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# Synthesis and biological evaluation of sulfonylurea and thiourea derivatives substituted with benzenesulfonamide groups as potential hypoglycemic agents

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# ABSTRACT

A novel class of sulfonylurea and thiourea derivatives substituted with benzenesulfonamide groups were designed and synthesized. The target compounds were assayed for the effects on the insulin release of isolated rat pancreatic islets and the glucose transport in adipocytes of rats. Some of them exhibited high potency. Compound **10** also had potent antiplatelet activity and showed an excellent property to protect collagen–epinephrine-induced mice mortality as well as plasma glucose-lowering activity in vivo. The preliminary pharmacological profile of compound **10** showed that it might be useful in the treatment of diabetics with cardiovascular and nephropathy complications.

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Patients with diabetes mellitus are at high risk for developing long-term complications including neuropathy, nephropathy, retinopathy, and cardiovascular disease.<sup>1</sup> Atherosclerotic macrovascular disease accounts for the majority of morbidity and mortality associated with diabetes.<sup>2</sup> Diabetic nephropathy, as a serious chronic diabetic microvascular complication, has become a single most important cause of end-stage renal disease.<sup>3</sup> So a more comprehensive approach aimed at reducing cardiovascular and nephropathic complications as well as controlling glucose excursion may prove more beneficial to diabetic patients.

The increased atherosclerotic risk in diabetic patients<sup>4</sup> was associated with many factors, mainly including hyperglycemia, insulin resistance, pro-inflammatory and prothrombotic status.<sup>5</sup> In particular, the prothrombotic status is related to endothelial dysfunction, impaired fibrinolysis, increased coagulation factors, and importantly, increased platelet reactivity.<sup>6,7</sup> Antiplatelet drugs may have therapeutic potential in prevention or treatment for diabetic cardiovascular complications. On the other hand, elevated glucose in diabetes has long been recognized to increase levels of arachidonic acid metabolites, many of which stimulate TXA<sub>2</sub>-PGH<sub>2</sub> receptor (TPr).<sup>8,9</sup> The TXA<sub>2</sub>/PGI<sub>2</sub> ratio of diabetics was significantly higher than that of healthy volunteers.<sup>10</sup> Earlier studies with TXA<sub>2</sub> receptor antagonists Bay U3405<sup>11</sup> and S-1452<sup>12</sup>in dia-

betic rats suggested that endogenous TXA<sub>2</sub> receptor agonists stimulate the development of diabetic nephropathy.<sup>13</sup>

There were four main pathways proposed for the mechanisms of hyperglycemia-associated tissue damage: the polyol pathway, the hexosamine pathway, protein kinase C activation, and the formation of advanced glycation end-products (AGE). But there was no apparent common element linking these mechanisms to each other. Clinical trials of inhibitors of these pathways in patients were also disappointing. A unifying mechanism proposed by Michael Brownlee<sup>14</sup> is the overproduction of superoxide by the mitochondrial electron transport chain. Increased production of ROS and oxidative stress is assumed to play a central role in the pathogenesis of diabetes complications.

S18886 (terutroban), a benzenesulfonamide-substituted tetrahydronaphthalene derivative, is an orally active, high potent, selective TPr antagonist.<sup>15</sup> It exerts potent antiplatelet and antivasoconstrictory effects via the TPr and antagonizes the actions of TXA<sub>2</sub>, as well as of other arachidonic acid products.<sup>16</sup> The antiatherosclerotic effect of S18886 has also been demonstrated in rabbits.<sup>17,18</sup> Several articles published recently had reported that S18886 can significantly inhibit atherogenesis in the non-diabetic and diabetic ApoE<sup>-/-</sup> mice.<sup>19,20</sup> It has also been reported that S18886 had the nephroprotective action in the diabetic ApoE<sup>-/-</sup> mice.<sup>20</sup> ApoE<sup>-/-</sup> mice are animals genetically deficient in TPr, so the mechanism of antiatherosclerotic and nephroprotective action of S18886 could be independent of platelet-derived TXA<sub>2</sub>.<sup>19</sup> Further studies indicated that S18886 can enhance the antioxidative defense.<sup>21</sup> The effect of inhibiting TPr can be attributed to a novel role

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of TPr in mediating oxidant stress in the kidney. TP receptors play an important role in atherogenesis and nephropathy which are closely related to diabetes.

Although the source of ROS is controversial<sup>22</sup> and the most elements in oxidant stress remains uncertain, the potent therapeutic potential of TPr antagonist in non-diabetic and diabetic atherosclerosis and early diabetic nephropathy is conceivable. With its important role in the oxidant stress, TPr antagonism can enhances the antioxidative defense, thereby effectively reducing ROS-induced downstream tissue damage in the development of diabetic cardiovascular and nephropathy complications.

Doltroban (BM 13,505) and sulotraban (BM 13,177) are two early reported platelet  $TXA_2$  receptor antagonists<sup>23</sup> The benzenesulfonamide is the common moiety in their structures and has been proven to be the pharmacophore of the TPr antagonists. S18886 also has the similar structural backbone with doltroban and sulotraban (as seen in Figure 1).

The sulfonylureas, for example, glimepiride and glibenclamide, were the most widely used oral antidiabetic drugs in clinic. Glimepiride also has blood platelet aggregation inhibiting property. So we hypothesized that TPr antagonistic and hypoglycemic activities may have some inherent relevancy with compounds with benzenesulfonamide and sulfonylurea structures.

The immediate medicinal chemistry strategy became the replacement of the benzoyl or heterocyclic formacyl groups, which are commonly present in sulfonylurea drugs, with benzenesulfonamide group which is the functional group of the blood platelet TXA<sub>2</sub> receptor antagonists. We also changed the urea group with its bioisosterist thiourea to check the change of activity. From these results, we designed a fused skeleton of doltroban and glimepiride structures (Scheme 1) with the expectation that the target compounds will show effects on diabetic complications such as nephropathy and atherosclerosis as well as hypoglycemic activity. We reported here the synthesis of new sulfonylurea and thiourea derivatives substituted with benzenesulfonamide groups and their in vitro and in vivo pharmacological characterization of hypoglycemic activit.

The target compounds were prepared as presented in Scheme 1. The synthesis started with the acetylation of phenylethylamine 1 with acetic anhydride in triethylamine under room temperature, to form *N*-acetylphenylethylamine **2**. Chlorosulfonation of **2** with chlorosulfonyl acid gave 4-(2-acetylaminoethyl) benzenesulfonyl

chloride 3, which was converted into the corresponding benzenesulfonamide 4 by stirring overnight in concentrated ammonia water. Hydrolysis of compound 4 with sodium hydroxide solution afforded the key intermediate 4-(2-aminoethyl) benzenesulfonamide 5. The benzenesulfonamide 5 reacted with substituted benzenesulfonyl chloride to form 4-(2-substituted benzenesulfonyl aminoethyl) benzenesulfonamide 6. The target compounds 7-25 were prepared by the reaction of compound 6 with isocyanate or isothiocyanate. It was reported that the reaction between compound **6** and isocyanate should be under the condition of strong base in anhydrous solvents.<sup>24</sup> We treated compound **6** with anhydrous potassium carbonate in refluxing anhydrous acetone for 6 h, to form potassium salt of compound **6**, then added the solution of isocyanate in anhydrous acetone dropwise to the potassium salt above, refluxed for another 6 h, we got the target compounds in reasonable vields (56.5–92.4%).<sup>25</sup>

The target compounds described in Scheme 1 were tested for in vitro insulin secretion activity with isolated pancreatic cells. The method was described in Ref. 26. The results were showed in Table 1.

The test demonstrated that insulin release of pancreatic cells increased by 5.49 times after glucose treatment and by 2.03 times after glimepiride (100 mol/L) compared with basal. The sulfourea derivatives with aryl substituents (**17–25**) showed very little activity. The urea compounds with phenylsulfonylamide groups (**7–16**) retained insulin release activity. But the amino- or methoxyl-substituted phenylsulfonylamides (**7**, **8**, and **11**) were less active, showed moderate activity. The halo-substituted compounds (**9**, **10**, **15**, and **16**) exhibited high potent. The compounds **10** and **9** with bromo or chloro atoms on the *para*-position of phenylsulfonylamide have the highest insulin release property, increased by 3.64 and 3.05 times, respectively, and higher than that of glimepiride at the same concentration (2.03 times).

Insulin can stimulate the glucose transport in adipocytes.<sup>27</sup> Sulfonylurea drugs have the effect to increase the glucose transport. The target compounds were also tested for the effect on glucose transport in adipocytes of rats in vitro (without/with 1.2 nM insulin) following the method described.<sup>28</sup> The results were showed in Table 2.

Glimepiride has the highest activity to increase the glucose transport in all sulfonylurea drugs. Treated with glimepiride (100  $\mu$ g/ml), glucose absorption of adipocytes increased by 2.5



Figure 1. The structures of Glimepiride, compound 10, Doltroban, Sulotraban, and S1886.



Scheme 1. Reagents and conditions: (a) (CH<sub>3</sub>CO)<sub>2</sub>O, Et<sub>3</sub>N, rt; (b) ClSO<sub>3</sub>H, 0 °C, 6 h; (c) NH<sub>3</sub>·H<sub>2</sub>O, rt, 12 h, 89.9%; (d) NaOH, H<sub>2</sub>O, reflux, 3 h, 78%; (e) ArSO<sub>2</sub>Cl, K<sub>2</sub>CO<sub>3</sub>, DCM–H<sub>2</sub>O; (f) K<sub>2</sub>CO<sub>3</sub>, anhydrous acetone, reflux 6 h then add isocyanate to reflux another 6 h, 56.5–92.4%; (g) K<sub>2</sub>CO<sub>3</sub>, anhydrous acetone, isothiocyanate as (f).

 Table 1

 Effects of compounds on insulin release of cells isolated from pancreatic islets of rats

Compound	Insulin secretiona (uIU/ml) Concn. (µmol/L)			
	1	10	100	
Control				25.12 ± 1.92
Vehicle (0.25% DMSO)				23.65 ± 1.89
Glucose (25 mmol/L)				129.85 ± 11.15
Glimepiride			48.14 ± 3.13 <sup>c</sup>	
7	$30.18 \pm 2.94^{b}$	36.67 ± 3.24 <sup>c</sup>	$28.52 \pm 2.27^{a}$	
8	23.57 ± 1.45	$27.67 \pm 1.21^{a}$	$37.32 \pm 2.22^{b}$	
9	$38.15 \pm 2.44^{\circ}$	41.29 ± 3.80 <sup>c</sup>	72.23 ± 5.38 <sup>c</sup>	
10	40.79 ± 3.08 <sup>c</sup>	65.21 ± 4.26 <sup>c</sup>	86.08 ± 6.45 <sup>c</sup>	
11	24.57 ± 2.14	24.47 ± 1.33	24.83 ± 1.64	
12	22.86 ± 2.25	$28.27 \pm 1.03^{a}$	31.29 ± 2.15 <sup>b</sup>	
13	$22.45 \pm 2.04$	22.70 ± 1.31	25.93 ± 1.75	
14	$32.15 \pm 2.44^{b}$	41.29 ± 3.80 <sup>c</sup>	52.23 ± 5.38 <sup>c</sup>	
15	$33.95 \pm 2.04^{b}$	$41.18 \pm 3.16^{\circ}$	55.03 ± 5.31 <sup>c</sup>	
16	$32.09 \pm 2.68^{b}$	45.01 ± 4.07 <sup>c</sup>	65.98 ± 6.25 <sup>c</sup>	
17	$28.94 \pm 2.04^{a}$	$32.10 \pm 2.16^{b}$	26.06 ± 1.60	
18	$27.99 \pm 1.35^{a}$	$27.71 \pm 1.84^{a}$	24.97 ± 1.79	
19	22.86 ± 2.53	24.98 ± 1.23	23.42 ± 2.18	
20	$29.46 \pm 2.29^{a}$	26.37 ± 1.64	26.07 ± 2.36	
21	24.05 ± 1.34	24.36 ± 1.12	26.09 ± 2.13	
22	22.25 ± 1.54	$24.67 \pm 2.02$	26.17 ± 2.21	
23	23.89 ± 2.57	23.01 ± 2.19	22.25 ± 2.01	
24	21.99 ± 2.03	24.24 ± 1.67	20.87 ± 1.91	
25	24.48 ± 1.43	23.50 ± 1.45	23.69 ± 1.06	

Each value represents means ± SD of four determinations. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001 compared to Vehicle (0.25% DMSO). P < 0.05 was taken as the level of significance.

times compared to basic absorption. Some of the compounds tested showed relatively high activity. The compounds **10**, **23**, **11**, and **9** (100  $\mu$ g/ml) increased glucose transport by 1.73, 1.59, 1.58, and 1.57 times, respectively. Some compounds (**8**, **10**, **16**, **19**, and **23**) showed synergistic effect with insulin to increase the glucose transport. The SAR was somewhat different to that of insulin release activity.

After determining the in vitro properties of compounds, compound **10** was chosen to in vivo experiments. In the first, the fasted C57BL/6J mice were used to assess the ability of the chosen compound to lower plasma glucose level.<sup>29</sup>The results were shown in Figure 2. Three doses (20, 7, and 2.5 mg/kg) of compound **10** obviously reduced the blood glucose level of the mice and showed the remarkable dose relevance. At the dose of 20 mg/kg, the glucose-lowering property was about to 50%, the duration is more than 6 h. The potency was similar to that of glimepiride.

The compound **10** was evaluated for its ability to inhibit platelet aggregation of human platelet-rich plasma (PRP) induced by arachidonic acid or adenosine diphosphate with the method described.<sup>30</sup> The concentrations that caused 50% inhibition of platelet aggregation ( $IC_{50}$ ) was obtained by regression analysis of the concentration versus inhibition of aggregation curve. The results were shown in Table 3.

 $IC_{50}$  of compound **10** and Asp to AA were 0.21 and 0.35  $\mu$ mol/L, and  $IC_{50}$  to ADP were 3.00 and 9.34  $\mu$ mol/L, respectively. The results showed that compound **10** had potent antiplatelet activity and was higher than Asp.

Protective effect of compound **10** against collagen–epinephrineinduced mice mortality was performed on Chinese Kunming mice following the method described.<sup>31</sup> The effects of compound **10** or Asp were evaluated by measuring the incidence of death within 15 min of injection. Results are expressed as percent survival. Each group was comprised of 11 mice.

The results were shown in Table 4. Three groups (40, 20, and 10 mg/kg) of compound **10** obviously reduced mice mortality and showed the remarkable dosage relevance. At the dose of 40 mg/kg of compound **10**, the percentage of protection was 72.7% while that of ASP (60 mg/kg) was 63.6%. So compound **10** showed remarkable protective effect against collagen–epinephrine-induced mice mortality.

The preliminary pharmacokinetic properties of compound **10** was determined in rats (Table 5). It had a small volume of distribution (Vd =  $0.78 \pm 0.36 \text{ L/kg}$ ), which suggested high plasma proteinbinding rate, similar to that of other sulfonylurea drugs (e.g., Glimepiride).<sup>32</sup> The oral half-life was  $1.77 \pm 0.21$  h, and the oral bioavailability was determined to be 52.96%. Compound **10** showed similar pharmacokinetic properties with other sulfonylurea drugs.<sup>33</sup>

# Table 2 Effects of compounds on glucose transport in adipocytes of rats *in vitro* (without/with insulin)

Compound	D-1- <sup>3</sup> H-Glucose (dpm) Concn (μM, without/with 1.2 nM insulin)				
	1	10	100		
Control				4501.2 ± 278.44/	
				5379.33 ± 227.19 <sup>b</sup>	
Vehicle (0.25% DMSO)				4427.1 ± 179.05/	
				5477.12 ± 136.44 <sup>b</sup>	
Insulin (10 nM)				16239.2 ± 1131.40°	
Glimepiride	$5061.2 \pm 579.10^{a}$	$7476.6 \pm 816.5^{\circ}/$	$11068.51 \pm 792.67^{\circ}$		
	6365.67 ± 378.57 <sup>dg</sup>	8593.72 ± 382.70 <sup>th</sup>	13255.41 ± 916.73 <sup>f,h</sup>		
7	4215.36 ± 343.88/	5616.15 ± 218.29 <sup>b</sup> /	6736.82 ± 545.69 <sup>b</sup> /		
	5087.78 ± 403.47	$6200.35 \pm 159.65^{\circ}$	$6812.33 \pm 428.54^{e}$		
8	4302.19 ± 328.31/	4573.80 ± 368.89/	5207.81 ± 389.64 <sup>a</sup> /		
	6123.02 ± 202.43 <sup>dh</sup>	$6516.90 \pm 505.34^{d,h}$	7244.87 ± 322.08 <sup>eh</sup>		
9	5869.85 ± 380.34 <sup>a</sup> /	6411.70 ± 252.69 <sup>b</sup> /	6960.26 ± 545.75 <sup>b</sup> /		
	5942.68 ± 226.19	$6358.77 \pm 467.25^{d}$	7013.28 ± 327.48 <sup>e</sup>		
10	4871.74 ± 171.54 <sup>a</sup> /	6512.65 ± 288.59 <sup>b</sup> /	7674.84 ± 220.06 <sup>c</sup> /		
	5996.43 ± 229.52	7979.94 ± 183.76 <sup>e,h</sup>	8486.79 ± 303.85 <sup>th</sup>		
11	5294.78 ± 473.18 <sup>a</sup> /	6547.93 ± 511.95 <sup>b</sup> /	7037.78 ± 409.57 <sup>c</sup> /		
	5587.26 ± 307.42	$6341.17 \pm 470.32^{d}$	$7447.72 \pm 486.62^{e}$		
12		Not tested			
13	4281.30 ± 19.56/	4174.59 ± 394.56/	4458.36 ± 256.10/		
	5374.21 ± 436.29	5533.15 ± 346.28	5297.11 ± 401.64		
14		Not tested			
15	4576.35 ± 400.97/	$5878.83 \pm 420.22^{a}$	6632.70 ± 251.62 <sup>b</sup> /		
	5217.31 ± 325.19	5933.89 ± 296.52	6521.12 ± 362.28 <sup>e</sup>		
16	4421.72 ± 151.54/	4861.54 ± 169.52 <sup>a</sup> /	6556.61 ± 284.52 <sup>b</sup> /		
	5523.89 ± 425.52	$6002.46 \pm 245.55^{d}$	7896.14 ± 273.72 <sup>eh</sup>		
17		Not tested			
18	4377.95 ± 266.81/	4385.12 ± 199.67/	4663.52 ± 284.63/		
	5319.50 ± 354.27	5644.89 ± 410.65	5761.23 ± 333.33		
19	4179.50 ± 322.49/	4118.78 ± 411.45/	4366.76 ± 231.86/		
	5708.43 ± 159.63	7555.71 ± 316.32 <sup>f,i</sup>	6987.19 ± 609.10 <sup>eh</sup>		
20	4587.26 ± 156.24/	4432.15 ± 355.65/	4763.58 ± 284.30/		
	5179.30 ± 441.02	5436.58 ± 284.56	5764.25 ± 333.16		
21		Not tested			
22	4125.36 ± 225.49/	4026.52 ± 387.24/	4335.26 ± 189.61/		
	5177.84 ± 334.94	5234.61 ± 358.10	5069.25 ± 423.67		
23	5687.31 ± 214.66 <sup>b</sup> /	6514.04 ± 371.32 <sup>b</sup> /	7061.15 ± 175.07 <sup>c</sup> /		
	5115.18 ± 201.45	5512.62 ± 454.58	7832.17 ± 282.40 <sup>eg</sup>		
24	4428.46 ± 295.45/	4401.01 ± 155.02/	4513.06 ± 197.33/		
	5627.67 ± 347.18	5115.32 ± 223.52	5624.51 ± 300.39		
25	4425.16 ± 223.31/	4381.35 ± 369.15/	4550.21 ± 292.13/		
	5026.34 ± 198.74	5377.15 ± 246.51	5573.24 ± 462.15		

Each value represents means ± SD of four determinations.

 ${}^{a}P < 0.05$ ,  ${}^{b}P < 0.01$ ,  ${}^{c}P < 0.001$  compared to vehicle (0.25% DMSO).

 $^{d}P < 0.05$ ,  $^{e}P < 0.01$ ,  $^{f}P < 0.001$  compared to vehicle (0.25% DMSO) with 1.2 nM insulin.

 ${}^{g}P < 0.05$ ,  ${}^{h}P < 0.01$ ,  ${}^{i}P < 0.001$  compared to the data without insulin.

P < 0.05 was taken as the level of significance.



**Figure 2.** Effect of compound **10** on fasted plasma glucose in C57BL/6 J mice, means  $\pm$  SEM, n = 10,  ${}^{\circ}P < 0.05$ ,  ${}^{\circ\circ}P < 0.01$ , versus control.

Table 3	
Effect of compound <b>10</b> on human platelet aggregation induced by AA and ADP	
	-

Compound	$IC_{50} (\mu mol/L)^{a}$	
	AA	ADP
10	0.21	3.00
Asp	0.35	9.34

<sup>a</sup> IC<sub>50</sub> values were determined from direct regression curve analysis.

In summary, we have developed a novel class of sulfonylurea and thiourea derivatives substituted with benzenesulfonamide groups as potential hypoglycemic agents. These compounds increased the insulin release of isolated rat pancreatic islets, improved the insulin-stimulated glucose transport in adipocytes of rats in vitro. Compound **10** with fused structure of glimepiride and doltroban or S18886, showed remarkable activities to the two in vitro models above. In vivo compound **10** has also shown efficacy to lower plasma glucose level of fasted C57BL/6J mice. Furthermore compound **10** had potent antiplatelet activity and showed an excellent property to protect collagen–epinephrine-in-

#### Table 4

Effect of compound  ${\bf 10}$  in the collagen–epinephrine-induced cerebral thrombosis mice model

Compound	Asp	10		
Dose (mg/kg)	60	10	20	40
Survival rate (%)	63.6 <sup>b</sup>	27.3ª	45.5 <sup>b</sup>	72.7 <sup>b</sup>

Data represent the survival percent in a group of 11 mice.

 ${}^{a}P < 0.05$ ,  ${}^{b}P < 0.01$ , compared with control.

# Table 5

Main PK parameters of compound **10** in rats ( $\bar{x} \pm s$ , n = 6)

Parameters	Value
Dose iv/op (mg/kg)	2.5/5.0
$t_{1/2}$ (iv, h)	1.91 ± 0.65
$t_{1/2}$ (op, h)	1.77 ± 0.21
Vd (L/kg)	0.78 ± 0.36
CL (L/h)	0. 36 ± 0. 22
$T_{\rm max}(h)$	0.83 ± 0.13
$C_{\rm max}$ (mg/L)	3.33 ± 0.80
AUCop (mg/L/h)	10.04 ± 2.43
F (%)	52.69

duced mice mortality. Although the mechanism by which compound **10** acts is not well established, the preliminary pharmacological profile of compound **10** showed that it may be related to TPr and might be useful in the treatment of diabetics with cardiovascular and nephropathy complications. Further evaluation of compound **10** is underway.

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- Selected spectroscopic data: Compound **10**: IR (KBr): 3259, 2927, 1690, 1336, 1161, 1090 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz,δ ppm): 7.37–7.88 (m, 8H, Ar-H), 6.29 (d, 1H, NH-C<sub>6</sub>H<sub>10</sub>CH<sub>3</sub>), 3.32 (m, 1H,), 3.30 (q, 2H, CH<sub>2</sub>), 2.78 (t, 2H, CH<sub>2</sub>), 0.81–1.69(m, 12H,); MS (ESI, *m/z*): 558 ([M+H]<sup>+</sup>, base peak), 580 ([M+Na]<sup>+</sup>); Anal. Calcd for C<sub>22</sub>H<sub>28</sub>BrN<sub>3</sub>O<sub>5</sub>S<sub>2</sub>: C 47.31, H 5.05, N 7.52; found: C 47.20, H 5.04, N 7.65.
- 26. The pancreatic tissue was removed from rats and digested with collagenase I for 5–8 min at 38 °C for 4–5 times. Dispersed pancreatic cells were washed with Hanks liquid (containing 10% fetal bovine serum). After centrifugation, the isolated cells were cultured in RPMI 1640 medium with the addition of 10% fetal bovine serum. Fibroblasts were taken out with iodoacetic acid incubated for 48 h. Final incubation were carried out for 6 h at 37 °C with glucose (25 mmol/L), test and standard compounds at different concentrations. The insulin concentration in 1640 medium was measured by radioimmunoassay method.
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- 28. Fat cells were separated from SD rats and identified by oil red O-staining. 0.5 ml adipocyte suspension  $(5 \times 10^5 \text{ cells/ml})$  was added into 2-ml polypropylene plastic tube and incubated for 30 min at 37 °C in 5% CO<sub>2</sub> incubator. Different concentrations of target compounds (0.25% DMSO) were added in different experimental groups and incubated at 37 °C for 30 min. In the presence or absence of 1.2 nM insulin the cells were incubated at 37 °C for 4 h. According to the different experimental groups, 2-Deoxy-D-[1-<sup>3</sup>H]glucose was added (final concentration 0.2 µCi/ml) and after incubated for 1.5 h, icecold 4 °C dimethyl silicone was added immediately. After centrifugation at 10,000 rpm for 2 min, lower layer culture was removed. One hundred microliters 5% SDS was added to crack the upper fat clumps. After 0.5 h, the lysis was added to scintillation vial with 5 ml scintillation liquid (4.0 g PPO, 0.4 g POPOP dissolved in 1 L xylene), standed in dark for 12 h and the radiation intensity (Bq) of <sup>3</sup>H was measured by liquid scintillation counting.
- 29. Fasted C57BL/6 J mice were dosed daily by oral gavage for 3 days. Treatments were compound 10 (20, 7, and 2.5 mg/kg), glimepiride (20 mg/kg) or vehicle (0.5% carboxymethylcellulose). On day 5, mice were weighed and the blood samples were collected from the cut tip of the tail vein at 0, 0.5, 1, 2, 4, and 6 h after dosing. Samples were assayed for glucose. Plasma glucose was measured using the glucose oxidase Trinder method.
- 30. Blood was collected from the cubitus artery of healthy volunteers, who were limosis and avoiding treatment with antithrombotic drugs 2 weeks before test, into plastic vessels containing 0.1 volume of 3.8% aqueous sodium citrate. Platelet-rich plasma (PRP) was then prepared by centrifugation at 1000 rpm for 10 min. Platelet-poor plasma (PPP) was prepared from the remaining blood after removing PRP, by centrifugation at 3000 rpm for 10 min. PRP was adjusted to a concentration of  $6.0 \times 10^{-5}$  cells/µl by using PPP. By using ADP (5 µmol/L) and AA (200 µmol/L) as an aggregating inducer, respectively, platelet aggregation of ASP and compound 10: 100, 10, 1, and 0.1 µmol/L) was assayed on a four-channel aggregometer (LBY-NJ2 type, Plysion Co., Ltd).
- 31. Chinese Kunming mice weighing about 30 g were grouped after overnight fasting (n = 11). Mice were dosed once daily by oral gavage for 7 days. Treatments were compound 10 (40, 20, and 10 mg/kg), ASP (60 mg/kg) or vehicle (0.5% carboxymethylcellulose). On day 7, at 1 h after dosing, 220 µg collagen and 8 µg epinephrine were injected intravenously for 20 s. The protective effects were calculated by measuring the incidence of death within 15 min of injection. Results are expressed as percent survival.
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