Form 3

# Report for Joint/Usage Research Program for Endocrine/Metabolism (Fiscal Year 2024)

Date：2025/4/26

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

| Principal Applicant | |
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| Institution | College of Molecular Medicine, Ziauddin University. |
| Position | Associate Professor |
| Name | Abdul Hameed |

We report on the results of joint research in fiscal year 2024 as below.

（Program No. 24006）

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| 1． Research Title | **Roles of Hispidulin as Potent Insulin Secretagogue and its Mechanism(s)** |
| 2． Purpose and Significance of the research project | **Purpose**: The present research project aims to identify natural, safer, efficacious insulin secretagogues with novel drug targets for the regulation of glucose-dependent insulinotropic effects. Hispidulin, a natural flavone, was identified as a new insulin secretagogue that enhances insulin secretion in response to high glucose and seems to be a better drug candidate than synthetic marketed drugs. In this project, the insulinotropic mechanism(s) of hispidulin was investigated in in C57BL/6 J mice islets and INS-1 832/13 cells.<br><br>**Significance of the research project**: Insulin secretory impairments and β-cell dysfunction is the predominant pathophysiology in non-obese type 2 diabetes patients, mostly found in Asia. Unfortunately, the current treatment options available for the β-cell functionality are glinides and sulfonylureas, which secrete insulin irrespective of glucose levels, posing continuous stress on β-cells, leading to insulin secretory impairments, hypoglycemia, and other adverse effects. Therefore, identification of new, safer insulin secretagogues that selectively act under high glucose conditions could reduce the side effects of already marketed drugs. This collaborative project aims to identify a novel, natural, and safe insulin secretagogue that acts on specific molecular drug targets in the presence of high glucose, thereby avoiding overstress on β-cells and offering a promising therapeutic alternative for a large number of diabetic patients.<br><br>The project's preliminary data revealed several promising findings that show a clear opportunity to address challenges with the current anti-diabetic drug discovery research. Most importantly, the project has established a valuable scientific bridge between Japan, a leader in biomedical research, and Pakistan, where scientific capacity is still under development. Overall, this initiative would play a crucial role in the development of potential anti-diabetic drugs and lay the foundation for future advancements. This research would empower young researchers in Pakistan to pursue further exploration and enhance their research capabilities to develop novel anti-diabetic therapies. |

| 3．Period of The Program | April 1, 2024 ～ March 31, 2025 |
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4．Project Members

| Name | Age | Sex | Affiliation | Position | Role |
|---|---|---|---|---|---|
| (Principal Applicant)<br>Abdul Hameed | 42 | M | Ziauddin University, College of Molecular Medicine | Associate Professor | PI of the project, supervised and monitored the project including Project conception to Data curations and analysis, result compilation, and report preparation. |
| (Research Collaborators)<br>Muhammad, Moazzam Tau-heed | 33 | M | Ziauddin University, College of Molecular Medicine | Graduate student | Performed experiments under supervision. |
| Falak Shahab | 31 | F | Ziauddin University, College of Molecular Medicine | Graduate student | Performed experiments under supervision. |
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※If additional space is required, please attach a separate sheet.

| 5．Collaborating Researcher of IMCR | Name of Laboratory | Diabetes and Metabolic Disorders | Name | Kohichi Matsunaga |
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6．Research Plans

Hispidulin was tested for its insulinotropic mechanism(s) in INS1-832/13 cells and isolated mouse islets under stimulatory glucose conditions, in the presence of agonists and antagonists targeting key insulin signaling pathways. The involvement of AKAP-9 was examined through siRNA-mediated knockdown and protein interaction analysis. Intracellular cAMP and PKAα levels were assessed using ELISA and Western blotting. The impact of AKAP-9 inhibition on hispidulin-induced glucose-stimulated insulin secretion was also evaluated. The effect of hispidulin on glucose tolerance, GSIS, and insulinogenic index was evaluated in diabetic mice.

Research Plans for the Proposed Project:

1. Cell culture and transfection validation
2. Effect of AKAP-9 knockdown and hispidulin treatment on insulin secretion and intracellular PKA activation
3. Role of hispidulin in insulin secretion kinetics using perifusion experiments
4. Role of hispidulin in insulin secretory events using TIRF microscopy
5. *In vivo* effect of hispidulin in diabetic mice

Research plans 1, 2, and 5 have been completed. Research plans 3 and 4 will be conducted soon by the Kohichi Matsunaga at the Institute for Molecular and Cellular Regulation (IMCR), Gunma University, Japan.

7 . Research results:

Please describe the details of the contribution of the joint research with IMCR in obtaining the results.

In the present study, hispidulin, a natural flavone, was evaluated to explore its insulin secretory mechanism(s). Hispidulin showed insulin secretory potential both in INS1832-13 cells and isolated mice islets exclusively at stimulatory glucose concentration. Using pharmacological approach, the insulin secretory mechanism of hispidulin was investigated by blocking the key signaling pathways using antagonists and observing their effect on hispidulin-induced insulin secretion. It was found that hispidulin showed no considerable effect on intracellular cAMP concentration, suggesting hispidulin's effect is neither in cAMP production nor inhibition of cAMP hydrolysis. Furthermore, hispidulin showed an additive effect in both forskolin and IBMX-induced insulin secretion, suggesting the hispidulin effect on the downstream signaling target. To further evaluate whether the additive effect of hispidulin is either PKA or epac2-dependent, H-89, a PKA inhibitor, and ESI-05, an epac2 inhibitor, were used. ESI-05 exerted no noticeable effect in the hispidulin-induced insulin secretion. However, H89 completely inhibited the hispidulin-induced insulin secretion, suggesting hispidulin may exert its effect in a PKA-dependent manner. Furthermore, using the molecular approach, hispidulin showed strong binding affinity with AKAP9-like protein in INS1-832-13 cells rather than PKA, suggesting AKAP-9 role in hispidulin-induced insulin secretion. To investigate this further, the effect of HD was further evaluated in AKAP-9 knockdown INS1-832-13 cells. Surprisingly, AKAP-9 knockdown further amplifies the glucose and HD-induced insulin secretion, suggesting that AKAP-9 knockdown leads to the stimulation of insulin secretion, and this effect is further augmented by HD, suggesting that inhibition of AKAP-9 activity, both by knocking down or inhibiting by HD seems to be responsible for the triggering hyperglycemic stimulation of insulin secretion. As our data showed, in contrast to most of the other AKAP proteins, AKAP-9 seems to be involved in the negative regulation of adenylate cyclase-cAMP-PKA signalosomes in INS1-832-13 cells, and inhibition of this negative regulation by knocking down and inhibiting HD, the glucose-stimulated insulin secretion is further enhanced. To further validate this hypothesis, the effect of HD was evaluated on intracellular PKAα signaling. Interestingly, it was observed that using HD, the intracellular PKAα signaling was further increased. This data suggests the HD inhibits the negative regulation of AKAP-9-cAMP-PKA signalosome by increasing the intracellular PKAα concentration, hence augmenting the glucose-induced insulin secretion. As AKAP-9 is a negative regulator of cAMP-PKA signalosome, further studies are needed to see the expression of AKAP-9 in diabetic pancreatic islets and how HDs interfere with and modulate the AKAP-9 expression to improve the diabetic condition. The acute *in vivo* results showed that hispidulin improved glucose tolerance and enhanced glucose-stimulated plasma insulin in diabetic mice. Taking all these data together, we conclude that HD potentiates insulin secretion predominantly through AKAP-9-cAMP-PKA signaling cascade, distal to the K-ATP channel coupled with stimulatory glucose; however, to authenticate the conclusive targets is still needed. Therefore, we extended this joint research project to perform further advanced experiments such as insulin secretion kinetics using perifusion experiments and insulin secretory events using TIRF microscopy, intracellular Ca2+ measurements, and, most importantly, its evaluation and validation in human islets.

８． Present status of academic conference presentations and research papers associated with the results of the joint research, and exchange of information on the joint research with the collaborating researcher at IMCR.
(As much as possible, please state papers that include the names of the collaborating researcher at IMCR or papers stating that the research was supported by the Joint Research Program with IMCR.
Regarding papers, please send a PDF file together with the report to the email address of the general affairs section of the Institute.) Office of General Affairs: kk-msomu4@ml.gunma-u.ac.jp

① Please list the publications that include the name of the collaborating researcher from IMCR and send a reprint of each publication to IMCR.
**Publication is in progress**

② Please list the publications that include a description that the research was supported by the Joint Research Program with IMCR and send a reprint of each publication to IMCR.
**Publication is in progress**

③ List up to 3 conferences (name of conference, date of conference, and title of the presentation).

We have presented and displayed our project data at international scientific meetings. The following are the details.

1. **Abdul Hameed*, Kohichi Matsunaga**. Hispidulin is an insulin secretagogue targeting the AKAP9-mediated PKA signaling pathway. IDF-WPR Congress 2023 / 15th Scientific Meeting of AASD during July 21-23, 2023 in **Kyoto Japan**.
2. **Abdul Hameed*, Kohichi Matsunaga.** Hispidulin is an insulin secretagogue targeting the AKAP9-mediated PKA signaling pathway. 17th European Diabetes and Endocrinology Congress during November 20-21, 2023 Dubai, UAE.
3. **Abdul Hameed*, Kohichi Matsunaga.** Hispidulin is an insulin secretagogue targeting the AKAP9-mediated PKA signaling pathway. 21st International Congress of Endocrinology (ICE) 2024 in Dubai during March 1-3, 2024.

④ Exchange of information exchange with collaborating researcher from IMCR (please list main points of communication).