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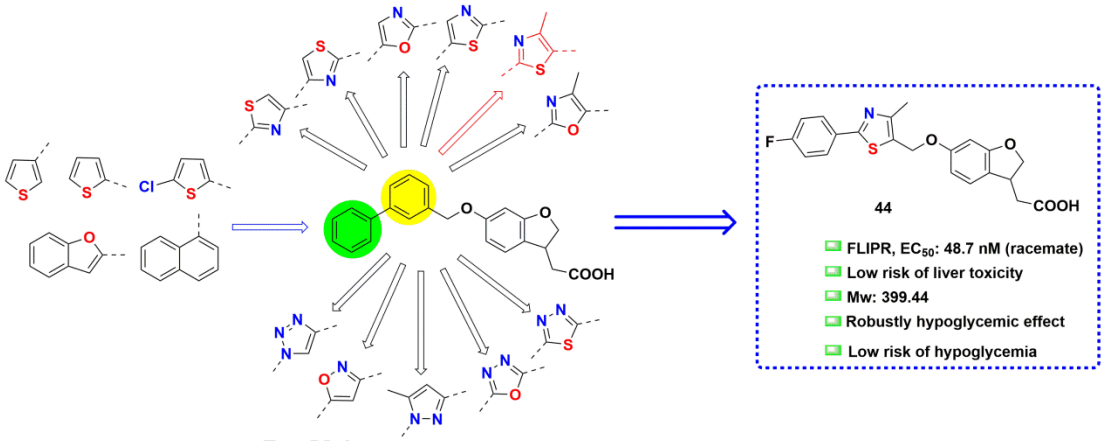
Graphical Abstract



11 polar heteroaromatics were designed and synthesized to improve the lipophilicity and liver toxicity of TAK-875.

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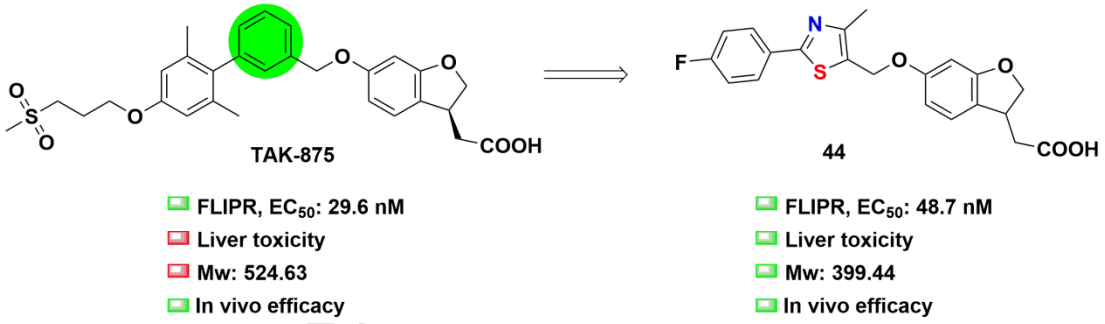
Abstract



The free fatty acid receptor 1 (FFA1/GPR40) has attracted interest as a novel target for the treatment of type 2 diabetes. Several series of FFA1 agonists including TAK-875, the most advanced compound terminated in phase III studies due to concerns about liver toxicity, have been hampered by relatively high molecular weight and lipophilicity. Aiming to develop potent FFA1 agonists with low risk of liver toxicity by decreasing the lipophilicity, the middle phenyl of TAK-875 was replaced by 11 polar five-membered heteroaromatics. Subsequently, systematic exploration of SAR and application of molecular modeling, leads to the identification of compound **44**, which was an excellent FFA1 agonist with robustly hypoglycemic effect both in

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normal and type 2 diabetic mice, low risks of hypoglycemia and liver toxicity even at the twice molar dose of TAK-875. Meanwhile, two important findings were noted. First, the methyl group in our thiazole series occupied a small hydrophobic subpocket which had no interactions with TAK-875. Furthermore, the agonistic activity revealed a good correlation with the dihedral angle between thiazole core and the terminal benzene ring. These results promote the understanding of ligand-binding pocket and might help to design more promising FFA1 agonists.

Keywords: FFA1 agonist, GPR40, Liver toxicity, Type 2 diabetes, Heteroaromatics, Dihedral angle.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a global epidemic characterized by impaired glucose homeostasis due to insulin deficiency and tissue resistance to insulin-stimulated glucose uptake and utilization.[1, 2] Despite an inspiring array of pharmacotherapies for T2DM, most of current hypoglycemic agents are associated with undesirable side effects such as body weight gain, risk of hypoglycemia, and gastric symptoms.[3-6] Hence, a novel drug with increased safety and durability in controlling blood glucose levels is still an urgent need for T2DM.[7, 8]

The G protein-coupled receptor 40 (GPR40)/free fatty acid receptor 1 (FFA1), the prominent ones of novel antidiabetic targets in the last decade, play a key role in amplifying glucose-stimulated insulin secretion (GSIS) on pancreatic β-cells without the associated risk of hypoglycemia.[9-12] The mechanism of action of FFA1 is mainly couples with the G protein α-subunit of the Gq family, which activates phospholipase C, leading to the production of inositol triphosphate and release of intracellular Ca^{2+} from the endoplasmic reticulum, which are associated with enhanced insulin secretion in a glucose concentration-dependent manner.[13, 14]

Recently, a variety of synthetic FFA1 agonists that contain acidic moieties have been reported (**Figure 1**),[15-23] of which the compounds TAK-875, AMG-837 and LY2881835 have reached to clinical trials. However, the known FFA1 ligands reported in the literature to date have been often hampered by relatively high molecular weight and lipophilicity even beyond the scope of "Rule-of-Five" (red mark in **Figure 1**), which is associated with poor water-solubility, metabolic instability, toxicity, high promiscuity, and correlates with a higher risk of attrition in clinical

trials.[24-28] Therefore, researches have suggested that clogP values should not exceed 4−5 in the optimization process.[24, 29] At present, a lot of classic strategies have been extensively adopted to decrease the lipophilicity such as introduction of hydrophilic group and scaffold hopping with polar skeleton.[25, 30-32] With this perspective in mind, our research was focused on decreasing the lipophilicity of the most advanced compound TAK-875, which was terminated in phase III studies due to concerns about liver toxicity in December 2013.[33-35] Our previous study also identified a series of phenoxyacetic acid derivatives as a more hydrophilic acidic moiety compared with the dihydrobenzofuran of TAK-875.[36] In this study, we envisioned that replacement of the middle benzene ring of TAK-875 with a polar five-membered heteroaromatics was a worthy approach for the decrease of clogP value, to potentially reduce the risk of liver toxicity. Hence, 11 heteroaromatics with the combination of different heteroatom number and location were designed and synthesized to explore the nature of ligand-binding pocket and select an optimal heteroaromatics for further SAR studies (**Figure 2**). Among them, compound **26**, with robust FFA1 agonistic activity (77.3 nM), was selected to replace the terminal benzene ring with a series of heterocycles in order to expand the SAR studies. Then, various substituents in the terminal benzene ring of **26** were also introduced to optimize the FFA1 agonistic activity. Based on the molecular modeling study and analysis of the SAR, we discovered that the methyl group of **26**, the most important fragment in our thiazole series, efficiently occupied a small hydrophobic subpocket which had no interactions with TAK-875. Furthermore, the agonistic activity revealed a good correlation with the dihedral angle between thiazole core and the terminal benzene ring. Of all the synthesized compounds, compound **44** with excellent FFA1 agonistic activity (48.7 nM) and antihyperglycemic effect, revealed a low risks of hypoglycemia and liver toxicity, was considered to be a promising drug candidate worthy of further investigation.

Put Figure 1 and Figure 2 here

2. Results and Discussion

2.1. Chemistry

The synthetic routes of intermediates **2a-g** are summarized in **Scheme 1**. The commercially

starting material benzoylhydrazide **1** was treated with chloroacetyl chloride to afford the desired product **2**, which was further converted to intermediate **2a** or **2b** by dehydrating with $POCl_3$ in acetonitrile at reflux for 4 hrs or cyclizing with Lawesson's reagent, respectively.[37, 38] Similar conditions to **2a** or **2b** provided the intermediate **2c** or **2d** from the common intermediate **4**, formed from commercially available 2-Aminoacetophenone **3** treated with chloroacetyl chloride. Cyclization of the starting material 2-Bromoacetophenone with ethyl thiooxamate generated the desired ester **1a**, which was followed by reduction using $NaBH_4$ and methanol,[39] and further chloridized to yield the desired intermediate **2e**. The synthesis of the intermediate **1b** was started from the intermolecular cyclization between phenylhydrazine and ethyl 2, 4-dioxopentanoate.[40] While the intermediate **1c** was prepared by ring-closure reaction using hydroxylamine hydrochloride and ethyl 2,4-dioxo-4-phenylbutanoate **10**, which was easily obtained in oxaloylation of acetophenone by Claisen condensation with diethyl oxalate in the presence of NaOEt.[41, 42] The obtained ester **1b** or **1c** was subsequently converted to compounds **2f** or **2g** according to the method for the synthesis of **2e**.

The chlorinated intermediates **2h-i** and **14a-u** were synthesized starting from various benzamide **11a-u** as shown in **Scheme 2**. The ethyl 2-chloroacetoacetate was condensed with benzamide **11a** or various thiobenzamide **12a-u** to form the oxazole **1d** or substituted thiazole **13a-u**.[43] The thiazole **1e** was obtained from the intermolecular cyclization between ethyl bromopyruvate and thiobenzamide, which was afforded from benzamide by treatment with Lawesson's reagent in high yield.[44] Next, the obtained ester **1d-e** or **13a-u** was reduced with $NaBH_4$ in the presence of methanol, followed by treating with thionyl chloride catalyzed by DMF, generated the chlorinated intermediates **2h-i** and **14a-u**.

The synthesis of the target compounds **20** to **50** is depicted in **Scheme 3**. The dihydrobenzofuran intermediate **15** was synthesized *via* published procedures.[15] Condensation of the obtained chlorinated intermediates **2a-i** or **14a-u** with **15** by using Williamson ether synthesis, followed by basic hydrolysis, afforded the desired carboxylic acids **20** to **27** and **29** to **50**. The synthesis of triazole core in **28** was accomplished by classical click chemistry method.[45] The starting material **15** was treated with 3-bromoprop-1-yne in acetone at the presence of potassium carbonate to give **16** in high yield. Azidobenzene **18** was obtained by treating aniline with $NaNO_2$ followed by NaN_3 in 50% acetic acid. The intermediates **16** reacted smoothly with

azidobenzene **18** at ambient temperature in the presence of catalytic amount of copper sulfate and sodium ascorbate in 80 % methanol, followed by basic hydrolysis, giving the target compound **28**.

Put Scheme 1, 2 and 3 here

2.2. Biological activities and discussion

2.2.1. FFA1 agonistic activity and SAR study

In order to explore an optimal polar heteroaromatic to replace the middle phenyl of TAK-875, the agonistic activities of the synthesized 11 heteroaromatic compounds with relative lower molecular weight and clogP values were investigated by a fluorometric imaging plate reader (FLIPR) assay in Chinese hamster ovary (CHO) cells expressing human FFA1 (**Table 1**). The oxdiazole core (**20**) with lowest clogP value (clogP = 1.705) was initially designed to decrease the lipophilicity, but the agonistic activities of **20** far more deviated the desired effect (6.31% agonistic activity at 300 nM). An approximately 2-fold increase in potency over the compound **20** was obtained only by replacing the oxygen atom of **20** with sulphur atom in compound **21**. We speculated that the improvement of the activity was associated with the larger hydrophobic effect and electronic distribution of sulphur atom than oxygen atom.[30] A similar improvement in potency was also observed by converting compound **22** to compound **23**, which was intended to increase the hydrophobic interaction with Gly_{139} and Val_{84} by removing the left-hand nitrogen of **21**, and this design strategy was directly inspired by the crystal structure of FFA1 bound to TAK-875.[46] However, position exchange of sulphur and nitrogen atom in **23** to afford **24** resulted in a significant drop of activity, attributed to the strict electrical interaction in the binding pocket of FFA1. On the basis of the above analysis, we replaced the right-hand nitrogen with methyl in **20** to further explore the electrical and hydrophobic effect with ligand-binding pocket, and the obtained compound **25** showed a 3-fold increase in potency compared with the parent compound **20**. Consistent with before-mentioned findings, replacement of the oxygen atom in **25** with sulphur atom gave compound **26**, a superior agonist with a 67.41% agonistic activity at 300 nM, showed the great potential for further optimization. Interestingly, switched the sites of sulphur and nitrogen atom and removed the methyl in **26** to afford **27** appeared to diminish the *in vitro*

agonistic activity. Meanwhile, other combinations of heteroatoms such as triazole (**28**), parazole (**29**) and isoxazole (**30**) were designed to decrease the lipophilicity, but none of them showed the desired potency, further confirming that the importance of electrical and hydrophobic effect in ligand.

Put Table 1 here

To better understand the robustly agonistic activity of **26** and the SAR for FFA1 agonists, a molecular modeling study based on the recently reported crystal structure of FFA1 (PDB accession code: 4PHU) was performed by using the Molecular Operating Environment (MOE) (**Figure 3**). As shown in **Figure 3A**, the interaction mode of compound **26** was nearly perfect overlap with TAK-875, and the carboxylate moiety of them showed the same hydrogen bonding interactions with Tyr_{91}, Arg_{183} and Arg_{2258} in FFA1. Interestingly, the methyl group of **26** occupied a small hydrophobic subpocket (**Figure 3**) which had no interactions with TAK-875. It was suggested that this hydrophobic interaction was crucial for the robustly agonistic activity of **26** rather than other five-membered heteroaromatic compounds evaluated above.

Put Figure 3 here

Based on these results above, we therefore selected this scaffold of **26** as our starting point for further modification. Our optimized efforts were directed to replace the terminal benzene ring with a series of heterocycles in order to expand the SAR studies (**Table 2**). Gratifyingly, the bioisosteres 3-thiophene derivative **32**, with lower lipophilicity, exhibited a slight improvement on potency in comparison with the parent **26**. However, the agonistic activity of **31** was significantly lower than **32** only by moving the sulphur atom from the 3-position to the 2-position of the thiophene core. Concerns about potential metabolic issues associated with the thiophene ring led us to introduce electron-withdrawing chlorine in thiophene ring to afford **33** while appeared to diminish the agonistic activity.[47] The bulkier heterocycles such as benzofuran and naphthalene have been reported to improve the agonistic activity of TAK-875 series,[17] however, a similar

optimization in **34** and **35** exhibited a markedly reduced agonistic activity in our thiazole system. One possible rational explanation is that the electron donors including oxygen and nitrogen atoms are available for interaction with low-lying σ* orbitals of the C−S bond, and the noncovalent sulfur interaction could restrict conformational flexibility of a molecule just as the intramolecular hydrogen-bonding interaction.[30, 48, 49] As shown in **Figure 4**, the thiophene ring (**31**) and the benzofuran (**34**) tend to be coplanar with the middle thiazole core, which might hindered the hydrophobic interaction highlighted in **Figure 3** owing to deviating the favorable conformation.

Put Table 2 and Figure 4 here

In order to select a suitable lead compound for further modification, the most potent compounds **26** and **32** were used to evaluate the oral glucose tolerance test (OGTT) in normal ICR mice (**Figure 6A**). Although the *in vitro* activity of **32** had more potential than **26**, the hypoglycemic effect of **32** was significantly lower than **26**. We speculated that the weaker *in vivo* effect of **32** may be attributable to a faster metabolism for thiophene ring of **32**.[47, 50]

These results prompted a comprehensive evaluation on various substituents in the terminal benzene ring of **26**, an orally bioavailable FFA1 agonist. As shown in **Table 3**, for the *ortho*-substituted compounds, the agonistic activity of **26** (2-H, 1.20 Å) ＞ **42** (2-F, 1.47 Å) ＞ **39** (2-Cl, 1.75 Å) ＞ **36** (2-Me, 1.80 Å) ＞ **45** (2-CF_3, 2.20 Å) suggested that the steric effect (Van der Waals radius) in the 2-position might influence agonistic activity of FFA1.[51] This phenomenon also appeared in the naphthalene ring (**35**), seen as cyclic analogue of 2-methyl benzene (**36**). These results further demonstrated the SAR of the present series was different from the TAK-875 series which obtained the best activity by introducing substituents in the 2-position of terminal benzene ring.[16-18, 52] Given the dihedral angle between thiazole core and the terminal benzene ring with substituents in the 4-position ＜ 3-position ＜ 2-position (**Table 3**), which revealed a good correlation with FFA1 agonistic activity: the agonistic activity of the *para*-substituted compounds (**38**, **41**, **44** and **47**) ＞ *meta*-substituted compounds (**37**, **40**, **43** and **46**) ＞ *ortho*-substituted compounds (**36**, **39**, **42** and **45**), respectively. Therefore, the abnormal SAR could be attributed to the larger space restriction induced by the larger dihedral angle, which

changed the favorable conformation especially blocked the hydrophobic interaction of methyl group on thiazole. With this beneficial experience for avoid substitution at *ortho*-position, the 3,5-disubstituted (**49**) and 3,4,5-trisubstituted methoxy group (**50**) were introduced to increase the solvation effects with the water molecules which are exposed outside the receptor, but the agonistic activity of them was inferior to **48** (4-methoxy group), implying that the polysubstituted methoxy groups may introduce an unfavorable steric interaction with the surface of FFA1. After evaluation of SAR in all the previously described areas, a clear SAR picture and the key pharmacophore of this chemical scaffold were developed and summarized in **Figure 5**.

Put Table 3 and Figure 5 here

2.2.2. Oral Glucose Tolerance Test evaluated in the normal mice

Based on these results above, the optimized compounds **38**, **41**, **43** and **44** were selected to evaluate the *in vivo* hypoglycemic effects in normal ICR mice by OGTT. The time-dependent changes of blood glucose and the area under the curve ($AUC_{0\text{-}2h}$) of the plasma glucose levels are shown in **Figure 6**. All of the selected compounds showed good hypoglycemic activity in accordance with *in vitro* agonistic activity. Among them, the hypoglycemic effect of compound **44** was in close proximity to the positive control TAK-875, the most advanced compound once in phase III studies.

Put Figure 6 here

2.2.3. Dose-response relationship of 44 explored in normal ICR mice

On the basis of the *in vivo* hypoglycemic effects, the most potent compound **44** (10, 30 and 60 mg/kg) was further investigated for the dose-response relationship of lowering blood glucose level in normal mice. As shown in **Figure 7A**, compound **44** demonstrated a dose-proportional decrease in blood glucose levels with comparable efficacy at 30 mg/kg compared to TAK-875 at 20 mg/kg during an OGTT. Furthermore, the plasma glucose curve of **44** tended to flat after 60 min at the dose of 60 mg/kg. This result might be at least in part rationalized by the low risk of

hypoglycemia showed in **44**.

Put Figure 7 here

2.2.4. Effects of 44 on the risk of hypoglycemia

Obtaining a positive result in pharmacological study, the risk of hypoglycemia was subsequently assessed in fasting normal ICR mice by oral administrating a high dose of **44** in comparison with glibenclamide to further confirm the above speculation. As shown in **Figure 7B**, glibenclamide (15 mg/kg) lowered plasma sugar levels far below normal fasting levels. In contrast, compound **44**, even at the high dose of 60 mg/kg, only slightly reduced fasting glucose levels in ICR mice, and the change of plasma glucose levels was much smaller than that caused by administration of glibenclamide. Thus, our results indicated that compound **44** revealed a low risk of hypoglycemia, a serious adverse effect to sulfonylureas, which are widely used as one of the first-line drugs for the treatment of T2DM.

2.2.5. Hypoglycemic effects of 44 explored in type 2 diabetic C57BL/6 mice

To further assess antihyperglycemic effects of **44** in the diabetic state, STZ-induced type 2 diabetic C57BL/6 mice were used to evaluate the OGTT of **44**, the most potent agonist among our synthetic compounds. As shown in **Figure 8**, the compound **44** was significantly improved the hyperglycemia state of diabetic mice as TAK-875. The results demonstrated that compound **44**, with a low risk of hypoglycemia, has a great potential for improving the diabetic state.

Put Figure 8 here

2.2.6. Effects of 44 on the risk of liver toxicity

For evaluating the risk of liver toxicity, a 30 days chronic study was subsequently carried out in normal ICR mice. In this study, vehicle, TAK-875 (20 mg/kg, 38 mmol/kg) and **44** (30 mg/kg, 75 mmol/kg) was orally administered to normal mice once daily for 30 days. At the end of study, several parameters indicative of liver function including alanine aminotransferase (ALT), aspartate

transaminase (AST) and total bilirubin (TBIL) in serum were measured. As shown in **Table 4**, the ALT and AST, the main biomarkers of liver injury, was significantly increased in TAK-875 group compared with vehicle group, the result demonstrated a relatively high risk of liver toxicity observed in TAK-875. Gratifyingly, the compound **44** group, even at the twice molar dose of TAK-875, showed comparable values of ALT and AST similar to that of vehicle group. These results suggested that compound **44**, with lower molecular weight and lipophilicity, successfully reduced the risk of liver toxicity compared to TAK-875.

Furthermore, in this chronic **44** treatment study, no particular side effects were observed at the compound **44** group, indicating the possibility that minimized off-target pharmacology and metabolic toxicity.

Put Table 4 here

3. Conclusion

With the aim of developing potent FFA1 agonists with reduced lipophilicity to decrease the risk of liver toxicity, we have identified a new series of thiazole FFA1 agonists by comprehensive evaluating 11 heteroaromatics with the combination of different heteroatom number and location. Subsequently, the optimal lead compound **26** was selected to replace the terminal benzene ring with a series of heterocycles, and various substituents in the terminal phenyl of **26** were also introduced to optimize the agonistic activity. Among them, the preferred compound **44** exhibits potent agonistic activity on FFA1 and produces a robustly hypoglycemic effect both in normal and type 2 diabetic mice. Besides, compound **44**, even at the high dose of 60 mg/kg, revealed a low risk of hypoglycemia. In further studies, a 30 days chronic **44** treatment study was carried out in normal mice, even at the twice molar dose of TAK-875, revealed a lower risk of liver toxicity compared with TAK-875, and no particular side effects were observed at compound **44** treated group. All of these results demonstrated that compound **44** was meaningful for further investigation, and the information obtained from the SAR studies in our thiazole series might help to design more active FFA1 agonists that are structurally related.

4. Experimental section

4.1. General chemistry

All starting materials, reagents and solvents were obtained from commercial sources and used without further purification unless otherwise indicated. Purifications by column chromatography were carried out over silica gel (200-300 mesh) and monitored by thin layer chromatography performed on GF/UV 254 plates and were visualized using UV light at 254 and 365 nm. Melting points were taken on a RY-1 melting-point apparatus and were uncorrected. NMR spectra were recorded on a Bruker ACF-300Q instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR spectra), chemical shifts are expressed as values relative to tetramethylsilane as internal standard, and coupling constants (*J* values) were given in hertz (Hz). LC/MS spectra were recorded on a Waters liquid chromatography-mass spectrometer system (ESI). Elemental analyses were performed by the Heraeus CHN-O-Rapid analyzer. TAK-875 was synthesized as previously reported.[15]

The physical characteristics, ^{1}H NMR, ^{13}C NMR, MS and elemental analysis data for all intermediates and target molecules, were reported in the supporting information.

4.2. Molecular modeling

Docking simulations were performed using MOE (version 2008.10, The Chemical Computing Group, Montreal, Canada). The crystal structure of FFA1 (PDB ID: 4PHU) was downloaded from the Protein Data Bank (PDB). Prior to ligand docking, the structure was prepared with Protonate 3D and a Gaussian Contact surface was draw around the binding site. Subsequently, the active site was isolated and the backbone was removed. The ligand poses was filtered using Pharmacophore Query Editor. The compound structures were placed in the site with Pharmacophore method and then ranked with the London dG scoring function. For the energy minimization in the pocket, MOE Forcefield Refinement was used and ranked with the London dG scoring function.

4.3. Biological methods

4.3.1. Ca^{2+} influx activity of CHO cells expressing human FFA1 (FLIPR Assay)

CHO cells stably expressing human FFA1 (accession no. NM_005303) were seeded into 96-well plates at a density of 15K cells/well and incubated 16 h in 5% CO_2 at 37 °C. After, the culture medium in the wells was removed and washed with 100 μL of Hank's Balanced Salt Solution.

Then, cells were incubated in loading buffer (containing 2.5 μg/mL fluorescent calcium indicator Fluo 4-AM, 2.5 mmol/L probenecid and 0.1% fatty acid-free BSA) for 1 h at 37 °C. Various concentrations of test compounds or γ-linolenic acid (Sigma) were added into the cells and the intracellular calcium flux signals were monitored by FLIPR Tetra system (Molecular Devices). The agonistic activities of test compounds on human FFA1 were expressed as [(A−B)/(C−B)]×100 (increase of the intracellular Ca^{2+} concentration (A) in the test compounds-treated cells and (B) in vehicle-treated cells, and (C) in 10 μM γ-linolenic acid-treated cells). EC_{50} value of selected compound was obtained with Prism 5 software (GraphPad).

4.3.2. Animals and Statistical Analysis of the Data

Male ICR mice (18-22 g) and male C57BL/6 mice (18-22 g) were purchased from Comparative Medicine Centre of Yangzhou University (Jiangsu, China), acclimatized for 1 week before the experiments. The animal room was maintained under a constant 12 h light/black cycle with temperature at 23 ± 2 °C and relative humidity 50 ± 10% throughout the experimental period. Mice were allowed ad libitum access to standard pellets and water unless otherwise stated, and the vehicle used for drug administration was 0.5% sodium salt of Caboxy Methyl Cellulose aqueous solution for all animal studies. All animal experiments were performed in compliance with the relevant laws and institutional guidelines, and our experiments have been approved by the institutional committee of China Pharmaceutical University.

Statistical analyses were performed using specific software (GraphPad InStat version 5.00, GraphPad software, San Diego, CA, USA). Unpaired comparisons were analyzed using the two-tailed Student's t-test, unless otherwise stated.

4.3.3. Oral Glucose Tolerance Test in male ICR mice

Normal ICR mice 10 weeks old were fasted overnight (12 h), weighted, bled *via* the tail tip, and randomized into 8 groups (n = 6). Mice were administrated orally with a single doses of vehicle, TAK-875 (10 $mL \cdot kg^{-1}$; 20 $mg \cdot kg^{-1}$; 38 $mmol \cdot kg^{-1}$), or selected compounds (10 $mL \cdot kg^{-1}$; 38 $mmol \cdot kg^{-1}$) and subsequently dosed orally with 30% glucose aqueous solution (3 $g \cdot kg^{-1}$) after half an hour. Blood samples were collected immediately before drug administration (-30 min), before glucose challenge (0 min), and at 15, 30, 45, 60 and 120 min post-dose. The blood glucose was

measured by blood glucose test strips (SanNuo ChangSha, China).

4.3.4. Dose-response relationship of 44 explored in male ICR mice

To investigate dose-response relationship of **44**, normal ICR mice 10 weeks old were fasted overnight (12 h), weighted, bled *via* the tail tip, and randomized into 5 groups (n = 6). Mice were administrated orally with a single doses of vehicle, TAK-875 (10 $mL \cdot kg^{-1}$; 20 $mg \cdot kg^{-1}$), or compound **44** (10 $mg \cdot kg^{-1}$, 30 $mg \cdot kg^{-1}$, 60 $mg \cdot kg^{-1}$) and subsequently dosed orally with 30% glucose aqueous solution (3 $g \cdot kg^{-1}$) after half an hour. Blood samples were collected immediately before drug administration (-30 min), before glucose challenge (0 min), and at 15, 30, 45, 60 and 120 min post-dose. The blood glucose was measured by blood glucose test strips (SanNuo ChangSha, China).

4.3.5. Effects of 44 on the risk of hypoglycemia

10 weeks old male normal ICR mice were fasted overnight and randomized into 3 groups (n = 6). Compound **44** (60 $mg \cdot kg^{-1}$), glibenclamide (15 $mg \cdot kg^{-1}$), or vehicle was orally administered, and blood was collected from tail vein immediately before administration (0 h) and at 30, 60, 90, 120 and 180 min after administration and measure blood glucose as described above.

4.3.6. Hypoglycemic effects of 44 explored in type 2 diabetic mice

Male C57BL/6 mice after 1 week adaptation were fed with high-fat diet (45% calories from fat, from Mediscience Ltd., Yangzhou, China) ad libitum for 4 weeks to induce insulin resistance and then injected intraperitoneally (i.p.) with low dose of STZ (10 $mL \cdot kg^{-1}$; 80 $mg \cdot kg^{-1}$). The mice were fed with high-fat-diet for another 4 weeks, and the development of diabetes was confirmed by measuring blood glucose levels. The mice with fasting blood glucose level 11.1 mmol/L or higher were considered to be diabetic and were used in the experiment as type 2 diabetic mice model.[53, 54]

Type 2 diabetic C57BL/6 mice were fasted overnight (12 h), weighted, bled via the tail tip, and randomized into 3 groups (n = 6), another group of normal fasting C57BL/6 mice was added as negative control. Mice were administrated orally with a single doses of vehicle, TAK-875 (10 $mL \cdot kg^{-1}$; 20 $mg \cdot kg^{-1}$), or compound **44** (10 $mL \cdot kg^{-1}$; 30 $mg \cdot kg^{-1}$) and subsequently dosed orally

with 20% glucose aqueous solution (2 g·kg^{-1}) after half an hour. Blood samples were collected immediately before drug administration (-30 min), before glucose challenge (0 min), and at 15, 30, 45, 60 and 120 min post-dose. The blood glucose was measured by blood glucose test strips (SanNuo ChangSha, China).

4.3.7. Risk of liver toxicity of 44 evaluated in normal ICR mice

Normal ICR mice were dosed daily with the vehicle, TAK-875 (10 mL·kg^{-1}; 20 mg·kg^{-1}) or compound **44** (10 mL·kg^{-1}; 30 mg·kg^{-1}) by gavage administration for 30 days. The body weights were measured every 5 days and the dosage was adjusted according to the most recent body weight. All animals were observed daily and any abnormal state were recorded. At the end of treatment, mice were fasted overnight (12 h), blood samples were drawn from orbit and centrifuged at 3500 rpm/min for 10 min to separate serum. ALT, AST and TBIL in serum were measured using automatic biochemical analyzer (Beckman Coulter, AU5811, Tokyo, Japan).

Acknowledgements

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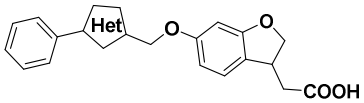
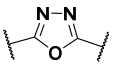
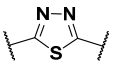
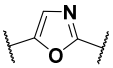
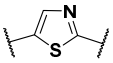
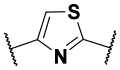
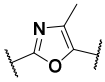
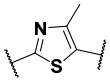
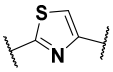
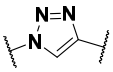
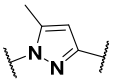
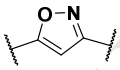
Table 1: *In vitro* activities and select physicochemical properties of 11 heteroaromatics **20** to **30**



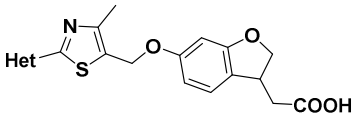
compd	Het	Act%(300nM)[a]	Act%(100nM)[b]	Mw	clogP[c]
TAK-875		76.01	65.32	524.63	4.697
20		6.31	1.54	352.35	1.705
21		13.65	3.36	368.41	2.507
22		10.15	2.34	351.36	2.932
23		19.76	5.26	367.42	3.597
24		0.23	0.16	367.42	3.597
25		21.17	7.15	365.39	3.403
26		67.41	54.81	381.45	4.298
27		5.67	0.15	367.42	3.597
28		0.89	0.76	351.36	2.702
29		1.23	0.98	364.40	3.73
30		1.67	0.84	351.36	3.232

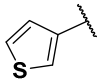
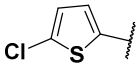
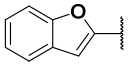
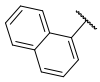
[a] Agonist activities mean values at a screening concentration of 300 nM were obtained from three independent experiments.

[b] Agonist activities mean values at a screening concentration of 100 nM were obtained from three independent experiments.

[c] clogP values were estimated with ChemDraw Ultra, version 12.0.

Table 2: *In vitro* activities and physicochemical properties of designed compounds **31** to **35**



compd	Het	Act%(100nM)[a]	hFFA1 EC_{50} (nM)[b]	Mw	clogP[c]
TAK-875		65.32	29.6	524.63	4.697
26		54.81	77.3	381.45	4.298
31		35.15	ND	387.47	4.189
32		56.47	68.5	387.47	3.979
33		8.34	ND	421.91	4.923
34		14.78	ND	421.47	5.068
35		8.53	ND	431.51	5.472

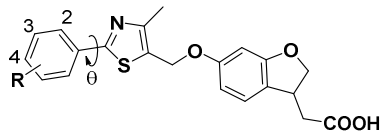
ND = Not determined.

[a] Agonist activities mean values at a screening concentration of 100 nM were obtained from three independent experiments.

[b] EC_{50} values for FFA1 activities represent the mean of three determinations.

[c] clogP values were estimated with ChemDraw Ultra, version 12.0.

Table 3: *In vitro* activities and dihedral angle of designed compounds **36** to **50**



compd	R	Act% (100nM)[a]	hFFA1 EC_{50} (nM)[b]	Calcd dihedral angle θ (deg)[c]
TAK-875		65.32	29.6	89.6
26	H	54.81	77.3	4.9
36	2-Me	10.68	ND	50.4
37	3-Me	47.16	118.5	12.6
38	4-Me	54.85	73.5	3.2
39	2-Cl	12.36	ND	46.7
40	3-Cl	42.97	137.5	13.3
41	4-Cl	53.35	85.6	7.8
42	2-F	25.76	ND	36.5
43	3-F	56.90	64.8	10.6
44	4-F	61.63	48.7	7.4
45	2-CF_3	7.76	ND	58.6
46	3-CF_3	35.68	ND	15.3
47	4-CF_3	50.35	96.7	4.8
48	4-OMe	46.78	123.4	4.3
49	3,5-diOMe	17.53	ND	6.3
50	3,4,5-triOMe	7.47	ND	16.6

ND = Not determined.

[a] Agonist activities mean values at a screening concentration of 100 nM were obtained from three independent experiments.

[b] EC_{50} values for FFA1 activities represent the mean of three determinations.

[c] Dihedral angle between thiazole core and the terminal benzene ring was calculated by Dihedral Plot in MOE, version 2008.10.

Table 4: Effects of **44** on the values of AST, ALT and TBIL

Groups	ALT (IU/L)	AST (IU/L)	TBIL (IU/L)
Vehicle	35.0±4.3	133.1±13.1	4.1±0.4
TAK-875 (20 mg/kg)	48.3±4.7$^{\#}$	181.1±15.3$^{\#}$	4.0±0.8
44 (30 mg/kg)	37.5±4.1*	141.8±14.8*	4.0±0.5

Results are expressed as mean ± SD for six mice in each group. $^{\#}p \leqslant 0.05$ compared to vehicle group by Student's t-test. $^{*}p \leqslant 0.05$ compared to TAK-875 group by Student's t-test.

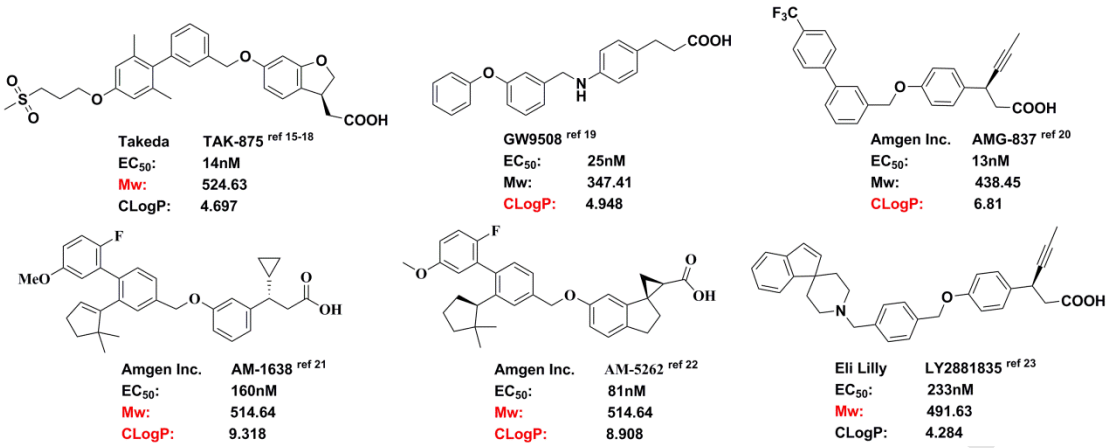


Figure 1: Selected examples of synthetic GPR40 agonists. clogP values are calculated with ChemDraw Ultra 12.0 using the "clogP" option.

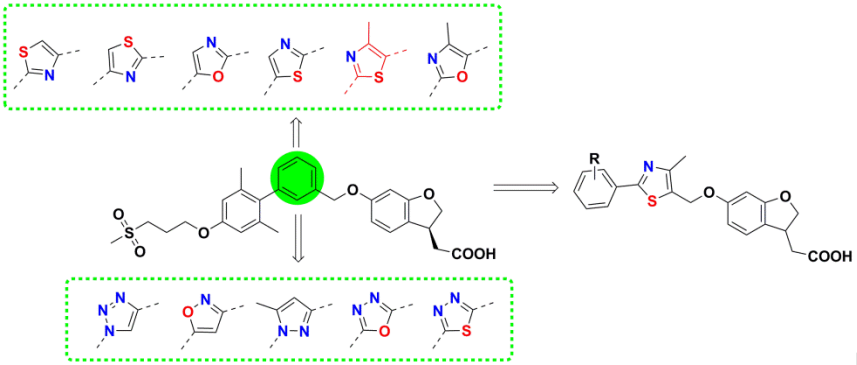


Figure 2: Our strategy to decrease the lipophilicity of TAK-875 by replacing the middle benzene ring with 11 heteroaromatics.

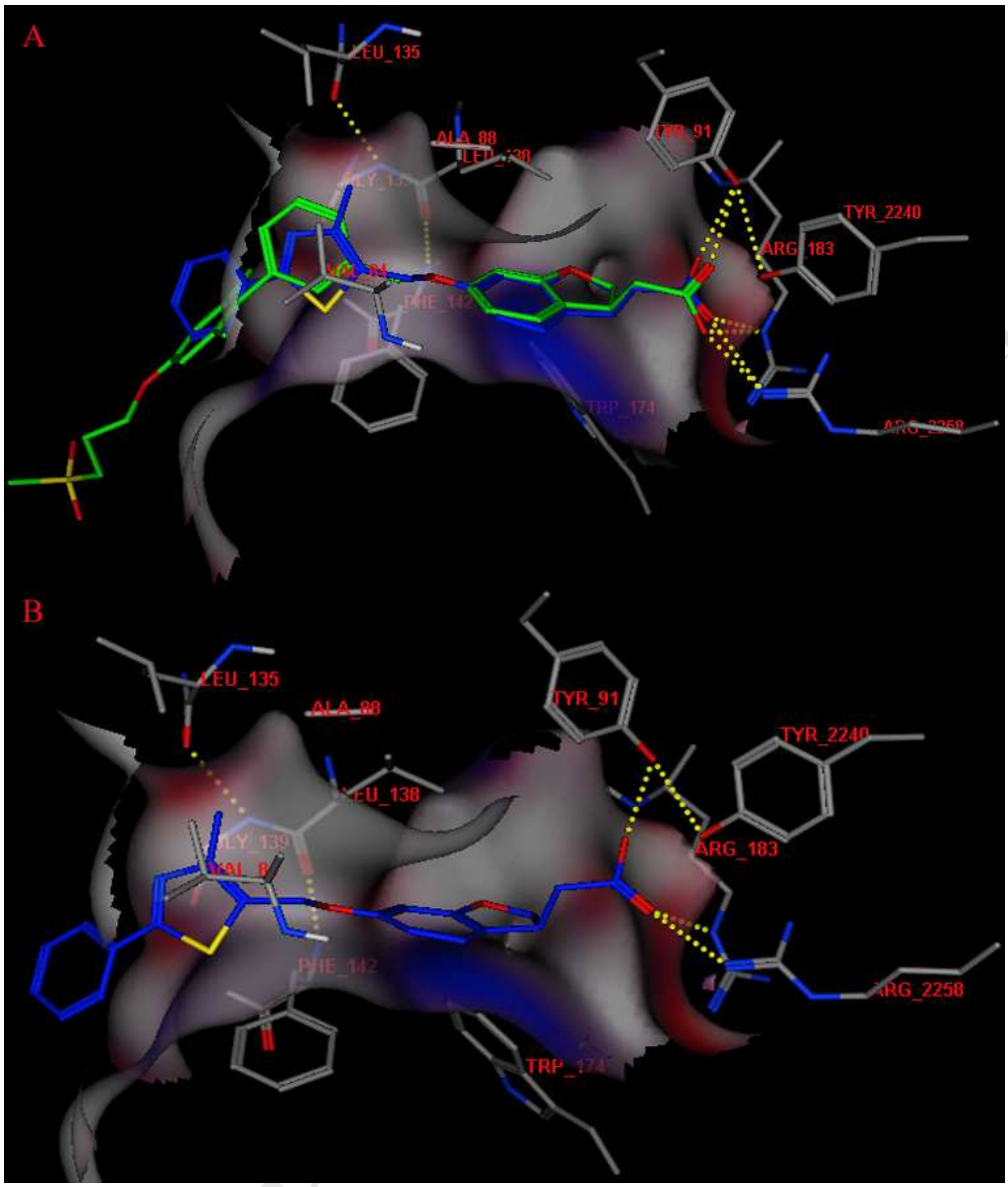


Figure 3: The interaction mode of compound **26** bound to FFA1. Key residues are labeled in red, and hydrogen bonding interactions are represented by yellow dashed lines. (A) Overlay of TAK-875 (green) and **26** (blue) bound to FFA1. (B) Compound **26** bound to FFA1.

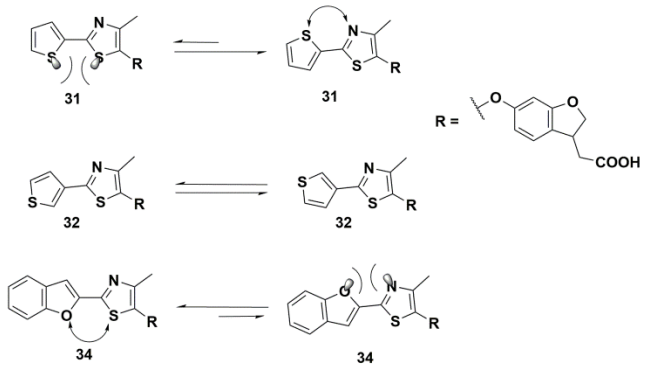
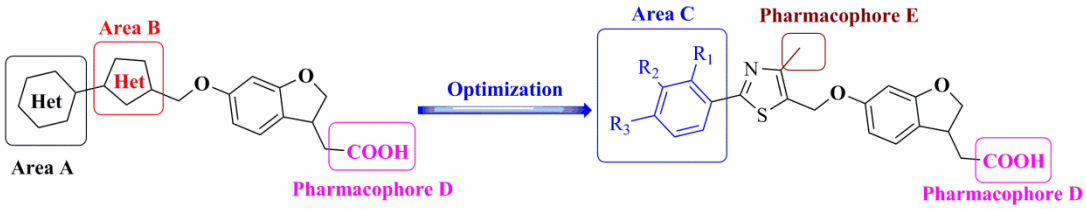


Figure 4: Presumed conformational preference based on the interaction between the lone pair (O and N) and C−S σ* orbitals (noncovalent N···S interaction and O···Sinteraction).



Area A: Other than the bulkier heterocycles, certain heterocycles are tolerated in this area. The kinds and location of heteroatom have a significant influence on the activity mediated by the noncovalent sulfur interaction. Among various heterocycles, 3-thiophene showed the strongest activity.

Area B: This area have a strict requirement for electrical distribution and hydrophobicity. Those properties were crucial for interactions around this area, even affect the interactions of terminal carboxyl with receptor by conformation "cross-talk". After a comprehensive evaluation on various heterocycles, thiazole with a methyl group in 4-position showed the strongest activity.

Area C: *Ortho*-substituents are not tolerated, as the size increases the potency decreases (H > F > Cl > Me > CF_3). Agonistic activity of a substituent in the $R_3 > R_2 > R_1$. Other than the steric effect, the electronic nature of substituents don't have a significant effect on the activity. The polysubstituted bulkier groups were also not tolerated even substituent in the R_3 and R_2.

Pharmacophore D: The carboxylate moiety, form hydrogen bonding network with receptor, was crucial for activity. Once the hydrogen bonding network was disturbed by unfavorable conformation, the agonistic activity will be significantly reduced.

Pharmacophore E: The methyl group, the most important fragment in the thiazole core, specifically occupied a small hydrophobic subpocket which had no interactions with TAK-875.

Figure 5: SARs and the key pharmacophore summary.

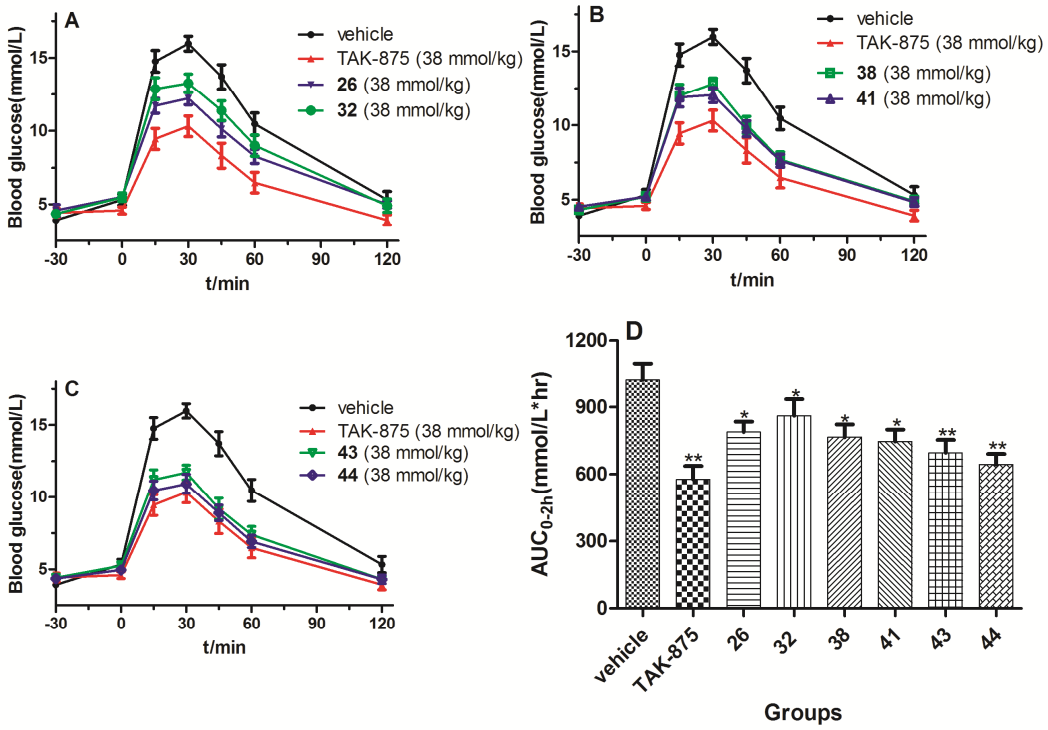


Figure 6: Effects of compounds on blood glucose levels during an OGTT in normal ICR mice. A, B and C represent time dependent changes of plasma glucose levels. D shows the $AUC_{0\text{-}2h}$ of blood glucose levels. Values are mean ± SEM (n = 6). *p≤0.05, **p≤0.01 compared to vehicle normal mice by Student's t-test.

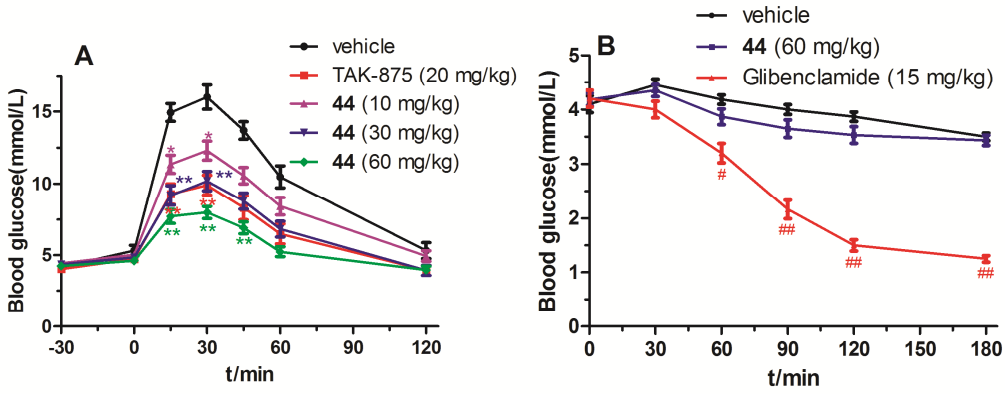


Figure 7: (A) Dose–response relationship of **44** explored in normal ICR mice. (B) Effects of **44** on fasting plasma glucose in normal mice. Values are expressed as mean ± SEM for six animals in each group. *p≤0.05, **p≤0.01 compared to vehicle normal mice by Student's t-test. $^{\#}$p≤0.05, $^{\#\#}$p≤0.01 compared to **44** treated mice by Student's t-test.

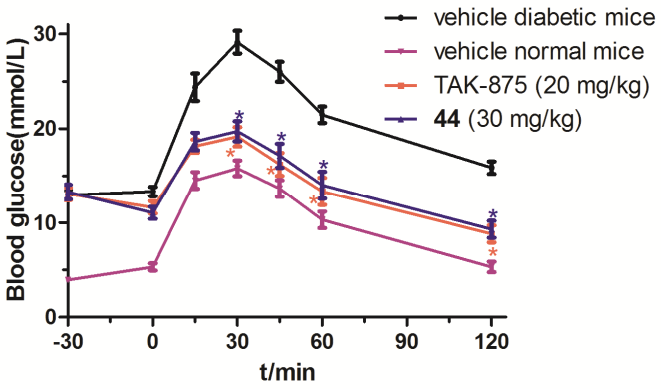
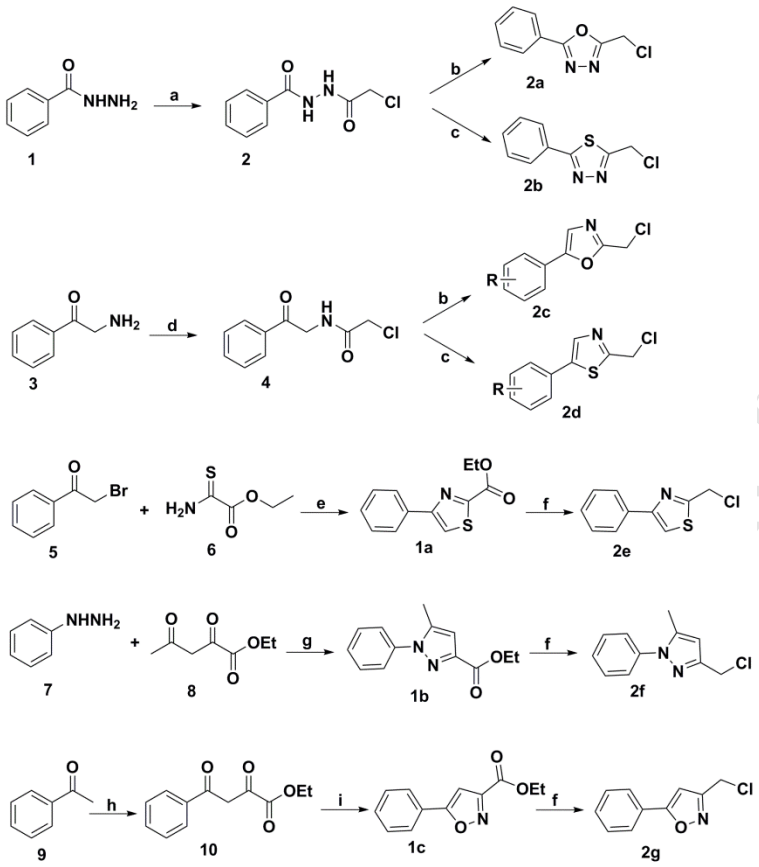
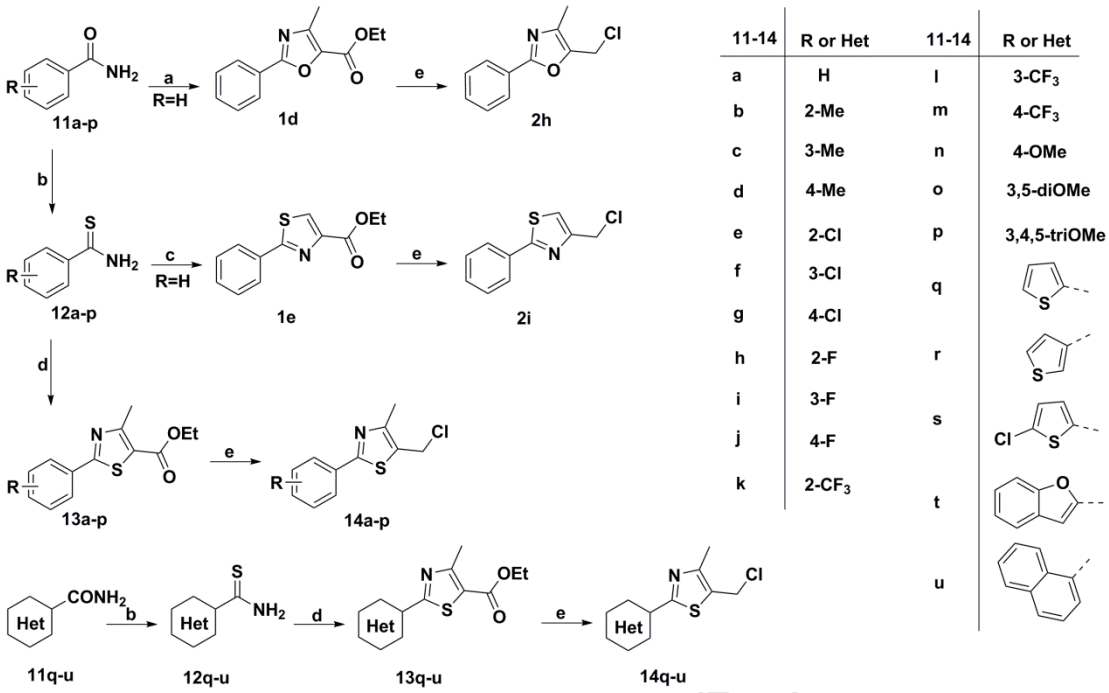


Figure 8: Effects of **44** on blood glucose levels during an OGTT in fasting type 2 diabetic C57BL/6 mice. Values are mean ± SEM (n = 6). *P≤0.05 compared to vehicle diabetic mice by Student's t test.

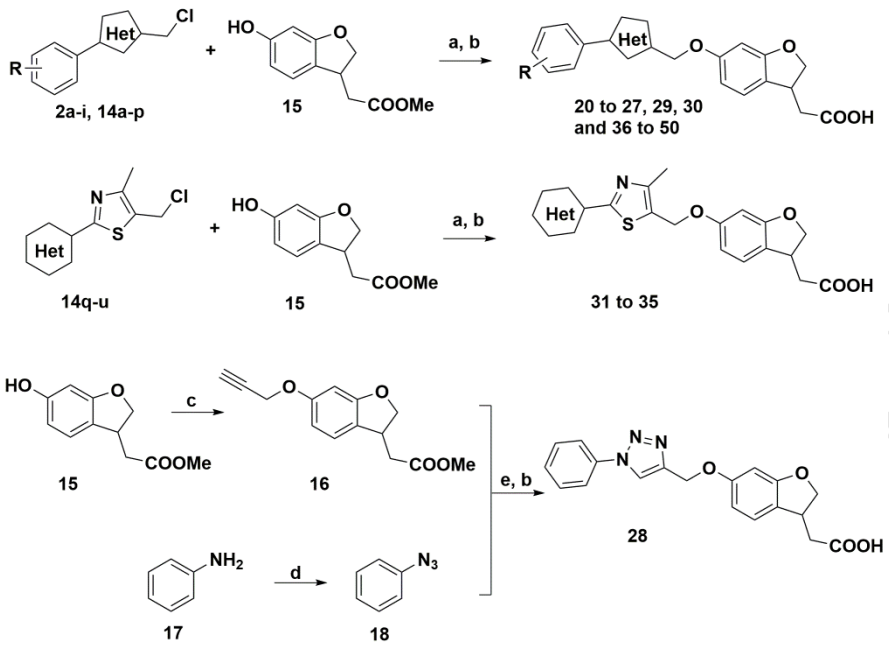


Scheme 1. Synthesis of intermediates **2a-g**. Reagents and conditions: (a) Choroacetyl chloride, NaOH, 0˚C, 1 h, 68.2%; (b) $POCl_3$, acetonitrile, reflux, 4 h, 66.5-76.1%; (c) Lawesson's reagent, THF, reflux, 4 h, 60.3-75.7%; (d) Choroacetyl chloride, trimethylamine, 0-rt, 16 h, 62.3%; (e) EtOH, reflux, 6 h, 74.3%; (f) (1) $NaBH_4$, MeOH, THF, reflux; (2) $SOCl_2$, CH_2Cl_2, DMF, 40 °C, 4h; (g) EtOH, reflux, 2 h, 40.3%; (h) Diethyl oxalate, NaOEt, 0-rt, 18 h, 76.7%; (i) Hydroxylamine hydrochloride, EtOH, reflux, 2 h; 60.7%.



11-14	R or Het	11-14	R or Het
a	H	l	3-CF_3
b	2-Me	m	4-CF_3
c	3-Me	n	4-OMe
d	4-Me	o	3,5-diOMe
e	2-Cl	p	3,4,5-triOMe
f	3-Cl	q	
g	4-Cl		
h	2-F	r	
i	3-F	s	
j	4-F		
k	2-CF_3	t	
		u	

Scheme 2. Synthesis of intermediates **2h-i** and **14a-u**. Reagents and conditions: (a) ethyl 2-chloroacetoacetate, EtOH, reflux, 16 h, 42.1%; (b) Lawesson's reagent, THF, reflux, 4 h; (c) ethyl bromopyruvate, EtOH, reflux, 4 h, 76.7%; (d) ethyl 2-chloroacetoacetate, EtOH, reflux, 6 h, 61.5 - 92.3%; (e) (1) $NaBH_4$, MeOH, THF, reflux; (2) $SOCl_2$, CH_2Cl_2, DMF, 40 °C, 4 h.



Scheme 3. Synthesis of target compounds **20** to **50**. Reagents and conditions: (a) K_2CO_3, acetone, KI, 45 °C, 12 h; (b) $LiOH \cdot H_2O$, THF/MeOH/H_2O, rt, 4 h; (c) 3-bromoprop-1-yne, K_2CO_3, acetone, reflux, 8 h; (d) $NaNO_2$, 50% acetic acid, then NaN_3, 0–5 °C, 84.2%; (e) ascorbate sodium, $CuSO_4$, 80% MeOH, rt, 20 h, 89.5%.

Highlights

- 11 heterocycles were synthesized to improve the lipophilicity of TAK-875.
- The methyl group in our thiazole core occupied a crucial hydrophobic subpocket.
- The agonistic activity revealed a good correlation with the dihedral angle.
- **44** revealed lower risk of liver toxicity compared with TAK-875.
- **44** showed lower risk of hypoglycemia compared to first-line drug glibenclamide.