

**Ranolazine: a Potential Anti-diabetic Drug**

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**Keywords: Ranolazine, Beta-cell, Apoptosis, Proliferation, Diabetes**

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## **ABSTRACT**

Diabetes is a life-long chronic disease that affects more than 24 million Americans. Loss of pancreatic beta-cell mass and function is central to the development of both type 1 (T1D) and type 2 diabetes (T2D). Therefore, preservation or regeneration of functional beta-cell mass is one of the essential strategies to treat diabetes [1]. In my study, I tested if ranolazine, a synthetic compound, has potential to prevent or treat diabetes. Diabetes were induced in mice by giving multiple low-doses of streptozotocin (STZ). Ranolazine was given twice daily via an oral gavage (20 mg/kg) for 5 weeks. Blood levels of glucose, insulin, and glycosylated hemoglobin (HbA1c) were measured. Glucose tolerance test was performed in control and treated mice. Pancreatic tissues were stained with hematoxylin and eosin or stained with insulin antibody for islet mass evaluation. INS1-832/13 cells and human islets were further used to evaluate the effect of ranolazine on beta-cell survival and related signaling pathway. Fasting blood glucose levels after the fourth week of STZ injections were lower in ranolazine treated group (199.1 mg/dl) compared to the vehicle group (252.1 mg/dl) ( $p < 0.01$ ). HbA1c levels were reduced by ranolazine treatment (5.33%) as compared to the control group (7.23%) ( $p < 0.05$ ). Glucose tolerance was improved in ranolazine treated mice ( $p < 0.05$ ). Mice treated with ranolazine had higher  $\beta$ -cell mass (0.25%) than the vehicle group (0.07%) ( $p < 0.01$ ). In addition, ranolazine improved

survival of human islets exposed to high levels of glucose and palmitate, whereas cell proliferation was not altered. In addition, ranolazine slightly increased the cAMP in MIN-6 cell and human islets. In conclusion, ranolazine may have therapeutic potential for diabetes by preserving beta-cell mass.

Keywords: Ranolazine, Beta-cell, Apoptosis, Proliferation, Diabetes

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## GLOSSARY OF TERMS

AMPK: 5' AMP-activated protein kinase

ATX-II: sea anemone toxin ATX-II

C<sub>max</sub>: peak plasma concentrations

CYP2D6: cytochrome P450 2D6

ER: endoplasmic reticulum

FBS: fetal bovine serum

FFA: free fatty acid

FPG: fasting plasma glucose

GK rat: Goto-Kakizaki rat

GLP-1: glucagon like peptide-1

GLUT2: glucose transporter 2

GSIS: glucose-stimulated insulin secretion

HbA1c: glycosylated hemoglobin

IL-1 $\beta$ : Interleukin-1 beta

iNOS: inducible nitric oxide synthases

i.p.: Intraperitoneal injection



JNK: c-Jun N-terminal kinases

K<sub>ATP</sub> channel: ATP-sensitive potassium channel

LV systolic pressure: left ventricular systolic pressure

MAPK: Mitogen-activated protein kinase

NCX: sodium-calcium exchanger

NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells

PDX-1: pancreatic and duodenal homeobox factor-1

SAPK: Stress-activated protein kinase

STZ: streptozotocin

T1D: type 1 diabetes

T2D: type 2 diabetes

ZF rats: Zucker Fatty Rats

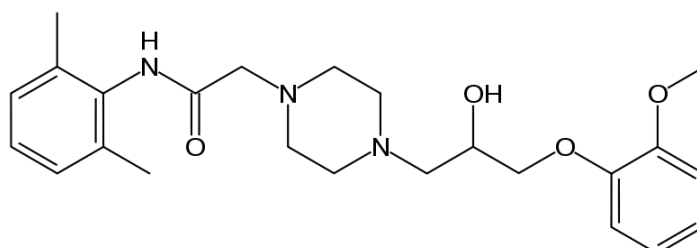
## REVIEW OF LITERATURE

### Ranolazine

Ranolazine is an anti-anginal drug sold under the name Ranexa. It is a piperazine derivative first developed by Syntex Laboratories (Edinburgh, UK). An early study found that it can reduce the derangement of myocardium induced by the occlusion of coronary artery in dogs in 1987 [19]. Since then, numerous studies have been done in vivo and in vitro to characterize the anti-anginal action of ranolazine [19].

### Chemical properties of ranolazine

Ranolazine is a racemic mixture containing enantiomeric forms (S-ranolazine and R-ranolazine). Its systematic name is (±)-N-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]-1-piperazine acetamide (Fig. 1). The molecular formula of ranolazine is  $C_{24}H_{33}N_3O_4$ [20] and its molecular weight is 427.54g/mol. It is a white or slightly yellow powder that is soluble in dichloromethane and practically insoluble in water. Generally, ranolazine can be stored for 5 years at 25°C. Ranolazine hydrochloride is often used in animal studies and clinical trials since it is soluble in water [21].



**Fig. 1. Chemical structure of ranolazine**

## **Metabolism of ranolazine**

Ranolazine is mainly metabolized in the liver by the cytochrome P450 3A enzyme (CYP2D6) and it can also be metabolized in the intestine [22]. The absolute bioavailability of ranolazine is high, which ranges from 35% to 50% [23]. Using radiolabelled ranolazine orally administered to the mice, it was found that 73% of the dose was excreted in urine, with unchanged ranolazine accounting for less than 5% of total excreted radioactivity in both urine and feces [23], suggesting that the compound is extensively absorbed and metabolized. Dose adjustment is required in patients with mild to moderate renal insufficiency or hepatic impairment because these conditions will lead to the reduction of ranolazine clearance. People with severe renal disease or hepatic impairment should not take ranolazine [22]. If used orally administered by immediate-release capsule, ranolazine has the elimination half-life of 1.4-1.9 hours, which is rather short. Taking advantages of extended release formulation, the elimination half-life of ranolazine can reach 7 hours on average, suggesting that the duration of its absorption is greatly extended [23]. On the basis of the data from clinical trials, the dose of ranolazine given to treat anti-anginal patients is 500 mg twice daily at the beginning of the treatment and is increased to the maximum recommendation dose of 1000 mg twice daily based on the symptoms [24]. At a dose of 1000 mg, the mean steady-state peak plasma concentrations ( $C_{max}$ ) was 2569ng/ml and 95% of  $C_{max}$  values were between 420 to 6080ng/ml [25].

## **Anti-anginal action of ranolazine**

Ranolazine is an anti-angina drug sold under the name Ranexa. As the first drug approved by FDA in over ten years to treat chronic angina, it is only used in patients

who have not achieved an adequate response with other anti-angina treatments [26]. Large amount of preclinical and clinical studies have been done on the anti-angina effect of ranolazine. The aim of angina treatment is to maintain the balance between myocardial oxygen demand and supply. Traditionally, the treatment of chronic angina includes increasing myocardial oxygen supply by vasodilation, reducing coronary artery obstruction and decreasing myocardial oxygen demand [27-29]. The agents used for these therapies include nitrates, calcium channel blockers and beta-adrenergic blockers [27-29].

Ranolazine exhibits a different mechanism of action from the common anti-anginal drugs. Increase in late  $I_{Na}$  due to the progression of ischemia will contribute to the elevation of intracellular sodium concentration, which in turn activates the cell membrane sodium-calcium exchanger (NCX) [30, 31]. A prolonged activation of NCX will lead to the intracellular  $[Ca^{2+}]_i$  overload and induce further ischemia [31]. Ranolazine was proposed to block the late  $I_{Na}$  and reverse the effect induced by late  $I_{Na}$ . Sea anemone toxin ATX-II (ATX-II) is used as an enhancer of late  $I_{Na}$  in the preclinical studies [32], which mimic the effect of ischaemia to increase intracellular  $[Na^+]_i$  [32] and intracellular  $[Ca^{2+}]_i$  [33]. Preclinical studies show that ranolazine is able to attenuate the ATX-II induced increase in late  $I_{Na}$  [32] and  $[Na^+]_i$ -dependent calcium overload [33]. The reduction of  $[Ca^{2+}]_i$  induced by ranolazine will decrease left ventricular systolic pressure (LV systolic pressure), peak LV  $+dP/dt$  and myocardial lactate release. The reduction of LV systolic pressure reduces the consumption oxygen and ATP for the muscle contraction, increases myocardial nutrition and oxygen supply through blood flow. The final effect of ventricular wall pressure reduction is balanced by oxygen demand and supply, thus preventing

ischemia[14]. Ranolazine also has beta-adrenergic effects [34]. Studies on rats showed that ranolazine dose-dependently inhibits the isoprenaline-induced decreases in diastolic arterial pressure and increases in heart rate through a weak beta 1- and beta 2- adrenoceptor antagonist effect [34]. It is also believed that ranolazine inhibits fatty acid  $\beta$ -oxidation and activates pyruvate dehydrogenase, which result in a greater use of glucose as a energy substrate. The switch of the substrate to glucose increase the ratio of ATP production to oxygen consumption, which leads to the improvement of myocardial function under conditions of reduced myocardial oxygen delivery during ischemia [35]. Results from several clinical trials testing the anti-ischemic effect of ranolazine during the last decade proved that ranolazine is well tolerated and effective in reducing the frequency of angina and increasing exercise duration [27, 29, 36, 37]. However, ranolazine needs to be further studied since the precise mechanisms of its anti-anginal effect are not fully understood.

### **Ranolazine and diabetes**

As diabetes is an established risk factor for cardiovascular disease (CVD), its management in patients with CVD is complicated because the cardiovascular safety of some oral glucose-lowering agents has been questioned [38].

The result of CARISA study, in which ranolazine has been shown to be effective in treating chronic angina in combination with commonly prescribed cardiovascular drugs, showed that ranolazine lowered HbA1c and significantly improved glycemic control in diabetic patients [15]. Specifically, the study showed that intake of 750 mg

twice daily reduced the HbA1c level in patients from  $7.65 \pm 0.20$  to  $7.14 \pm 0.13\%$  ( $p=0.008$ ). The HbA1c level of patients treated with 1000mg twice daily decreased from  $7.92 \pm 0.21$  to  $6.93 \pm 0.13$  ( $p=0.0002$ ) [15]. In the MERLIN-TIMI 36 randomized controlled trial, exploratory data analysis showed that ranolazine treatment is associated with a decline in HbA1c in patients with diabetes and chronic angina [17]. After comparing the HbA1c levels in among 4918 patients, it was found that ranolazine significantly reduce HbA1c levels in patients with diabetes [17]. It also reduce the incidence of new patients with fasting blood glucose  $>110\text{mg/dl}$  or  $\text{HbA1c} \geq 6\%$  who are not hyperglycemia before the study. [17]. The HbA1c levels in patients with diabetes treated with ranolazine for 4 months was decreased from 7.5% to 6.9% ( $P<0.001$ ) [17].

To further confirm the anti-diabetic effect of ranolazine, the data of patients with diabetes from MERLIN trial was further evaluated. In that study, HbA1c levels of 6 to 8% and fasting plasma glucose (FPG)  $< 150$  was defined as moderate hyperglycemia; HbA1c level of  $\geq 8-10\%$  and  $\text{FPG} \geq 150-400\text{mg/dl}$  was defined as severe hyperglycemia. Results showed that there were significant reductions in HbA1c levels in both patients with HbA1c levels of 6 to 8% ( $P<0.045$ ) and HbA1c levels of  $\geq 8-10\%$  ( $P<0.001$ ) [18]. The average FPG levels of the ranolazine-treated patients with  $\text{FPG} \geq 150-400\text{mg/dl}$  were reduced by  $25.7\text{mg/dl}$  ( $P=0.001$ ) compared with those in placebo treated patients [18].

Collectively, these human studies provide strong evidence that ranolazine has significant anti-diabetic effects in diabetic patients. However the underlying mechanism for this action by ranolazine is unknown. Recently, it was shown that

ranolazine can promote glucose-stimulated insulin secretion in cultured rat and human pancreatic islets [16].

## **Pancreatic beta-cell and diabetes**

Diabetes is a chronic disease that affects more than 20 million Americans. There are three types of diabetes, which includes gestational diabetes, T1D, and T2D. T2D is the most common diabetes, which often occurs in adulthood. However, the rate of T2D in teens and young adults is rising since the obesity rate is increasing. T1D, also known as juvenile diabetes before, often occurs in children, teens and young adults. Nearly all T1D patients make little or no insulin because of beta-cell failure. Patients need to be injected with insulin everyday. Gestational diabetes occurs in pregnant women who never had diabetes before. Pregnant women with gestational diabetes need careful medical supervision to control their blood glucose level.

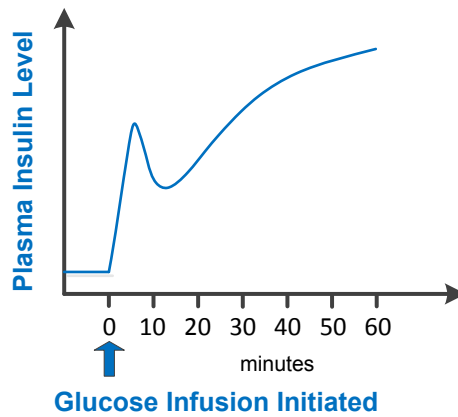
## **Pancreatic beta-cell and insulin release**

Beta-cells are insulin secreting cells that account for 65-80% of total cells in the islets of Langerhans in pancreas. Other cells in the islets of Langerhans are alpha cells that produce glucagon (15-20% total islet cells), delta cells that produce somatostatin (3-10% total islets cells), PP cells that secrete pancreatic polypeptide (3-5% total islets cells), and epsilon cells that release ghrelin (<1% total cells).

Insulin, a hormone produced by beta-cells, is critical for regulating energy metabolism and maintaining blood glucose homeostasis in the body. Pancreatic beta-cells sense changes in plasma glucose concentration and respond by

releasing corresponding amounts of insulin. As shown in Fig. 2 [39], insulin secretion in response to elevated blood glucose in a biphasic pattern. The initial spike of insulin release is referred to as the first phase response, lasting only about 4 to 10 minutes [40], which is followed by a gradual increasing rate of insulin release to a plateau which is referred to the second phase release[41]. Glucose-stimulated insulin secretion (GSIS) involves two signaling pathways, the ATP-sensitive potassium channel ( $K_{ATP}$ ) channel-dependent and  $K_{ATP}$  channel-independent pathways. The  $K_{ATP}$  channel-dependent GSIS has been well characterized. In brief, Glucose transported into beta-cells goes through glycolysis and mitochondrial oxidation. These events causes increases in intracellular ATP/ADT ratios, which sequentially leads to closure of  $K_{ATP}$  channels, depolarization of beta-cells [42], and opening of voltage-gated L-type  $Ca^{2+}$  channels on the plasma membrane,  $Ca^{2+}$  influx, and exocytosis of insulin-containing granules [43, 44]. The  $K_{ATP}$  channel-dependent pathway is the major pathway mediating the first phase of GSIS [45]. The mechanism of the second phase of GSIS, which is largely  $K_{ATP}$  channel-independent, is not fully understood, but protein kinase A, phospholipase A2, nitric oxide, cyclic GMP, phosphatidylinositol 3-kinase, and pertussis toxin-sensitive G-proteins are reportedly involved [45].





**Fig. 2. Glucose-stimulated biphasic insulin release**

### **Diabetes and beta-cell apoptosis**

Diabetes is a metabolic disorder that is marked by abnormally high levels of blood glucose. It is estimated that at least 25.8 million or 8.3% of Americans presently suffer from diabetes, and 79 million people have pre-diabetes [2]. While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 [3]. Among diabetic patients, about 5% of them are diagnosed as T1D, which usually occurs in children and young adults [2]. T1D is an autoimmune disease that is induced by the organ-specific immune destruction of the insulin-secretion pancreatic beta-cell in the islets of Langerhans. The autoimmune destruction of beta-cell will

lead to the absolute insulin deficiency and hyperglycemia. T1D is considered the consequence of a combination of genetic predisposition and environmental factors [46, 47]. T2D is the most common form of diabetes. It is the result of insulin resistance and lack of sufficient insulin secretion, which lead to abnormally high levels of blood glucose. Family history and genes are the most important risk factor for T2D [48]. Unhealthy life styles such as low physical activity and poor diet, which may lead to excess body weight and obesity, also greatly increase the risk of development of T2D [48].

As a hormone that maintain blood glucose homeostasis in the body, insulin helps regulates the glucose uptake of muscle, fat, and liver cells. Insulin resistance is a condition in which insulin is less effective in lowering blood glucose. During the insulin resistance, beta-cells will produce more insulin to help glucose enter cells in order to maintain the glucose homeostasis. Insulin resistance can be caused by both hereditary and life style of individuals. Most people with insulin resistance will not suffer from T2D due to compensatory hyperinsulinemia [49]. However, a small portion of people will develop hyperglycemia due to the cellular dysfunction of beta-cell, which finally lead to the onset of T2D. In the early stage of T2D, insulin resistance has already reached its maximum extent for the disease while the compensatory hyperinsulinemia can still maintain glucose tolerance [50, 51]. With the disease progression, the body cannot produce enough insulin to maintain glucose homeostasis due to cellular dysfunction and apoptosis of beta-cell, and overt diabetes develops [49, 52, 53]. Therefore, the reduced insulin secretion caused by the progressive decline of beta-cell mass and function is central to the glycemic control over time. Indeed, those with T2D always manifest increased beta-cell

apoptosis and reduced beta-cell mass [6, 7, 9]. For example, in obese and lean individuals with T2D, there are 63% and 41% reduction in beta-cell mass, respectively, as compared to people without diabetes. [54]. As such, a strategy that promotes beta-cell survival and mass can potentially provide a therapeutic means to prevent the onset of diabetes [10].

Pancreatic beta-cell mass are regulated by the balance of proliferation and death of the pancreatic beta-cells. Evidence shows that beta-cells are regenerated by self-replication mechanism, and thus beta-cell replication is important for maintaining beta-cell mass in diabetic patients [11, 12]. Pancreatic beta-cell apoptosis is the main reason for the loss of beta-cells in T1D and also contributes to the loss of beta-cell mass in deregulated metabolism-mediated T2D [9, 13]. While the causes of pancreatic beta-cell apoptosis in T1D are well understood, the causes of beta-cell apoptosis in T2D are complex and not fully understood. It seems that the reduction of beta-cell mass and function are the results of genetic predisposition, but the rate of the beta-cell loss is affected by concomitant environmental conditions [52]. A large body of literature has shown that hyperglycemia and hyperlipidemia, which often occurs in the pathogenesis of T2D, can induce beta-cell apoptosis and reduce beta-cell mass via multiple mechanisms [13].

### **Glucotoxicity and glucolipotoxicity play the central role for beta-cell function in T2D**

It is well known that chronic hyperglycemia and hyperlipidemia contribute to the deterioration of beta-cell function in T2D, which is referred to as glucotoxicity and lipotoxicity, respectively. Long-term exposure of beta-cells to elevated levels of

glucose and free fatty acid (FFA) will impair glucose-induced insulin secretion, reduce insulin gene expression and at the same time decrease beta-cell mass [55, 56].

### **Glucotoxicity**

The apoptosis of beta-cell caused by hyperglycemia can be mediated by chronic oxidative stress, endoplasmic reticulum (ER) stress and cytokines such as Interleukin-1 beta (IL-1 $\beta$ ) [57]. Oxidative stress is thought to be the central mechanism of glucotoxicity in pancreatic beta-cells [55, 56, 58]. Under the condition of hyperglycemia, excessive glucose lead to the production of ROS and increased oxidative stress in beta-cell [59, 60]. The beta-cell is susceptible to oxidative stress since the expression and activity of anti-oxidant enzymes in the cells are very low [13]. Oxidative stress can cause the reduction of MafA and pancreatic and duodenal homeobox factor-1 (PDX-1) DNA-binding activity, decrease in *PDX-1* mRNA expression and activation of the c-Jun N-terminal kinases (JNK) pathway [58]. As a transcriptional activator for insulin gene expression and regulated by glucose concentration, MafA is beta-cell-specific and may be involved in beta-cell development and function [61]. Pancreatic and duodenal homeobox factor-1 (PDX-1) is crucial for the development of the pancreas by regulating genes of insulin, glucose transporter 2 (GLUT2), and glucokinase [58]. Studies show that treatment with antioxidants protects beta-cell against apoptosis caused by oxidative stress, partially reverses PDX-1 and MafA binding activity, and improves glucose homeostasis in vivo [56, 58].

Cytokines are also mediators of glucose toxicity in T2D [62-64]. High glucose concentrations contribute to the increasing release of IL-1 $\beta$ , which will in turn activate several pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and Mitogen-activated protein kinase (MAPKs) such as p38 and JNK, leading to the production of nitric oxide (NO), DNA fragmentation, and ultimately apoptosis of beta-cell [62-64].

Endoplasmic reticulum (ER) stress is also considered an important mechanism that is involved in glucotoxicity. As one of the most important organelles to beta-cell, ER is responsible for not only the synthesis, initial post-translational modification, folding and maturation of pro-insulin, but intracellular calcium homeostasis [65]. Because of their high rate of insulin synthesis, beta-cells are susceptible to ER stress. ER stress induced by chronic high glucose in beta-cell leads to beta-cell dysfunction and death [65].

### **Lipotoxicity**

Lipotoxicity also plays important roles in the progression of T2D. As an essential fuel for beta-cell in the normal state, FFA will become toxic to beta-cells when they are chronically exposed to excessive levels of FFA. Prentki and Corkey proposed that elevated FFA in the presence of abundant levels of glucose lead to the accumulation of cytosolic citric, which is the precursor of malonyl-CoA. The inhibition of carnitine-palmitoyl-transferase-1 by malonyl-CoA can further result in the inhibition of beta-oxidation and accumulation of long-chain fatty acid CoA in the cytosolic, which is toxic to beta-cell [66]. Increasing evidence showed that 5' AMP-activated protein kinase (AMPK) is involved in the lipotoxicity since AMPK is stimulated by palmitate

and inversely correlated with glucose in beta-cell [67]. The presence of hyperlipidemia will activate AMPK which in turn activate the synthesis of FFA [68], leading to further accumulation of FFA in the cytosol. The elevated level of FFA will lead to the impairment of insulin gene expression and insulin secretion. Free fatty acid induces beta-cell apoptosis in the presence of high glucose through ceramide formation, altered lipid partitioning, generation of oxidative stress and ER stress[56, 69]. Several major signaling pathways are regulated by ceramide including pro-apoptotic MAPKs such as SAPK/JNK and P38 [56, 70]. It has been reported that the exposure of beta-cell to elevated levels of glucose and/or FFA stimulates inducible nitric oxide synthases (iNOS), activates caspase-3, which ultimately lead to the apoptosis of beta-cell [69, 70].

### **Glucolipotoxicity**

Glucotoxicity and lipotoxicity synergistically lead to the beta-cell dysfunction and apoptosis. Hyperglycemia can enhance lipotoxicity, which is referred as glucolipotoxicity. Many studies have been done to investigate the glucolipotoxicity in vitro and in vivo. Jacqueminet S et al. [71] investigated the insulin secretion of rat islets after long-term exposure to palmitate (0.5mM) in the presence of either low (2.8mM) or high glucose (16.7mM) concentration. Results showed that insulin expression was unchanged in the presence of palmitate at low glucose concentration while markedly decreasing in the presence of palmitate at high glucose concentration. However, Ernest Sargsyan and Peter Bergsten reported that lipotoxicity is glucose-dependent only in INS-1E cells but not in human islets and MIN6 cells [72]. In the study, both cell lines and human islets were cultured in the presence of palmitate (0.5mM) at low (5.5mM) or high (25mM) glucose for 48 hours.

Palmitate induced apoptosis in both cell lines and human islets at low glucose level. However, high glucose only potentiated palmitate-induced apoptosis in INS-1E cells but not in MIN-6 cell and human islets. The results were further explained by the reduction of palmitate oxidation by only 30% in human islets and MIN6 cells but by 80% in INS-1E cells after high glucose treatment. Since palmitate oxidation was reduced by glucose treatment, more FFA will be in the cytosol of the cell, which leads to the lipotoxicity. They concluded that lipotoxicity can occur without requirement of high levels of glucose.

Several in-vivo studies investigated the glucolipotoxicity. El-Assaad W et al. reported that elevated levels of FFAs and hyperglycemia synergistically cause islet beta-cell apoptosis because high glucose inhibit beta-oxidation and lead to the accumulation of lipid in the body [73]. Harmon JS et al. [74] conducted a study on Zucker diabetic fatty rat and found that a reduction triacylglycerol content in islets did not prevent the decrease of insulin mRNA levels while the prevention of hyperglycemia is associated with decreased islet TAG and insulin mRNA levels. Phuong Oanh T. Tran and Vincent Poitout [75] investigated whether high-fat diet induced hyperlipidemia will affect insulin secretion and beta-cell function in hyperglycemic vs. normoglycemic rats. Hyperglycemia Goto-Kakizaki rat (GK) rats and normoglycemia Wistar rats were fed a high-fat diet for 6 weeks. Results showed that high-fat feeding significantly impaired glucose-stimulated insulin secretion (GSIS) in islets from GK rats while there was no effect in Wistar rats. In addition, insulin treatment completely reversed the impaired GSIS from GK rats [75]. These data suggest that hyperglycemia may be required for the harmful effects of chronic hyperlipidemia in vivo.

As discussed above, hyperglycemia and hyperlipidemia, which are common in T2D patients, are toxic to beta-cells that lead to the reduction of beta-cell mass and deterioration of beta-cell function. As such, a search for potential medication that can promote beta-cell survival in this environment is important for preventing and treating T2D.

### **Ranolazine is a potential medication for diabetes treatment**

Due to the important role of beta-cell mass in the progression of T2D, one of the therapeutic strategies is to promote beta-cell survival. As an anti-angina drug approved by FDA in 2006, ranolazine can reduce ischemia-induced diastolic dysfunction by reducing the late sodium current  $I_{Na}$  [14]. Ranolazine was also found to inhibit the transient, late components of  $I_{Na}$  and various kinds of  $K^+$  channels in pituitary tumor GH<sub>3</sub> cells and NG108-15 neuronal cells [76]. Interestingly, in a clinical trial ranolazine was found to lower the FPG and HbA1c in patients with cardiovascular disease and diabetes [18]. However, as potential medication for diabetic patients, no research has been done to investigate whether ranolazine can preserve beta-cell mass under the situation of glucolipotoxicity, which mimics T2D.

### **High fat diet and STZ-induced T2D models**

Streptozotocin (STZ) (**Error! Reference source not found.**) is a chemical compound that is toxic to beta-cells. FDA approved it for using it to treat metastatic cancer of the pancreatic islets. However, it is only used under the circumstances that the cancer cannot be removed by surgery [77]. STZ is specifically toxic to beta-cell



since it is transported through GLUT2, which is expressed relatively high in beta-cells [78, 79]. Glucose transporter 1 (GLUT1) is expressed in glucagon producing alpha-cells [80]. Besides the use for pancreatic cancer treatment, STZ is also used to generate experimental diabetic animal models by selectively causing beta-cell destruction. It was shown that a single dose of STZ (30, 35, 40mg/kg body weight) after 24 hours fasting causes beta-cell necrosis and diabetes within 48 hours after injection [81, 82]. Like and Rossini later developed a mouse model more closely resembling T1D by intraperitoneal injection of multiple, low-doses of STZ (40mg/kg body weight) to mice for 5 consecutive days [83]. Mice treated with STZ exhibited symptoms of insulin deficiency, hyperglycemia, polydipsia, and polyuria, which mimic human T1D. The multiple low-doses of STZ treatment only gradually and partially damaged the pancreatic islet beta-cells, which is associated with beta-cell inflammation and apoptotic beta-cell. The multiple low-doses of STZ administration is one of the most widely used approach for generating insulin-deficient-dependent diabetic animal models [84].

As the most common type of diabetes mellitus, T2D animal models by STZ injection are recently established. Nakamura T et al. generated a T2D mouse model that mimics the non-obese type 2 diabetes, which reflects a majority of diabetic patients among Asian races. They administered STZ and nicotinamide (120 or 240mg/kg, STZ/NA120 or STZ/NA240) twice with an interval of 2 days to C57BL/6J mice [85]. Nicotinamide partially prevent the STZ-induced hyperglycemia, body weight loss and histological damage of pancreatic beta-cells. They obtained the mouse model that had mildly impaired glucose tolerance, almost normal fasting PG concentration,

normal body weight gain, normal insulin secretion, and decreased pancreatic insulin content (20 to 40% of normal animals).

Elizabeth R. Gilbert et al has reported a new nongenetic mouse model of T2D [86]. It was shown that middle-aged C57BL/6N mice, which are equivalent to 40 years old humans, develop T2D by high fat feeding for 4 weeks followed by STZ (40mg/kg) injections for 3 consecutive days. These mice developed insulin resistance and displayed reduced beta-cell mass. Okamoto T et al. generated T2D rat models with a low dosage of STZ (30mg/kg body weight, i.p.) [87]. Specifically, STZ treated Zucker Fatty (ZF) Rats (STZ-ZF) developed hyperglycemia, hyperlipidemia, hyperinsulinemia, and impaired GSIS. However, the morphology of pancreatic islets in STZ-ZF rats was not altered. A rat model of T2D was also developed by Reed MJ et al. [88]. Seven weeks old male Sprague-Dawley rats were fed high-fat diet for 2 weeks. The fat feeding induced insulin resistance of the Sprague-Dawley rats. After the fat feeding, STZ (50mg/kg) was intravenous injected into the rats. Fat-fed/STZ rats have the metabolic syndromes characteristics of T2D.

## **SUMMARY**

According to the data of National diabetes fact sheet, 8.3% of the population in America is suffering from diabetes [2]. It is clear that beta-cell mass is important for the glycemic control in both T1D and T2D. Long-term exposure of beta-cells to elevated levels of glucose and FFA will impair glucose-induced insulin secretion, reduce insulin gene expression and at the same time decrease beta-cell mass, which is referred to glucotoxicity and lipotoxicity. Glucotoxicity and glucolipotoxicity

play the central role for beta-cell function in T2D. Ranolazine is an anti-anginal drug, which was approved by FDA in 2006. During the studies of the anti-angina effects of ranolazine, it was unexpectedly shown that this drug has potent anti-diabetic action by lowering HbA1c in chronic angina patients [15-18]. However, it is unclear how ranolazine exerts such an anti-diabetic effect. In the present study, we use in vivo and in vitro methods to access the anti-diabetic effect of ranolazine.

### **SIGNIFICANCE**

According to the American diabetes association, 8.3% of Americans presently suffer from diabetes [2]. While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 [3]. Preservation or regeneration of pancreatic beta-cell mass plays the central role for effective treatment for both T1D and T2D. Except for the anti-angina effect, several clinical trials showed that ranolazine lowered HbA1c and blood glucose levels in patients with diabetes [15-18]. The objective of the research is to test (1) whether ranolazine can ameliorate blood glucose in diabetic mice; (2) whether and how ranolazine stimulates proliferation and/or prevents apoptosis of pancreatic beta-cells. The results from these studies will help define the mechanism underlying the anti-diabetic action of this drug, which could potentially lead to the development of new medicine to treat human diabetes, a growing health problem in the US and world-wide.

## INTRODUCTION

Diabetes is a metabolic disorder that is marked by abnormally high levels of blood glucose. It is estimated that at least 25.8 million or 8.3% of Americans presently suffer from diabetes, and 79 million people have pre-diabetes [2]. While the availability of novel drugs, techniques, and surgical intervention has improved the survival rate of individuals with diabetes, the prevalence of diabetes is still rising in Americans, with the number of people with diabetes projected to double by 2025 [3]. In both Type 1 diabetes (T1D) and Type 2 Diabetes (T2D), loss of beta-cell mass and function through apoptosis is central to the deterioration of glycemic control over time [4]. While peripheral insulin resistance is common during obesity and aging in mice and people, its progression to T2D is largely due to insulin secretory dysfunction and significant apoptosis of functional beta-cells [4-8], leading to an inability to compensate for insulin resistance. Indeed, those with T2D always manifest increased beta-cell apoptosis and reduced beta-cell mass [6, 7, 9]. As such, a strategy that promotes beta-cell survival and mass can potentially provide therapeutic means to prevent the deterioration of diabetes [10].

Pancreatic beta-cell mass is regulated by the balance of proliferation and death of pancreatic beta-cells. Evidence shows that beta-cells are regenerated largely by self-replication mechanism, suggesting that beta-cell replication is important for maintenance of beta-cell mass in diabetes patients [11, 12]. Pancreatic beta-cell apoptosis is the main reason for the loss of beta-cells in T1D and also contribute to the loss of beta-cell mass in deregulated metabolism-mediated T2D [9, 13]. While the causes of pancreatic beta-cell apoptosis in T1D are well understood, the etiology of beta-cell apoptosis in T2D is complex and not fully understood. A large body of

literature has shown that hyperglycemia and hyperlipidemia, which often occurs in the pathogenesis of T2D, can induce beta-cell apoptosis and reduce beta-cell mass via multiple mechanisms [13].

Due to the important role of beta-cell mass in the progression of T2D, one of the therapeutic strategies is to promote beta-cell survival. As an anti-angina drug approved by FDA in 2006, ranolazine can reduce ischemia-induced diastolic dysfunction by reducing the late sodium current [14]. Increased late  $I_{Na}$  due to the progression of ischemia will contribute to the further ischemia by activating the NCX [30, 31]. The anti-angina effect of ranolazine has been demonstrated by several clinic trials [15-18]. Interestingly, it was found that ranolazine also lower glycosylated hemoglobin (HbA1C) and fasting blood glucose level in chronic angina patients [15-18]. However, the mechanism by which ranolazine mitigates hyperglycemia and diabetes is unknown.

## Materials and Methods

### Reagents and materials

RPMI-1640 medium and supplements were purchased from Sigma-Aldrich (St. Louis, MO); CMRL-1066 medium was from Mediatech, inc. (Herndon, VA); fetal bovine serum (FBS) was obtained from Thermo Scientific (Logan, Utah). Ranolazine was synthesized by the Department of Medicinal and Bio-Organic chemistry of CV Therapeutics, Inc (now Gilead Inc, CA); HbA1c kit was from Semens Healthcare Diagnostics Inc (NY); cyclic AMP EIA Kits were purchased from Caymen Chemical (Ann Arbor, MI); CellTiter-Blue® cell viability assay kits were from Promega (Madison, WI); glucometer, cell proliferation ELISA, and BrdU reagents were purchased from Roche Applied Science (Indianapolis, IN); rat Insulin ELISA kits were from Mercodia Developing Diagnostics (Uppsala, Sweden). For in-vitro study, 10mM stock solution of ranolazine was made in dimethyl sulfoxide and immediately frozen at -80 °C before use and then diluted with cell culture medium for the experiments.

### Animal studies

The protocol of the study was approved by the Institutional Animal Care and Use Committee At Virginia Tech. Eight-week old, male C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed in an animal room maintained on a 12 hours light/dark cycle under constant temperature (22–25° C) with *ad libitum* access to food and water. Mice were randomly divided into 3 groups with 12 mice per group with drug treatment group given ranolazine (20 mg/kg) and control group given the same volume of vehicle twice a day by oral gavage.

Ranolazine was diluted by ddH<sub>2</sub>O before the oral gavage. This dose of ranolazine used in this study was determined according to the drug bioavailability in mice and the therapeutic blood concentration in humans taking this drug. After 1 week, STZ (40mg/kg) dissolved in 0.1mM cold sodium citrate buffer (pH 4.5) was given to the mice by i.p. for 5 days to induce diabetes. Control mice received same amount of citrate buffer. After the procedure, mice remained on ranolazine treatment or vehicle until the end of this experiment. Body weight and food intake were recorded weekly throughout the study.

### **Plasma glucose, insulin, and HbA1c measurements**

Before ranolazine and STZ treatment, baseline fasting blood glucose levels in tail vein blood sample were measured using a glucometer (Roche) to assure that the mice were euglycemic. After STZ injection, the levels of blood glucose were measured weekly to assess the onset of diabetes (fasting blood glucose >250 mg/dl) [89]. Plasma insulin concentration was measured by a ELISA kit. Blood HbA1c levels were measured by a HbA1c measure system (Siemens Healthcare Diagnostics Inc, NY) in mice fasted for 4 hours.

### **Glucose tolerance tests**

Glucose tolerance tests were done at the end of the animal study. Mice were fasted for 4 hours and then a single bolus of glucose (2 g/kg body weight) was given to the mice by i.p. injection. Glucose levels were measured using a glucometer (Roche) at 0, 15, 30, 60, and 120 min after glucose administration.

## **Immunohistochemistry and islet morphometry**

Six mice from each group were euthanized, and the pancreata were dissected, fixed in 4% (vol/vol) formaldehyde buffer (pH 7.2), and embedded in paraffin. A series of tissue sections (5- $\mu$ m thickness and 500  $\mu$ m interval) were prepared, mounted on glass slides, and immunofluorescently stained with a guinea pig anti-insulin antibody (Abcam, Cambridge, MA) for determining beta-cell mass. The beta-cell area was measured using images acquired from serial insulin-stained pancreatic sections. The beta-cell mass were calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue and multiplied by the pancreas weight as previously described [90].

## **Cell and Islet culture**

INS1-832/13 cells and MIN6 cells (a kind gift from Dr. Christopher B. Newgard at Duke University) were cultured in RPMI-1640 medium (11.1mM glucose, 10% FBS, 1mM sodium pyruvate, 10mM HEPES, 2mM L-glutamine, 50 $\mu$ M beta-mercaptoethanol, 100 units/ml penicillin/streptomycin) at 37°C and 5% CO<sub>2</sub>). The medium was changed every other day until the cells became 80% confluent. Human islets were isolated from cadaver organ donors in the Islet Cell Resource Centers and provided through the Integrated Islet Distribution Program, which is funded by National Institute of Health and the Juvenile Diabetes Research Foundation International and administrated by Administrative and Bioinformatics Coordinating Center (City of Hope Medical Center (Duarte, CA). The islet purity was 80-90 % and viability was 90-97 %. The islets were maintained in CMRL-1066 medium containing 10% FBS.



### **Cell proliferation assay**

INS1-832/13 cells were incubated with various concentrations of ranolazine or exendin-4 in RPMI-1640 medium containing 5.5 mM glucose and 5% FBS at 37°C. Forty-four hours later, the cultures were continued for an additional 4 h in the presence of BrdU (10 $\mu$ M). Cell proliferation was then assayed by measuring BrdU incorporation into DNA with an ELISA kit (Roche Applied Science, Indianapolis, IN). Cells were incubated with exendin-4 served as positive controls.

### **Viability assay**

INS1-832/13 cells were cultured in RPMI-1640 medium containing no glucose and 10% FBS with various concentrations of ranolazine or vehicle in the presence or absence of STZ (3.3 mM) for 24 hours. Human islets were cultured in CMRL-1066 medium containing 5.5 mM glucose and 5% FBS with various concentrations of ranolazine or vehicle in the presence of palmitate (1mM) and glucose (20mM) for 7days. Medium was refreshed every other day. At the end of the 6<sup>th</sup> day, CellTiter-Blue® reagent was added to the medium and incubate for 24 hours, beta-cell viability was tested using a fluorometer (560<sub>ex</sub>/590<sub>em</sub>). CellTiter-Blue® reagent measures the metabolic capacity of the cells. The results represent the viable cells present in the multi-well plate.

### **Intracellular cAMP assay**

Human islets (around 200 islets/tube), MIN-6 cells and INS1-832/13 cells were exposed to various concentrations of ranolazine or vehicle for 10 min. Forskolin (10 $\mu$ M) was used as a positive control. The supernatant was rapidly aspirated, and

the intracellular cAMP extraction and quantification were performed using a cAMP EIA Kit (Caymen Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions.

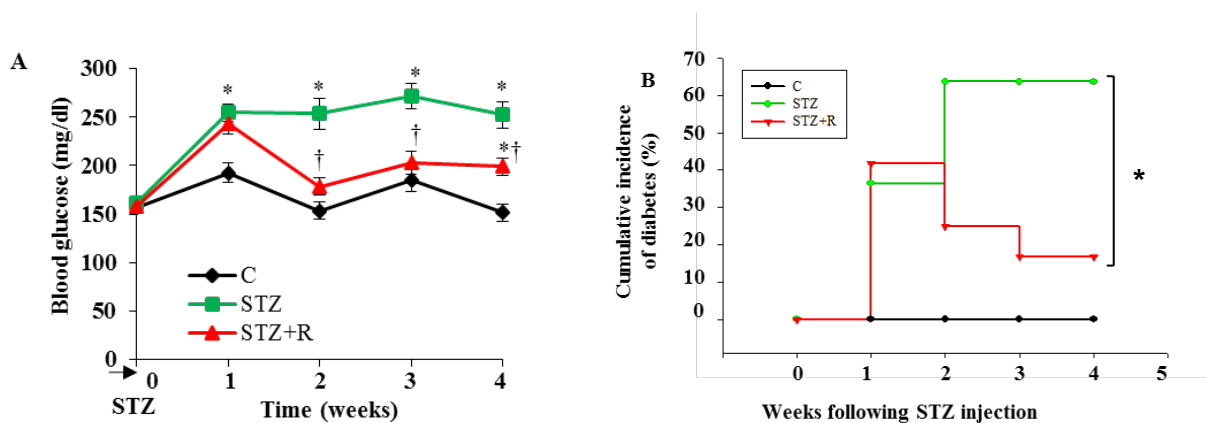
### **Data analysis**

All data were analyzed by one-way when designated using a SAS<sup>®</sup> program and are expressed as mean±SE. Differences between groups were assessed using Dunnett's test. Cumulative incidence of diabetes was analyzed by the Mann-Whitney rank sum test. A p-value < 0.05 was considered significant.

## RESULTS

### Ranolazine ameliorates STZ-induced hyperglycemia in diabetic mice.

Our study shows that ranolazine ameliorates hyperglycemia in STZ-treated diabetic mice (Fig. 3A). One week after the STZ injection, STZ alone treated group has significantly higher blood glucose level than the control group. However, ranolazine didn't decrease the blood glucose level in mice compared with STZ alone treated group. From the second week after STZ injection, mice treated with STZ and ranolazine have significantly lower blood glucose level than mice treated with STZ-alone (Fig. 3A). The incidence of diabetes is also lower in the STZ and ranolazine treated group compared with STZ alone treated group. By the 4<sup>th</sup> week after STZ injection, there are only 16.7% of mice were diabetic in ranolazine treated group, which is 50% less than the STZ-alone treated group (Fig. 3B) ( $p < 0.05$ ).

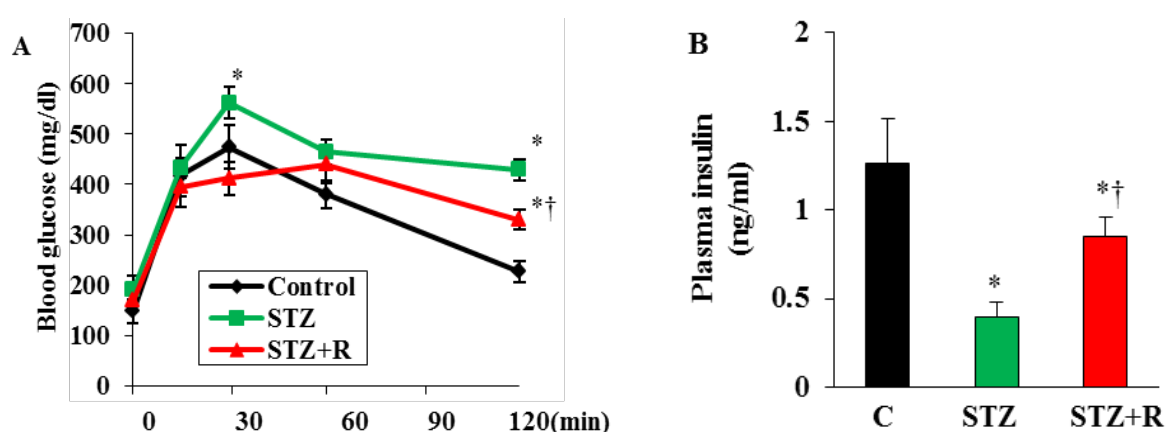


**Fig. 3. Ranolazine treatment prevent diabetes in mice**

Mice were treated with ranolazine (R, 20 mg/kg) through oral gavage for 1 weeks prior to administration of STZ (40 mg/kg) for 5 consecutive days. The mice in the control were injected saline. The levels of blood glucose drawn from the tail vein of fasted mice were measured weekly for 4 weeks following STZ injection (A). Incidence of diabetes was analyzed by the Mann-Whitney rank sum test (B). Data are expressed as mean $\pm$ SE ( $n=8$  for the control, and  $n=12$  each for STZ and

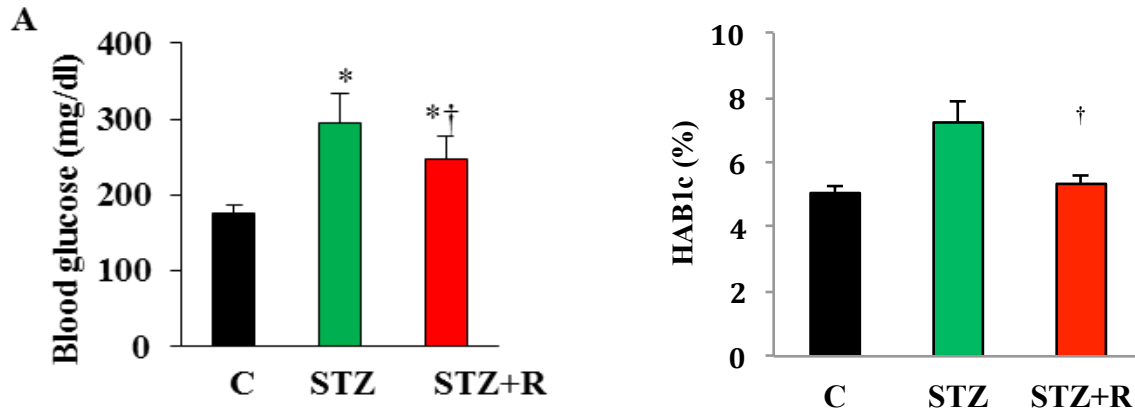
STZ+R groups). \*,  $p < 0.05$  vs. control; †,  $p < 0.05$  vs. STZ alone-treated mice. C: healthy control; STZ-streptozotocin; R=ranolazine.

The result from glucose tolerance test shows that diabetic mice treated with ranolazine had improved glucose tolerance which was associated with significantly higher blood insulin levels compared to those in mice treated with STZ alone (Fig. 4). Notably, after drug treatment withdrawal for 10 days, mice treated with ranolazine still had lower glucose levels than the STZ-alone treated mice (Fig. 5A). Additionally, the levels of HbA1c, which is the indicator of average blood glucose over 2-3 months, was significantly lower in ranolazine treated mice compared to those in STZ-alone treated group (Fig. 5B), further confirming the anti-diabetic action of this compound.



**Fig. 4. Ranolazine improves glucose tolerance and blood insulin level in mice**

Mice were fasted for 4 h followed by i.p. injection of 2g/kg of D-glucose for glucose tolerance test (A), or blood withdrawal for measuring plasma insulin levels by ELISA (B). Data are expressed as mean $\pm$ SE (n=4 for C; n=6 for STZ and STZ+R, respectively). \*,  $p < 0.05$  vs. control; †,  $p < 0.05$  vs. STZ alone-treated mice. R: ranolazine (20 mg/kg).

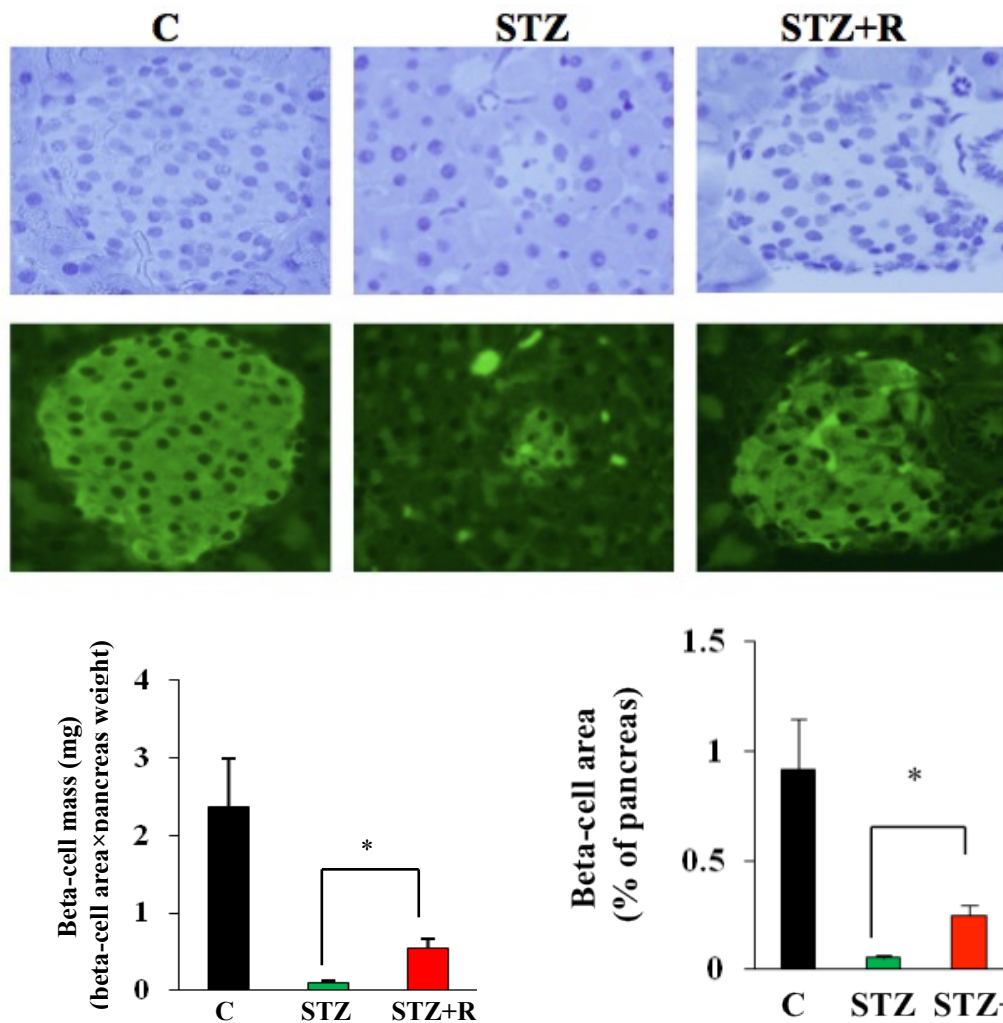


**Fig. 5. Blood glucose and HbA1c levels 10 days after drug withdrawal**

Data are expressed as mean±SE (n=4 for C; n=6 for STZ and STZ+R, respectively). \*, p<0.05 vs. control; †, p<0.05 vs. STZ-alone treated mice. R: ranolazine (20 mg/kg). C: healthy control; STZ-streptozotocin; R=ranolazine.

**Ranolazine preserves pancreatic islets beta-cell mass in diabetic mice.**

It is well known that STZ causes diabetes by selectively destroying islet beta-cells [79]. We therefore reasoned that Ranolazine might prevent diabetes in animals by protecting beta-cell from apoptosis and/or enhancing beta-cell regeneration. As shown in Fig. 6, oral administration of ranolazine preserved pancreatic islet beta-cell mass from STZ destruction.



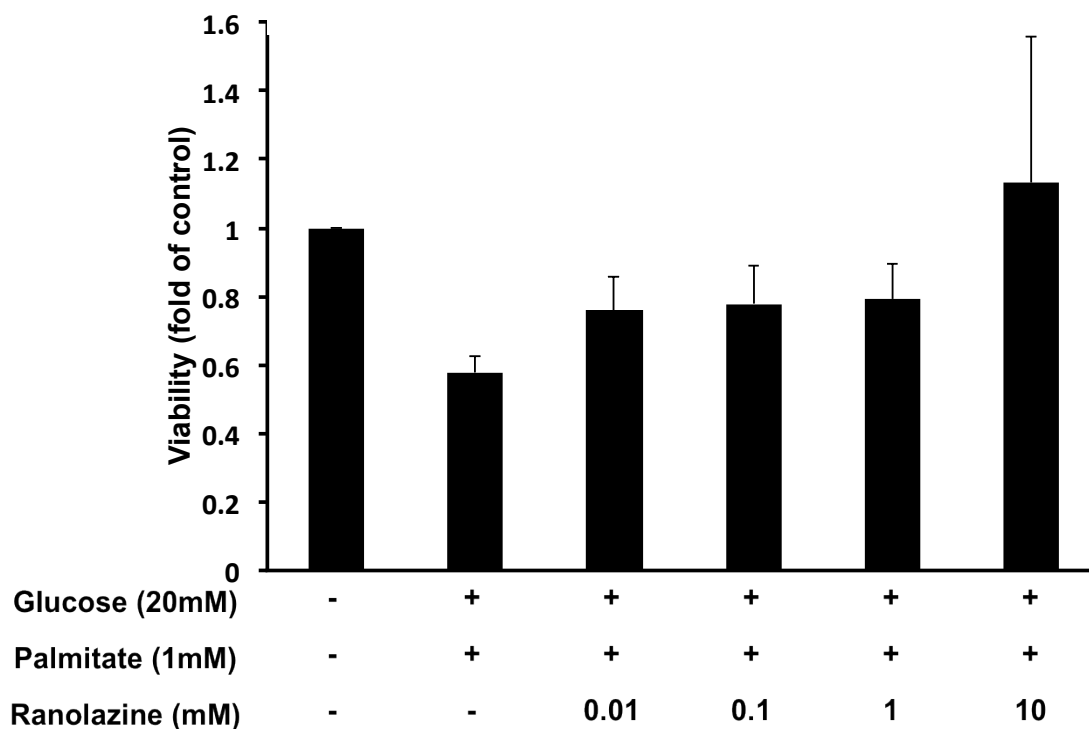
**Fig. 6. Ranolazine improves islet beta-cell mass in STZ-induced diabetic mice**

Pancreatic sections from control (C) or STZ diabetic mice given ranolazine (R) or vehicle were stained with hematoxylin and eosin (up) or immunostained for insulin (down). Pancreatic beta-cell mass were evaluated by fluorescence microscopy. Representative photographs from 6 mice in each group are shown. Data are expressed as mean±SE. \*,  $p < 0.05$  vs. control, †,  $p < 0.05$  vs. STZ-alone treated mice.

**Ranolazine improves viability of human islets exposed to chronic glucolipotoxicity.**

We further examined whether ranolazine has a protective effect on human islets under glucolipotoxicity, which mimics the situation of T2D. Human islets were incubated with 1mM palmitate and 20mM glucose in the presence or absence of

ranolazine. In the control group, islets were treated with 5.5 mM glucose and vehicle. As expected, the viability of human islets cultured in 1mM palmitate and 20mM glucose had lower viability as compared to control islets as determined by measuring reduction of resazurin to resorufin (Fig. 7). Human islets treated with 1mM palmitate and 20mM glucose have lower viability than the islets in control group. Although the effect is not significant, we can see that ranolazine can increase the viability of human islets under the glucotoxicity environment.

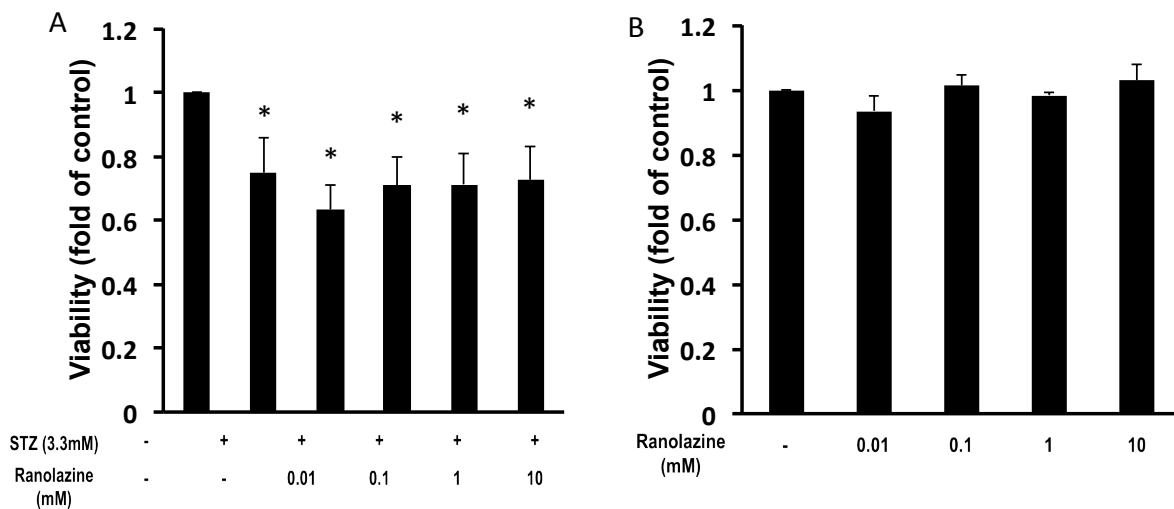


**Fig. 7. Effect of ranolazine on the viability of human Islets**

Human islets were incubated with palmitate (1mM) and glucose (20mM) in the presence or absence of various concentrations of ranolazine for 7days. Viability of human islets was measured, and data are expressed as means  $\pm$  SE from three experiments each performed in triplicate.

### The effect of ranolazine on clonal beta-cell viability.

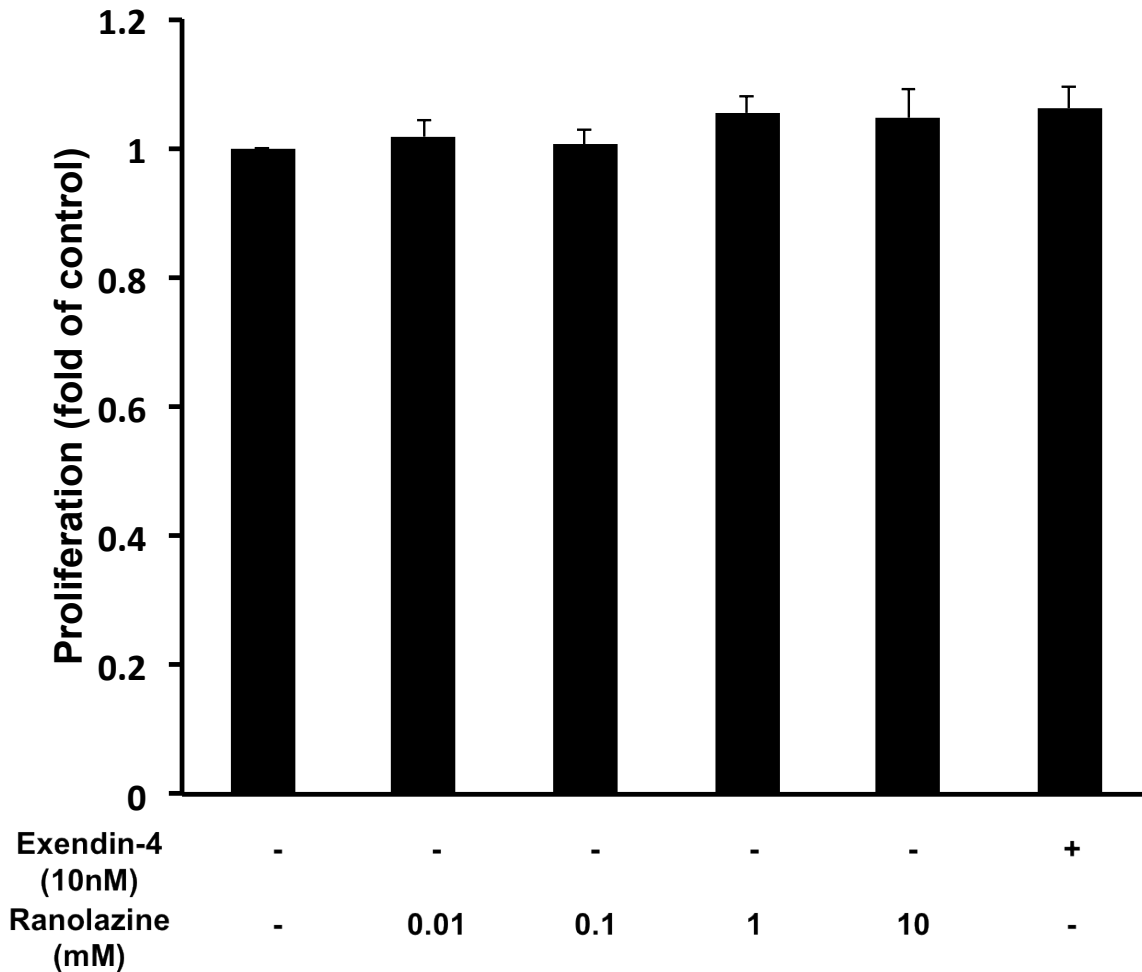
To determine whether ranolazine can protect beta-cell apoptosis induced by STZ in vitro, INS382/13 cells were incubated with 3.3mM STZ and various concentrations of ranolazine for 24 hours, followed by cell viability assay. STZ significantly decrease the viability of the cells compared with vehicle alone-treated group (Fig. 8). However, ranolazine didn't increase the viability of the cells exposed to STZ (Fig. 8). We also examine whether ranolazine can increase cell viability in normal condition. Results showed that there was no significant difference between control cells and ranolazine treated cells.



**Fig. 8. Effect of ranolazine on the viability of clonal beta-cells.**

A. INS382/13 cells were incubated with various concentrations of ranolazine or vehicle in the presence or absence of STZ (3.3mM) for 24hrs. B. INS382/13 cells were incubated with various concentrations of ranolazine or vehicle. Viability of cells was measured. Data are expressed as means  $\pm$  SE from three experiments each in triplicate. \*,  $p < 0.05$  vs. vehicle alone-treated cells.



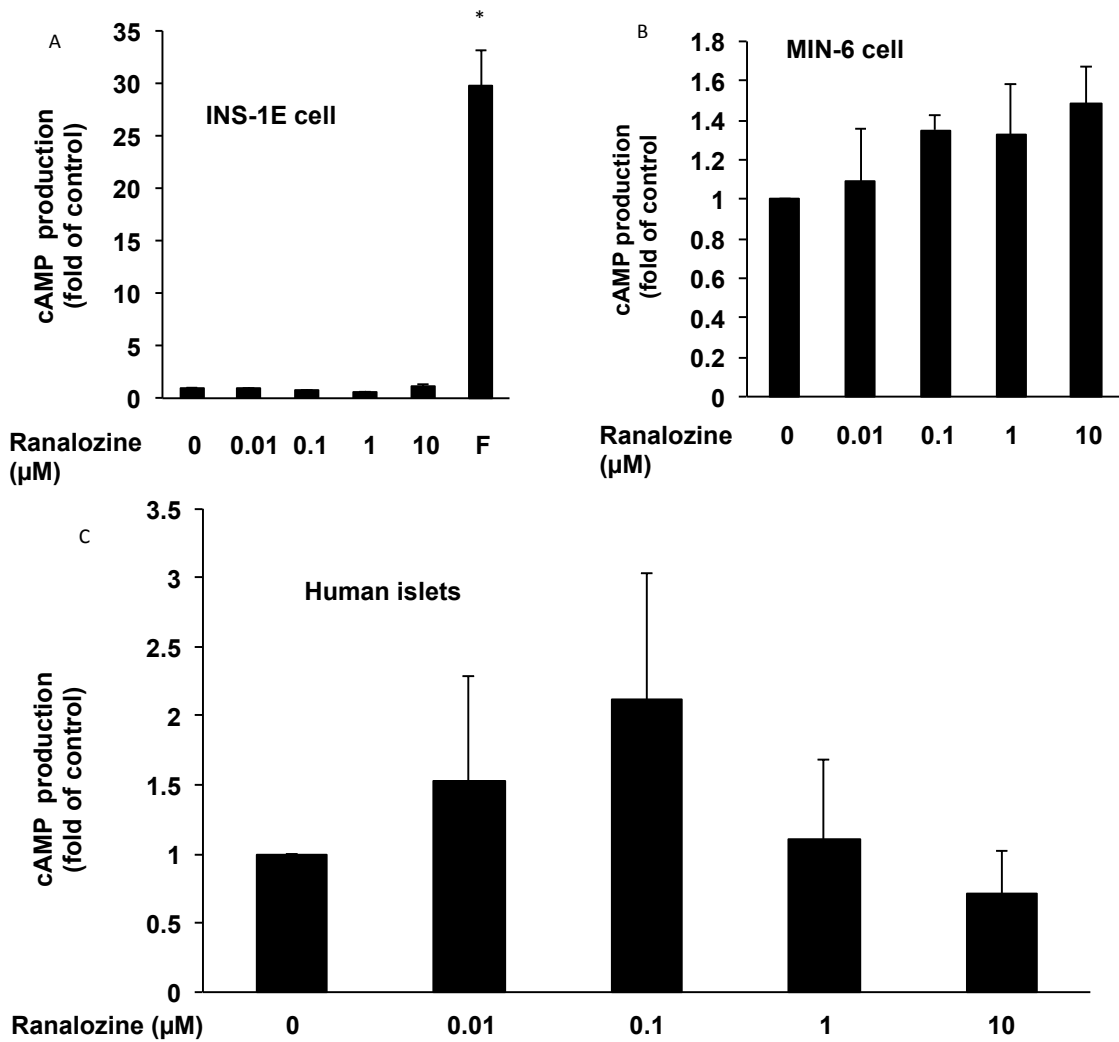


**Fig. 9. The effect of Ranolazine on the proliferation of beta-cells**

INS382/13 were incubated with various concentrations of ranolazine for 48hrs. DNA synthesis of INS382/13 cells were measured. Data are expressed as means  $\pm$  SE from six experiments each performed in triplicate. There is no significant difference between groups.

**Ranolazine has no effect on beta-cell proliferation.**

To examined whether ranolazine can stimulate the proliferation of beta-cells. Exendin-4, the analog of glucagon-like peptide-1, which was shown to induce beta-cell proliferation [91], was used as a positive control. As shown in Fig. 9, ranolazine had no significant effect on proliferation of INS1-832/13 cells. Exendin-4 slightly increases the proliferation of IN1S382/13 cells, but this effect was not significant (Fig. 9).



**Fig. 10. Effect of ranolazine on intracellular cAMP accumulation**

Human islets were incubated with various concentrations of ranolazine or forskolin (10 μM) for 10 minutes. The concentrations of intracellular cAMP levels were measured, and data was expressed as means ± SE from three experiments each in duplicate.

### **The effect of Ranolazine on intracellular cAMP production in beta-cell lines.**

To evaluate whether ranolazine stimulates intracellular cAMP generation in INS-1E , MIN6 cells, and human islets, cells or islets were exposed to various concentrations of ranolazine or vehicle for 10 min. Forskolin (10  $\mu$ M) was used as a positive control. The results show that ranolazine had no effect on intracellular cAMP content in INS1E cells (Fig. 10A), but moderately increased intracellular cAMP levels in MIN6 cells (Fig. 10B). It seems to have a biphasic effect on cAMP production in human islets, stimulating cAMP release at lower doses while inhibiting it at higher concentrations (Fig. 10C).

## DISCUSSION

Ranoazine is a FDA approved anti-angina drug that was found to lower plasma glucose and HbA1C in chronic angina patients with diabetes [15-18]. However, no study was done with animal models or in vitro beta-cells to further investigate the anti-diabetic effect of ranolazine. In the present study, we investigated the anti-diabetic effect of ranolazine on diabetic mice and in vitro beta-cells, providing evidence suggesting that ranolazine may be a potential anti-diabetic compound.

Since ranolazine has the ability to lower plasma glucose levels in chronic angina patients with diabetes in previous clinical studies [15-18]. We used C57BL6 mice to further test the anti-diabetic effect of ranolazine. Eight-week old, male C57BL6 was induced to be diabetic by the STZ administration. In the study, mice was injected STZ (40mg/kg) for five consecutive days, which cause mild to moderate levels of hyperglycemia by causing partial destruction of beta-cells. According to the study before [83], administering multiple low-dose STZ (40mg/kg body weight) to mice, which is same as our study, will trigger the symptoms of T1D. The symptoms include insulin deficiency, hyperglycemia, polydipsia, polyuria and most importantly, inflammation reaction that further lead to the destruction of pancreatic beta-cell. It was shown that C57BL mice are resistant to STZ-induced insulinitis [92]. In the animal study, we only developed a mild level destruction of beta-cell in C57BL mice. However, patients from several ranolazine clinical trials suffered from T2D.

The diabetes symptom of the mice model we created is not exactly the same as the T2D patients. Insulin resistance is the first symptom of T2D. In the early stage of T2D, compensatory hyperinsulinemia can still compensate for insulin resistance and

maintain the normal plasma glucose level. The decreased beta-cell mass and impairment of insulin secretion will ultimately lead to diabetes [52]. It is different from T1D, which is caused mainly by the autoimmune-mediated destruction of pancreatic beta-cells. However, pancreatic beta-cell mass is also important for the development of T2D since beta-cells secrete insulin. The diabetic mice model helps us determine whether ranolazine can potentiate insulin release, maintain euglycemia, and preserve beta-cell mass. Our study showed that ranolazine significantly lowered the blood glucose levels in STZ-treated mice. In addition, beta-cell mass was preserved in the ranolazine-treated group. While this study demonstrated that ranolazine has an anti-diabetic effect possibly by preserving functional beta-cell mass in insulin-deficient diabetic mice, whether it provides a similar protective effect on T2D remains to be determined.

The glucose tolerance test showed that mice treated with ranolazine have better glucose tolerance. Glucose tolerance test is always performed to evaluate pancreatic beta-cell function and insulin resistance [94]. Ranolazine may improve blood glucose levels by improving insulin release and insulin sensitivity. We further tested plasma insulin levels. It showed that mice treated with ranolazine indeed have higher plasma insulin levels than STZ-alone treated mice. However, it is unclear whether ranolazine directly enhanced endogenous insulin secretion in mice.

Glucose-stimulated insulin secretion is largely KATP-pathway dependent. Ranolazine was found to inhibit the inwardly rectifying K<sup>+</sup> channel in pituitary tumor GH3 cells and NG108-15 neuronal cells [76]. Inhibition of KATP will cause depolarization of plasma membranes of beta-cells, which results in the increase in Ca<sup>2+</sup> influx into the cells through L-type Ca<sup>2+</sup> channels and ultimately leads to

activation of insulin release [43, 44]. KATP of beta-cell is also a subclass of the inwardly rectifying K (+) channel. However, it is unclear whether ranolazine inhibits the KATP channel on beta-cells in vivo, thereby directly enhancing insulin secretion [93]. This question which needs further investigation.

The increased insulin release of beta-cell could be due to preserved functional beta-cell mass in diabetic mice. Interestingly, the blood glucose levels of mice in ranolazine treated group were still significantly lower than STZ-alone treated group after drug withdrawal for 10 days. We speculated that ranolazine might have improved beta-cell mass and/or function. Therefore, we performed histological and immunological staining of pancreatic islets. Results showed that mice treated with ranolazine have significantly higher beta-cell mass than mice treated with STZ alone. Therefore, ranolazine may directly or indirectly protect beta-cells.

As previously mentioned, ranolazine was found to inhibit the inwardly rectifying K (+) channel in pituitary tumor GH<sub>3</sub> cells and NG108-15 neuronal cells [76]. In beta-cells, knock out of K (ATP) channels may exert beneficial effects on oxidant-induced beta-cell death, which is dependent on the cytosolic Ca<sup>2+</sup>. The increase of cytosolic Ca<sup>2+</sup> can up-regulate antioxidant enzymes, which may protect beta-cells from oxidative stress [95]. Another explanation is that the increased insulin secretion or increased insulin sensitivity resulted from ranolazine treatment led to lower blood glucose level, which provides a better environment for beta-cells in vivo. Many studies have shown that hyperglycemia causes glucotoxicity of beta-cells, thereby contributing to the pathogenesis of diabetes [57]. If ranolazine increases insulin secretion or insulin sensitivity, pancreatic beta-cells may less likely be exposed to constant high levels of glucose.

To further understand how ranolazine improved blood glucose level and beta-cell mass in diabetes mice model, we used beta-cell lines and human islets to evaluate the protective effect of ranolazine on beta-cells. First we excluded the possibility that ranolazine exhibited the anti-diabetic effect through protecting beta-cells from necrosis and apoptosis caused by STZ. STZ is transported into beta-cells through GLUT2 [78, 79, 96] and causes DNA alkylation in the cells. The damage of DNA will further activate poly ADP-ribosylation and leads to the formation of superoxide radicals. In addition, STZ liberates toxic amount of NO that also damage DNA. Eventually, beta-cells go through necrosis after the treatment of STZ [96]. Exposure of INS1-832/13 cells to STZ in the presence of ranolazine showed no protective effect on STZ toxicity, suggesting that ranolazine may not directly protect beta-cells from STZ toxicity. In the animal study, multiple low-doses of STZ administration was used and this may triggered the inflammatory reaction which cause the apoptosis of beta-cell [83]. The possible mechanisms of the protective effect of ranolazine are that ranolazine suppressed the immune response of the mice.

Much research has been done on the effect of hyperglycemia and hyperlipidemia on beta-cells, which is referred as glucolipotoxicity. Glucolipotoxicity is recognized as a contributing factor for pancreatic beta-cell apoptosis. Plasma FFA concentration under normal living condition in lean healthy people are in the range of ~300-400  $\mu\text{M}$ . Among obese and T2D people however, the circulating concentrations of FFA are ~500-800  $\mu\text{M}$  [97]. To further evaluate whether ranolazine can prevent apoptosis of islet cells exposed to glucolipotoxicity, which is always associated with T2D, we incubated human islets and INS-1E cells with high glucose and palmitate. In the study, we treated the beta-cells with 1mM palmitate and 20mM glucose, the doses

that are often used to mimic hyperglycemia and hyperlipidemia in vivo. Results showed that ranolazine may partially reverse the toxic effect of hyperglycemia and hyperlipidemia on human islets, suggesting that ranolazine may protect beta-cells from glucolipotoxicity. However, the effect need to be further confirmed since there is no significant differences between groups. Human islets contain not only beta-cells but also other cell types such as alpha cells producing glucagon (15-20% total islet cells), delta cells producing somatostatin (3-10% total islets cells), PP cells producing pancreatic polypeptide (3-5% total islets cells), epsilon cells producing ghrelin (<1% total cells), and vessel cells. Therefore, further experiments are needed to determine whether ranolazine indeed specifically protects beta-cells, instead of other cell types, from apoptosis.

Research showed that pancreatic beta-cells are formed by self-replication [11, 12], which is emerged as a very important drug target for diabetes treatment. To determine whether the preservation of beta-cell mass by ranolazine in diabetic mice is through stimulating the proliferation of beta-cells, we tested whether ranolazine can stimulate beta-cell proliferation in vitro. Although large amount of studies showed that the incretin hormone glucagon-like peptide-1 (GLP-1) and its analog exendin-4 (Ex-4) promote beta-cell growth and expansion [98-100], our study showed no such effect. The result of the study also showed that ranolazine had no effect on beta-cell proliferation.

Cyclic AMP is a second messenger that is involved in the intracellular signal transduction in many cells. It is synthesized from ATP by adenylyl cyclase on the inner side of plasma membrane. It has been established that increased cAMP levels in beta-cell potentiate insulin exocytosis. The stimulation of insulin release by cAMP



is independent of intracellular  $\text{Ca}^{2+}$  concentration [101]. Recent studies also showed that activation of cAMP signaling exerts an anti-apoptotic effect on beta-cells. Guim Kwon et al. reported that cAMP dose-dependently prevents palmitate-induced apoptosis in beta-cells [102]. Study from Ulupi S. Jhala et al. [103] showed that GLP-1 promoted islet-cell survival through the activation of cAMP. To further investigate the mechanism of protective effect of ranolazine on beta-cells in vivo, we tested whether ranolazine potentiates cAMP level in beta-cells and islets. The results showed that ranolazine only moderately increased cAMP production in MIN-6 cells and human islets. Therefore, the second messenger cAMP may not play a major role in the anti-diabetic effect of ranolazine.

In summary, we have found that in the STZ treated mice model ranolazine lowered blood glucose levels and the incidence of diabetes, improved circulating insulin levels, and preserved beta-cell mass, these results suggest that ranolazine may be a novel anti-diabetic drug in addition to its anti-anginal action. However, the exact mechanism for its anti-diabetic action needs further investigation.

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