic drugs in near future.

3. Period of The Program	April 1, 2018 ~ March 31, 2019

4. Project Members

Name	Age	Gender	Institution/Department	Position	Role
(Principal Applicant) Dr. M. Hafizur Rahman	45	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	30	M	International Center for Chemical and Biological Sciences/ Dr. Panjwani Center for Molecular Medicine and Drug Research	Graduate student	Performed experiments under supervision of PI.
Kiran Maryam	29	F	International Center for Chemical and Biological Sciences/ Dr. Panjwani Center for Molecular Medicine and Drug Research	Graduate student	Performed experiments under 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 mice islets. Freshly isolated mice islets were incubated in different glucose concentrations in the presence of test compound with or without agonist/antagonist of K-ATP channel/Ca^{2+} channel/adenylate cyclase/cAMP/PKA/PKC and secreted insulin was measured by insulin ELISA kit. Research plans are followings:

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, PLC-PKC, and MEK-ERK1/2 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 achieved. 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 have designed a collaborative research project on Hymecromone (HCM) and Eupatorin (EPT) for their roles in insulin secretion. Going forward, Hymecromone purified from *Hippophae rhamnoides* was investigated for further in-depth insulinotropic mechanism(s). Mice islets in the size-matched group were treated in the following order, basal and stimulatory glucose, with or without HCM and/or pharmacological agonists/antagonists. The insulin content was measured using ELISA. 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, whereas EPT showed optimum activity at 50 μM; therefore, this respective dose was used for mechanistic studies.

HCM (200 μM) stimulated insulin secretion only at high glucose concentrations both in mice islets and MIN6 cells different from that of tolbutamide, a standard insulin secretagogue. At 16.7 mM glucose, HCM showed additive effects in tolbutamide-induced insulin secretion suggests that the insulin secretory mechanisms of HCM are different from sulfonylurea. HCM partially inhibited inward rectifier K^+ currents in MIN6 cells, whereas complete inhibition was found in tolbutamide. When islets were incubated with HCM and diazoxide to keep K-ATP channel open, HCM-induced insulin secretion was not inhibited completely compared with that of 16.7 mM glucose alone. Verapamil, an L-type Ca^{2+} channel blocker, showed no effect on insulin secretion by HCM at 3 mM but showed a significant inhibitory effect at 16.7 mM glucose. SQ22536, an adenylate cyclase inhibitor, has no effect on HCM-induced insulin secretion at 16.7 mM glucose. Subsequently, HCM showed no effect in IBMX- (a phosphodiesterase inhibitor) and forskolin (adenylate cyclase activator)-mediated insulin secretion indicates that HCM functions in the cAMP pathway. H-89, a PKA inhibitor, showed significant ($P<0.001$) inhibitory effect on HCM-induced insulin secretion at 16.7 mM glucose suggest that the effect of insulin secretion *per se* PKA-dependent. HCM-mediated insulin secretion was also moderately inhibited by PKC inhibitor, calphostin-C. Molecular docking studies revealed the best binding affinity of HCM with PKA; however, little interaction with PKC. In conclusion, Hymecromone potentiates insulin secretion predominantly through cAMP-PKA signaling pathway, distal to the K-ATP channel coupled with stimulatory glucose.

For Eupatorin, we found that Eupatorin (50 μM) potentiates insulin secretion only at 16.7 mM glucose and little to no effect at 3 mM glucose. Very interestingly, at 16.7 mM glucose EPT showed better insulin secretory activity than tolbutamide. Interestingly, we observed that EPT showed additive effect in IBMX- and/or forskolin-induced insulin secretion. Subsequently, we used inhibitor for PKA (H-89) and EPAC2 (MAY0132) alone and in combination. We found that in the presence of MAY0132, the EPT-induced insulin secretion was decreased to almost half. However, in the presence of H-89, the EPT-induced insulin secretion was inhibited almost completely. Furthermore, when we used MAY0132 and H-89 in combination, the EPT-mediated insulin secretion was less than that of insulin secretion by 16.7 mM glucose. These data suggest that cAMP signaling pathway is crucially involved in the EPT-induced insulin secretion. The EPT-induced insulin secretion was partially inhibited by PKC inhibitor, calphostin-C. Furthermore, the *in silico* interactions estimated with PKA and PKC were in good agreement with our *in vitro* findings.

Taken all these data together, we can conclude that Hymecromone and Eupatorin potentiate glucose-stimulated insulin secretion through cAMP-PKA glucose-dependent signaling cascade. However, further extensive electrophysiological, insulin secretory kinetic and *in vivo* studies are underway to derive the conclusive mechanism of HCM- and EPT-induced insulin secretion. Therefore, we extended this joint research project to perform further advanced experiments such as insulin secretion kinetics, morphological docking analysis, and intracellular Ca^{2+} and cAMP measurements to explore HCM- and EPT-induced insulinotropic mechanisms and pin-point their targets.

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

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]. Eupatorin and Hymecromone data were presented in the fourteenth biennial conference of Pakistan Society for Biochemistry and Molecular Biology (PSBMB), December 9-12, 2018, Karachi, Pakistan. In this abstract, 4 authors from Dr. Izumi group have been included. Below is the title of the abstract and author's name. (**Annexure A**)

Abstract title: Roles of Eupatorin and Hymecromone as Potent Insulin Secretagogues and their Mechanisms

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

[2]. An abstract of potential findings on Hymecromone in insulin secretion has been accepted for poster session for the 62nd Annual Meeting of Japan Diabetes Society to be 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. (**Annexure B**)

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**

[3]. An abstract of potential findings on Hymecromone also presented as invited lecture by M. Hafizur Rahman in the 9th ANRAP International Seminar in Karachi, Pakistan, during January 25 - 27, 2019. (**Annexure C**)

Abstract title: Natural Products for Anti-diabetic Research: ICCBS Experiences

[4]. An abstract of potential findings on Eupatorin in insulin secretion has also been presented as poster session in the 9th ANRAP International Seminar in Karachi, Pakistan, during January 25 - 27, 2019. (**Annexure C**)

Abstract title: Eupotarin potetiates insulin secretion in glucose-dependent cAMP-PKA amplifying pathway

Authors: M. Israr Khan, Abdul Hameed, Achyut Adhikari, Muneeb Ali, Zaheer Ul-Haq, and M. Hafizur Rahman

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