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Design and Synthesis of Rosiglitazone-Ferulic Acid-Nitric Oxide Donor Trihybrids for Improving Glucose Tolerance

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Graphical Abstract



relative to rosiglitazone.

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for improving glucose tolerance

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Abstract:

Glucose intolerance is associated with metabolic syndrome and type 2 diabetes mellitus (T2DM) while some new therapeutic drugs, such as rosiglitazone (Rosi), for T2DM can cause severe cardiovascular side effects. Herein we report the synthesis of Rosi-ferulic acid (FA)-nitric oxide (NO) donor trihybrids to improve glucose tolerance and minimize the side effects. In comparison with Rosi, the most active compound **21** exhibited better effects on improving glucose tolerance, which was associated with its NO production, antioxidant and anti-inflammatory activities. Furthermore, **21** displayed relatively high stability in the simulated gastrointestinal environments and human liver microsomes, and released Rosi in plasma. More importantly, **21**, unlike Rosi,

had little stimulatory effect on the membrane translocation of aquaporin-2 (AQP2) in kidney collecting duct epithelial cells. These, together with a better safety profile, suggest that the trihybrids, like **21**, may be promising candidates for intervention of glucose intolerance-related metabolic syndrome and T2DM.

Keywords: Glucose intolerance, rosiglitazone, nitric oxide, ferulic acid, type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized as glucose intolerance, insulin resistance and insufficient insulin production, leading to hyperglycemia [1]. Although several classes of new drugs, such as dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium/glucose cotransporter 2 (SGLT-2) inhibitors and the peroxisome proliferator-activated receptor γ (PPAR γ) agonists have been developed for the treatment of T2DM [2, 3], their cardiovascular safety remains a major concern in clinical applications [4]. Rosiglitazone (Rosi), a thiazolidinedione-based PPAR γ agonist, can increase insulin receptor sensitivity and has been used for treatment of T2DM in the clinic for several years. However, due to its potential risk of heart failure, it was restricted only for prescription by the FDA in 2013 [5]. Currently, the exact mechanisms underlying the heart failure of Rosi are still not clear. Previous studies have suggested that the side effect of Rosi may be attributed to the body weight gain and fluid retention at least in some patients [6-17]. Further studies have indicated that Rosi stimulates the expression of aquaporin 2 (AQP2), which is responsible for water reabsorption in the renal collecting duct, implicating fluid retention [18-24]. Hence, development of new compounds to inhibit AQP2 expression may reduce the Rosirelated side effect to improve its safety.

Chronic hyperglycemia is associated with oxidative stress, reactive oxygen species (ROS) production, which can exhaust endogenous thiol and tetrahydrobiopterin (BH₄) [25], deteriorating the redox imbalance and endothelium dysfunction by reducing nitric oxide (NO) generation [25-28]. Furthermore, a reduction in NO bioavailability can result in platelet aggregation, atherosclerosis and hypertension, leading to heart failure in T2DM patients [28]. In addition, NO can increase energy expenditure and enhance glucose and lipid metabolism by

improving insulin resistance [29-32]. Actually, conjugates by coupling an NO donor, such as diazeniumdiolate (NONOate), organic nitrate or furoxan with nateglinide (**2a** and **2b**), glibenclamide (**3a** and **3b**), or tolbutamide (**4**), as shown respectively in Figure 1, can display comparable or higher anti-diabetic activity with reduced adverse effects by slow releasing NO [33-35]. In addition, ferulic acid (FA) is an antioxidant with anti-hypertensive [36] and anti-platelet aggregation [37] activities. Importantly, FA can also improve glucose tolerance [36]. Accordingly, to improve the efficacy and safety of Rosi, we synthesized trihybrids of Rosi-FA-nitrate or Rosi-FA-diethylamine diazeniumdiolate (DEA/NO) and biologically evaluated for their effects on glucose tolerance.



Figure 1. Chemical structures of rosiglitazone and representative NO donating anti-diabetic agents.

2. Results and discussion

2.1 Chemistry

Synthesis of the target compounds **9a-d**, **15a-d**, **21**, **26a-d** and their corresponding intermediates **8a-d**, **14a-d**, **20** and **25** is outlined in Scheme 1. For **8a-d**, the starting material FA was reacted with dibromoalkanes to give monobromo intermediates **6a-d**, which were treated with AgNO₃ in acetonitrile to generate corresponding nitrates **7a-d** [38]. The nitrates were then condensed with chloromethyl chloroformate to form **8a-d** (Scheme 1A). For **14a-d**, FA was esterified to give methyl ester **10**, which underwent similar reactions as for **6a-d** and **7a-d**,

successively, to offer nitrates 12a-d [39]. The nitrates were demethylated under basic conditions to give acids 13a-d [39], which were treated with chloromethyl chlorosulfate in the presence of catalytic n-Bu₄NHSO₄ in H₂O/CH₂Cl₂ to yield **14a-d** (Scheme 1B). For **20**, *tert*-butyl ester of FA 16 was firstly synthesized according to the reported method [36]. Intermediate 17 was generated by the same procedure as for the synthesis of **8a-d**, which was then reacted with the potassium salt of Rosi in the presence of catalytic NaI to generate 18. After removal of *tert*-butyl in 18 by trifluoroacetic acid, 19 was obtained, which was treated with chloromethyl chlorosulfate to yield chloromethyl ester 20 (Scheme 1C). Intermediate 25 was synthesized using similar synthetic approach above to build carboxylic chloromethyl ester 23 at first, followed by coupling with the Rosi potassium salt and de-tert-butylation, successively (Scheme 1D). Finally, the target compounds 9a-d and 15a-d were synthesized by coupling potassium salt of Rosi with corresponding chloromethyl-containing intermediates 8a-d and 14a-d, respectively, in the presence of catalytic amount of NaI in DMF (Schemes 1A, 1B and 1E). The target compound 21 was produced referring to the published procedures with minor modification (Scheme 1C) [40]. Briefly, 20 was coupled with DEA/NO in the presence of 15-crown-5 in anhydrous THF under argon protection at 0 °C. The target compounds 26a-d were generated by the condensation of 25 with 7a-d in the presence of DCC/DMAP, respectively (Scheme 1D).

Scheme 1. Synthetic routes of compounds 9a-d, 15a-d, 21 and 26a-d^a



^{*a*}Reagents and conditions: (a) dibromoalkanes, TEA, DMF, 50 °C, overnight; (b) AgNO₃, acetonitrile, protected from light, reflux, overnight; (c) chloromethyl chloroformate, pyridine, CH₂Cl₂, 0–25 °C; (d) rosiglitazone potassium salt, DMF, catalytic NaI, argon protected, 25 °C; (e) MeOH, catalytic H₂SO₄, reflux, 8 h; (f) LiOH, MeOH/THF/H₂O, 25 °C, overnight; (g) Na₂CO₃, H₂O, 25 °C, 20 min, and then chloromethyl chlorosulfate, n-Bu₄NHSO₄, CH₂Cl₂, 0–25 °C, overnight; (h) trifluoroacetic acid, CH₂Cl₂, 25 °C, overnight; (i) DEA/NO, anhydrous THF, 15-crown-5, 0 °C, argon protected, 3 h; (j) DCC/DMAP, CH₂Cl₂, 25 °C, overnight.

The structures of all target compounds were characterized by ¹H NMR, ¹³C NMR and high resolution MS. The purity of compounds was determined by HPLC and all compounds with > 95% purity were used in biological evaluations.

2.2 Biological evaluations

2.2.1 Suppressive effect on cellular glucose output.

Trihybrids 9a-d, 15a-d, 21 and 26a-d (10 μ M) were first tested for their suppressive effect on cellular glucose output by determining glucose uptake rate in hepatic cells [41]. Human nontumor hepatic L-02 cells were treated with dexamethasone (1 μ M) to induce insulin resistance and cultured in serum-free medium in the presence of vehicle (0.1% DMSO) or each compound for 48 h. During the last 24-h culture, the cells were stimulated with 1 nM insulin and the glucose contents in the supernatants were measured for calculating glucose uptake rates. As shown in Table 1, treatment with 9b, 15a, 15b or 21, like Rosi, significantly suppressed the hepatic glucose output in these cells as compared with the vehicle-treated model group and the suppressive effect of 21 was significantly greater than that of Rosi. Preliminary analysis of structure-activity relationship revealed that the suppressive activity of glucose output (presented as glucose uptake percent rate) of trihybrid 21 (47.8 \pm 2.8) bearing the diazeniumdiolate as an NO donor moiety was several fold higher than trihybrids with the nitrate as an NO donor moiety **9a-d** (7.1 ± 0.8 to 12.2 ± 1.2), **15a-d** (7.1 ± 1.4 to 11.5 ± 2.6) and **26a-d** (6.8 ± 2.8 to 10.1 ± 2.8). The better effect of 21 was probably attributed to higher levels of NO produced by diazeniumdiolate(s) than nitrate(s) [38, 42-47]. In addition, the effects of 9a-d were similar to 15a-d and slightly better than 26a-d, which might be attributed to the various linker(s) used in these trihybrids, leading to different oil-water partition coefficients and abilities to penetrate the cells.

Table 1. Trihybrids' suppressive glucose output in insulin resistant L-02 cells.

Comp.	Glucose uptake (%)	Comp.	Glucose uptake (%)

control	$13.8 \pm 1.9^{***}$	15b	$11.1 \pm 2.3^*$
model	4.5 ± 0.7	15c	7.1 ± 1.4
Rosi	$11.8 \pm 2.4^{**}$	15d	8.5 ± 2.7
9a	9.6 ± 2.2	21	47.8 ± 2.8 ^{***#}
9b	$12.2 \pm 1.2^{**}$	26 a	10.0 ± 2.4
9c	7.1 ± 0.8	26b	7.8 ± 1.7
9d	8.0 ± 2.6	26c	10.1 ± 2.8
15a	$11.5 \pm 2.6^{**}$	26d	6.8 ± 2.8

Data are expressed as mean \pm SD of each compound from five separate experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. the model group. [#]P < 0.01 vs. the Rosi, determined by Student T-test.

Subsequently, the effects of the active compounds **9b**, **15a**, **15b** and **21** on 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) uptake were tested in insulin resistant murine 3T3-L1 adipocytes [48]. The 2-NBDG is a fluorescent glucose analogue and sensitively detected by fluorospectrometer [49]. Murine 3T3-L1 preadipocytes were cultured sequentially in different media to induce their differentiation. The mature adipocytes were treated with vehicle (control) or TNF- α (4 ng/mL) daily for 4 days to induce their insulin resistance [48]. Subsequently, the insulin resistant adipocytes were pre-treated with vehicle (model) or 10 μ M **9b**, **15a**, **15b**, **21** or Rosi and challenged with 100 μ M 2-NBDG at 37 °C for 30 min in the dark in the presence or absence of exogenous insulin. After being washed, the contents of intracellular fluorescent signals were detected. In comparison with that in the control, the levels of 2-NBDG uptake in the model group were reduced by 49% in the presence of insulin, demonstrating insulin resistance (Figure 2). Pre-treatment with Rosi, **9b**, or **21**, but not **15a** or **15b**, significantly increased the levels of insulin-stimulated 2-NBDG uptake in insulin-resistant adipocytes (Figure 2A). The most active compound **21** significantly improved 2-NBDG uptake with an EC₅₀ value



of $1.08 \pm 0.13 \,\mu\text{M}$, which was better than Rosi (EC₅₀ = $1.72 \pm 0.23 \,\mu\text{M}$). Hence, **21** was selected for further evaluation.

Figure 2. Compound **21** significantly improves 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes. The induced mature adipocytes were treated with TNF- α (4 ng/ml) to induce insulin resistance. Subsequently, the insulin-resistant adipocytes were treated in triplicate with vehicle (model) or the indicated compound (10 μ M) for 48 h and challenged with 100 μ M 2-NBDG in the absence (basal uptake) or presence of insulin (insulin stimulated uptake) for 30 min. The relative levels of 2-NBDG uptake in individual groups of cells were determined. Data are expressed as the mean ± SD of each group from three separate experiments and the levels of 2-NBDG uptake in individual groups of cells. (B) Effect of each moieties in **21** on 2-NBDG uptake. ###P < 0.001 vs. the control group. *P < 0.05, ***P < 0.001 vs. the model group. The difference among the groups was analyzed by one-way ANOVA and post hoc Tukey's test.

Given that **21** is composed of Rosi, FA and DEA/NO moieties, we next evaluated the effect of each moiety on 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes. Pre-treatment with **21**, Rosi, **10** (ferulic acid methyl ester), or **27** (obtained by removal of DEA/NO from **21**, Supporting Information, Scheme S1) at 10 μ M significantly increased the insulin-stimulated 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes and the effect of **21** was higher than that of Rosi, **10**, or **27**

(Figure 2B). Hence, individual moieties of **21** can promote the insulin-stimulated 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes.

2.2.2 Compound 21 improves glucose tolerance in mice.

Diabetic *db/db* mice spontaneously develop diabetes with severe glucose tolerance impairment. To determine the effect of 21 on the glucose tolerance, adult db/db mice were randomized and treated with vehicle (0.5% CMC-Na, model), Rosi at 27 µmol/kg (~10 mg/kg) and 21 at 13.5 μ mol/kg (~10 mg/kg) or 27 μ mol/kg (~20 mg/kg) daily by gavage for 18 days (n = 8 per group). A group of control C57BL/6 mice received the same dose of vehicle. While the control mice maintained euglycemic throughout the observation period the model group of mice displayed high levels of fasting blood glucose and their fasting blood glucose levels increased with time (Figure 3A). In contrast, treatment with Rosi or 21 at 9 days post treatment stopped the up-trend of fasting glucose and resulted in gradually reduced levels of fasting blood glucose in mice. More importantly, the levels of fasting blood glucose in the mice that had been treated with 21 were significantly lower than that of those with Rosi. Further analysis indicated that treatment with Rosi or 21 significantly reduced the AUC values of glucose tolerance and the effect of 21 was significantly stronger than that of Rosi in mice at 12 days post treatment (Figure 3B and Figure S1 of the Supporting Information). Given that Rosi treatment can result in body weight (BW) gain [14], we tested the BW values in individual mice. While treatment with Rosi significantly increased the BW, treatment with 21 at 13.5 or 27 µmol/kg for 18 days, the mean BW gain (the average BW on day 18 minus the average of initial BW on day 1) of db/db mice was lower than that of Rosi at 27 µmol/kg by 39% and 52%, respectively (Figure S2 and Table S1 of the Supporting Information). Thus, the significantly reduced levels of fasting blood glucose and AUC values of glucose tolerance by 21 indicated that treatment with 21 significantly improved glucose tolerance in diabetic mice.

Additionally, the serum levels of triglyceride (TG), low-density lipoprotein (LDL) and highdensity lipoprotein (HDL) were tested. The db/db mice treated with compound **21** (27 μ mol/kg) exhibited lower levels of serum triglyceride and higher levels of HDL in comparison with those by treatment of CMC-Na, respectively, suggesting that **21** might affect lipid regulations to some extent (Figure S3 of the Supporting Information).



Figure 3. Treatment with **21** improves glucose tolerance in mice. Diabetic db/db mice were randomized and treated with vehicle 0.5% CMC-Na (model), Rosi or **21** by gavage daily for 18 consecutive days. A group of C57BL/6 mice served as the control. Their fasting blood glucose levels were determined every three days and oral glucose tolerance OGTT was examined at 12 days post treatment, followed by calculating the AUC values. Data are expressed as mean \pm SEM of each group (n=8). (A) The dynamic changes in the levels of fasting blood glucose. (B) The AUC values of OGTT. ^{###}*P* < 0.001 vs. the control; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. the model; ^{§§§}*P* < 0.001 vs. the Rosi-treated group, calculated by One-way ANOVA and post hoc Tukey's test.

2.2.3 Compound **21** slowly produces NO, contributing to the enhanced 2-NBDG uptake in insulin-resistance adipocytes.

Compound **21** contained the NO donor of O^2 -esterified DEA/NO, which did not undergo spontaneous decay [50, 51]. Next, we analyzed the contribution of NO to the enhanced 2-NBDG uptake in insulin-resistance adipocytes using a fluorescent NO detector of 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM-2DA) [52]. As shown in Figure 4, DEA/NO released NO rapidly [53] while **21**-produced NO gradually increased with time, which were completely abrogated by treatment with carboxy-PTIO (C-PTIO), an NO scavenger [54].

However, there was no detectable NO in the cells treated with Rosi. To determine the role of NO in the **21**-enhanced 2-NBDG uptake, the insulin-resistant adipocytes were treated with vehicle (model), Rosi (10 μ M), **21** (3 and 10 μ M) or/and C-PTIO (20 μ M) for 48 h, respectively and challenged with 2-NBDG in the presence or absence of insulin for 30 min. The levels of 2-NBDG uptake in individual groups of cells were determined (Figures 4E and F). Treatment with C-PTIO alone did not change the levels of 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes. Treatment with **21** at both concentrations significantly enhanced the insulin-stimulated 2-NBDG uptake, which was mitigated by the addition of C-PTIO. Therefore, the NO produced from O^2 -esterified DEA/NO contributed to the **21**-enhanced insulin-stimulated 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes.

2.2.4 Compound 21 induced adipogenesis.

Rosi is a crucial stimulator of adipogenesis [55]. To determine the effect of **21** on adipogenesis, pre-adipocyte 3T3-L1 cells were induced for differentiation in the presence or absence of **21** or Rosi and the degrees of adipogenesis were measured by AdipoRed staining and fluorescent microscopy. We found that treatment with **21**, like Rosi, significantly increased the contents of intracellular triglyceride in 3T3-L1 cells (Figure 3), suggesting that **21** retained the capacity of Rosi to stimulate adipogenesis [56] The detailed results are shown in Figure S4 of the Supporting Information.



Figure 4. The NO produced by 21 contributes to the 21-enhanced insulin-stimulated 2-NBDG uptake in insulin-resistant 3T3-L1 adipocytes. (A-D) The levels of NO production. Adipocytes were treated the indicated compounds for varying periods and exposed to DAF-FM-2DA (10 μ M) in pH 7.4 PBS at 37 °C for 30 min. The fluorescent signals in individual groups of cells

were measured. (E-F) The levels of 2-NBDG uptake. Adipocytes were treated with indicated compounds for 48 h and challenged with 2-NBDG in the presence or absence of insulin for 30 min. The levels of 2-NBDG uptake in individual groups of cells were measured. Data are expressed as mean \pm SD of each group of cells from three separate experiments. **P < 0.01, ***P < 0.001 vs. the model group, calculated by One-way ANOVA and post hoc Tukey's test. ##P < 0.01, ###P < 0.001, &P < 0.05, &P < 0.01, &AP < 0.001, calculated by Student's t-test analysis.

2.2.5 Compound 21 has potent antioxidant activity.

Oxidative stress and high levels of ROS production are associated with insulin resistance [26, 48, 57-61]. Thus, reduction in the ROS levels will be a useful strategy for improving glucose tolerance [48]. Accordingly, the antioxidant activity of individual compounds was determined in the insulin-resistant adipocytes. While high levels of ROS were detected in the insulin-resistant adipocytes, treatment with Rosi (10 μ M), **21** (3 or 10 μ M) for 48 h significantly decreased the levels of ROS production, which were mitigated by the presence of C-PTIO (20 μ M) in the insulin resistant 3T3-L1 adipocytes (Figure 5A). Further analysis indicated that treatment with 10 μ M **21** significantly increased the levels of malondialdehyde (MDA, a common lipid peroxidation product) in the insulin-resistant adipocytes (Figures 5B-D). More importantly, these effects of **21** were significantly stronger than that of Rosi. Collectively, **21** exhibited potent antioxidant activity in mature 3T3-L1 adipocytes.



Figure 5. Compound **21** exhibits potent antioxidant activity in the insulin-resistant 3T3-L1 adipocytes. Following induction of insulin resistance by treatment with TNF- α , adipocytes were treated with vehicle (model) or individual compounds at the indicated dose in the presence or absence of C-PTIO (20 μ M) for 48 h. The control cells did not received TNF- α treatment and treated with vehicle alone. The levels of intracellular ROS were determined after staining with DCFH-DA and the levels of intracellular GSH, SOD and MDA were determined using specific kits. Data are expressed as mean \pm SD of each group from three individual experiments. ^{###}*P* < 0.001 vs. the control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. the model group. [§]*P* < 0.05, ^{§§}*P* < 0.01, ^{§§§}*P* < 0.001 vs. the Rosi-treated group, calculated by One-way ANOVA and post hoc Tukey's test. ^{&&&&}*P* < 0.001 calculated by Student's t-test.

2.2.6 Stability of 21 in different media.

To examine in vitro stability, **21** was incubated at 37 °C in the simulated gastric fluid (pH 1.5), intestinal fluid (pH 6.8), human liver microsomes and Sprague-Dawley (SD) rat plasma, respectively. At 4 and 24 h post incubation, **21** remained reasonably intact in the simulated gastric fluid (~97 and ~80%) and intestinal fluid (~97 and ~75%), as well as at 0.5 and 2 h in human liver microsomes (~94% and ~85%) (see Supporting Information, Tables S2 and S3). However, incubation of **21** in rat plasma reduced its contents by ~23% and ~53%, but increased levels of Rosi from ~20% to ~39% at 0.5 and 24 h post incubation (Figure 6). These results suggest that **21** may function as a prodrug of Rosi and mainly release it in plasma.



Figure 6. Metabolic hydrolysis of **21** in SD rat plasma. Compound **21** (100 μ M) was incubated in SD rat plasma at 37 °C for 0.5, 2, 4, 7, 16 or 24 h. After the addition of triple volumes of acetonitrile, the contents of **21** and Rosi in the organic phase were analyzed by HPLC. Data are expressed as mean ± SD from three separate experiments.

2.2.7 Compound 21 has a better safety profile.

21 does not reduce hematocrit (Hct) value in db/db mice. Treatment with Rosi can lead to water retention, which reduces the Hct values and be associated with increased risk of heart failure, particularly in T2DM patients [10, 62]. To determine the safety profile of 21, we first examined the Hct values in individual groups of mice. Treatment of Rosi at 27 μ mol/kg, but not

21 at 13.5 or 27 μ mol/kg, significantly reduced the Hct value in *db/db* mice, compared with the model group (Figure 7A), a hall mark of increased water retention in mice [10, 63].

21 protects the cardiovascular system. TNF- α is an important factor for insulin resistance and the pathogenesis of heart failure [64-66], and blood cardiac troponin I (cTnI) level is a biomarker of myocardial damage [67-69]. Further analysis indicated that treatment with **21** at 27 μ mol/kg, but not others, significantly reduced the levels of serum TNF- α in *db/db* mice (Figure 7B). This suggest that **21** may have anti-inflammatory activity, which is important for improving glucose tolerance and protecting from hyperglycemia-related cardiac complication [70]. Furthermore, treatment with **21**, like Rosi, significantly decreased the levels of serum cTnI in *db/db* mice and the effect of **21** was significantly stronger than that of Rosi and tended to be dose-dependent (Figure 7C). The cardiac protective effect of **21** may be attributed to NO and FA, which have been demonstrated to protect cardiac tissues from ischemic injuries [71, 72].



Figure 7. Serum hematocrit values (A), and concentrations of TNF- α (B) and cTnI (C). *db/db* Mice were treated with indicated compounds for 18 days. The tail vein blood was collected and analyzed to determine the hematocrit values, and the concentrations of TNF- α and cTnI by using the corresponding commercial ELISA kits. Results are expressed as mean \pm SD for six individual experiments. *###P* < 0.001 vs. model group (A), *#P* < 0.1, *##P* < 0.01 and *###P* < 0.001 vs. control group (B and C), **P* < 0.01, ***P* < 0.01, ***P* < 0.001 by One-way ANOVA.

In addition, treatment with **21** significantly inhibited the ADP- and AA-induced platelet aggregation in platelet-rich plasma (PRP) with IC₅₀ values of 2.14 ± 0.28 and 0.69 ± 0.11 mM, which were much lower than that of Rosi (IC₅₀ = 5.69 ± 0.62 and 6.34 ± 0.74 mM) (see

Supporting Information, Table S4). Given that inhibition on ether-a-go-go-related gene (hERG) channel can induce long QT syndrome [73-76], a severe cardiac adverse effect of drugs, the potential inhibitory effects of **21** and Rosi on hERG channel were evaluated by patch-clamp in Chinese hamster ovary cells that had been stably expressed for the hERG. Compound **21** displayed significantly less inhibition (IC₅₀ > 40.0 μ M) on hERG activation than Rosi (IC₅₀ = 29.5 μ M) (see Supporting Information, Table S5).

21 has lower in vivo acute toxicity. To further investigate the safety profile of **21**, the acute toxicity of both Rosi and **21** was tested on ICR mice at indicated doses as shown in Table 2. The animals were observed for abnormal behavior and mortality up to two weeks post treatment. Mice treated with Rosi at the lowest dose (500 mg/kg) all survived. However, treatment with Rosi at the highest dose (5000 mg/kg) killed all the mice. Thus, the median lethal dose (LD₅₀) value of Rosi was calculated to be 2584.7 mg/kg. Comparatively, for **21**, all mice survived at the dose of 2000 mg/kg, and three of ten mice died at the highest dose (5000 mg/kg) on day 14, suggesting the LD₅₀ value of **21** exceeds 5000 mg/kg.

	Dose (mg/kg)	Number of mice	e Total mortality	LD_{50}^{a} (mg/kg)			
	5000	10	10				
	4000	10	7				
Rosi	3000	10	5				
	2000	10	3	2584.7			
	1000	10	1				
	500	10	0				
^a 95% confidence limits: 1952.8–3325.7 mg/kg.							
Y	5000	10	3				
21	4000	10	1	> 5000			
	2000	10	0				

Table 2. Acute toxicity of Rosi and 21 in ICR mice.^a

^aMice were treated once with Rosi or **21** at indicated doses via oral gavage on day 1, and the health status of each mouse was observed daily for 14 days. The total mortality number on day 14 was listed.

2.2.8 Compound 21 inhibits the membrane translocation of aquaporin-2.

Although the mechanism underlying the Rosi-induced water retention has not been fully understood [18], previous studies have indicated that Rosi can stimulate the apical membrane translocation of intracellular vesicle AQP2, promoting water reabsorption in the kidney collecting duct epithelial cells [19, 21, 23]. To understand the molecular mechanisms underlying the action of 21, rat kidney inner medullary collecting duct (IMCD) cells were transfected for the expression of human AQP2 and the cells were treated with vehicle (control), 10 or 50 μ M 21, Rosi, 100 μ M forskolin (FK, a positive control) for 30 min. The membrane-associated proteins were biotinylated and the levels of membrane-associated biotinylated and total AQP2 were determined by Western blot. As shown in Figure 8, there is no obvious difference in the levels of both glycol- and nature AQP2 expression among the different groups of cells. In comparison with that of the control, treatment with FK or Rosi significantly increased the relative levels of membrane-associated biotinylated AQP2, particularly for the glycol-AQP2, in the cells, consistent with a previous report [21]. In contrast, the relative levels of membrane-associated biotinylated AQP2 in the 21-treated cells were significantly lower than that of Rosi-treated cells and treatment with 10 µM 21 almost had no stimulatory effect on the membrane translocation of AQP2.

Finally, immunofluorescent confocal analysis indicated that while little apical membraneassociated AQP2 was detected in the control cells, clearly bright anti-AQP2 staining was observed in the membranes (upper panels) and apical membrane line (bottom panels) of the FKor Rosi-treated cells. In contrast, treatment with 50 μ M 21 induced low levels of membrane translocation of AQP2 and treatment with 10 μ M 21 resulted in no membrane translocation of AQP2 in the cells. The mild stimulatory effect of 21 should not significantly increase water reabsorption in the kidney collecting duct and may explain why treatment with 21 had less increase the BW than Rosi in mice.



Figure 8. Compound **21** has little effect on the membrane translocation of AQP2 in IMCD cells. IMCD cells were transfected with the plasmid for expression of human AQP2. The cells were treated with vehicle (control) or FK (100 μ M), Rosi (10 or 50 μ M), or **21** (10 or 50 μ M) for 30 min. After the membrane-associated proteins were biotinylated and the membrane-associated biotinylated and total AQP2 in the different groups of cells were determined by Western blot. In addition, the different groups of cells were stained with FITC-anti-AQP2 and the distribution of AQP2 in the different groups of cells were photoimaged under fluorescent confocal microscope. Data are representative images or expressed as the mean ± SD of each group from three separate experiments. (A) Western blot analysis of biotinylated and total AQP2. (B) Quantitative analysis of ratios of membrane-associated biotinylated to total AQP2. (C) Immunofluorescence analysis of AQP2 distribution in the cells. The upper panels: connected cells. The bottom panels: The apical membrane. *###P* < 0.001 vs. the control group calculated by One-way ANOVA and post hoc Tukey's test. **P* < 0.05 and ****P* < 0.001 calculated by t-test.

3. Conclusions

Given that treatment with Rosi is associated with cardiovascular side effects, particularly in T2DM patients [5-13, 15], we designed and synthesized new trihybrids of Rosi, FA and an NO donor. Our data indicated that treatment with **21** not only suppressed the insulin-stimulated glucose output in non-tumor hepatocytes and promoted 2-NBDG uptake in 3T3-L1 adipocytes, respectively, but also ameliorated glucose intolerance in diabetic mice without significantly increasing body weights. The anti-diabetic effects of **21** were better than Rosi and associated with its ability to stimulate NO production, reduce oxidative stress and decrease serum TNF- α and cTnI levels in mice. Furthermore, **21** displayed relative stability in the simulated gastrointestinal fluids and human liver microsomes, and released Rosi mainly in plasma. More importantly, while treatment with Rosi stimulated the membrane translocation of AQP2, **21** had little such stimulatory role in IMCD cells. These, together with a better safety profile, suggest that the trihybrids, like **21**, may be promising candidates for intervention of glucose intolerance-related metabolic syndrome and T2DM.

4. Experimental section

4.1 Chemistry.

Nuclear magnetic resonance (NMR) spectra were obtained from a Bruker Avance 300 (¹H, 300 MHz; ¹³C, 75 MHz) or a Bruker Avance 500 (¹H, 500 MHz; ¹³C, 125 MHz) spectrometer at 300 K using TMS as an internal standard. Mass spectrometry (MS) spectra were recorded on a Mariner mass spectrometer. Melting points (mp) were measured by a Mel-TEMP II apparatus and uncorrected. TLC was performed on silica gel GF/UV 254, and column chromatography was conducted by silica gel (200–300 mesh). The purities of target compounds were characterized by HPLC analysis (Shimadzu DGU-20A3R) and HRMS (Agilent Technologies LC/MSD TOF). The target compounds **9a–d**, **15a–d**, **21** and **26a–d** with a purity of >95% were used for subsequent experiments. Intermediates **6a–d**, **7a–d**, **11a–d**, **12a–d**, **13a–d** and **16** were synthesized as previously described [36, 38, 39].

General Procedure A for the Preparation of Intermediates 8a–d and 17. Chloromethyl chloroformate (0.22 mL, 2.5 mmol) was added dropwise to the stirring solution of 7a–d or 16 (1 mmol) in CH_2Cl_2 (10 mL) at 0 °C, respectively, followed by the addition of pyridine (0.20 mL, 2.5 mmol). The reaction mixture was gradually warmed up to room temperature and was

monitored by TLC. After 1 h, the reaction was quenched by pouring into water (50 mL) and extracted by CH_2Cl_2 (3 × 20 mL). The combined organic layer was washed successively by water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried by anhydrous sodium sulfate, filtered, and evaporated in vacuum to give intermediates **8a–d** or **17** which was used immediately without further purification.

General Procedure B for the Preparation of Intermediates 14a–d, 20 and 23. Na₂CO₃ (424 mg, 4 mmol) was added to the stirring suspension of intermediates 13a–d, 19 or 22 (1 mmol) in water (8 mL) at room temperature. The reaction mixture turned transparent gradually in 20 min, and then dichloromethane (CH₂Cl₂, 16 mL) and n-Bu₄NHSO₄ (68 mg, 0.2 mmol) were added successively. After the reaction was cooled to 0 °C, chloromethyl chlorosulfate (0.12 mL, 1.2 mmol) was added dropwise. Then, the reaction mixture was gradually warmed up to room temperature and stirred overnight. The reaction solution was diluted in CH₂Cl₂ (30 mL) and washed successively by water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried by anhydrous sodium sulfate, filtered, and evaporated in vacuum to give intermediates 14a–d, 20 or 23 which was used immediately without further purification.

General Procedure C for the Preparation of Intermediates 19 and 25. Trifluoroacetic acid (7.8 mL, 105 mmol) was added to the stirring solution of 18 (2.32 g, 3.5 mmol) or 24 (1.9 g, 3.5 mmol) in CH_2Cl_2 (30 mL) at room temperature. The reaction mixture was stirred overnight and monitored by TLC. After completion of the reaction, the mixture was evaporated in vacuum. The crude product was purified by column chromatography (MeOH/CH₂Cl₂ = 1:10) to yield the title compound, respectively.

General Procedure D for the Preparation of Compounds 9a–d, 15a–d, 18 and 24. The intermediates 8a–d, 14a–d, 17 or 23 (1 mmol) was dissolved in DMF (10 mL) and added dropwise to the stirring mixture of rosiglitazone potassium salt (316 mg, 0.8 mmol) in DMF (5 mL) with catalytic amount of NaI at room temperature under argon protection. The reaction mixture was stirred overnight and then poured into water (80 mL). The mixture was then extracted by EtOAc (3×20 mL) and washed successively by water (3×20 mL) and brine (3×20 mL). The organic layer was dried by anhydrous sodium sulfate, filtered, and evaporated in vacuum. The crude product was purified by column chromatography (MeOH/CH₂Cl₂ = 1:200) to yield the title compound, respectively.

General Procedure E for the Preparation of Target Compounds 26a–d. Intermediate 25 (200 mg, 0.41 mmol) was dissolved in CH_2Cl_2 (10 mL) and 7a–d (0.41 mmol), DCC (102 mg, 0.49 mmol) as well as DMAP (3 mg, *cat.*) were added successively. The reaction mixture was stirred overnight at room temperature and extracted by CH_2Cl_2 (30 mL) and water (30 mL). The organic phase was successively washed by water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried by anhydrous sodium sulfate, filtered, and evaporated in vacuum. The crude product was purified by column chromatography (MeOH/CH₂Cl₂ = 1:200) to yield the title compound, respectively.

(*E*)-3-(*Nitrooxy*)*propyl* 3-[3-methoxy-4-((((5-(4-(2-(methyl(pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl)methoxy)*carbonyl*)*oxy*)*phenyl*]*acrylate* (**9***a*). This title compound was obtained in 11% yield as a light yellow solid: mp 56–58 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.13 (ddd, 1H, *J* = 4.5 Hz, 2.4 Hz, 0.9 Hz, ArH), 7.64 (d, 1H, *J* = 15.9 Hz, CH=), 7.44–7.41 (m, 1H, ArH), 7.16–7.15 (m, 1H, ArH), 7.12–7.09 (m, 4H, ArH), 6.84 (d, 2H, *J* = 9.2 Hz, ArH), 6.54 (ddd, 1H, *J* = 6.7 Hz, 5.4 Hz, 0.8 Hz, ArH), 6.49 (d, 1H, *J* = 8.7 Hz, ArH), 6.38 (d, 1H, *J* = 16.0 Hz, CH=), 5.67–5.62 (m, 2H, CH₂), 4.59 (t, 2H, *J* = 5.8 Hz, CH₂ONO₂), 4.49 (dd, 1H, *J* = 9.4 Hz, 4.0 Hz, CHS), 4.31 (t, 2H, *J* = 6.6 Hz, CH₂O), 4.15 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.95 (t, 2H, *J* = 5.7 Hz, CH₂N), 3.87 (s, 3H, CH₃O), 3.47 (dd, 1H, *J* = 14.2 Hz, 4.0 Hz, CH), 3.11 (s, 3H, CH₃), 3.17–3.06 (m, 1H, CH), 2.14 (p, 2H, *J* = 6.2 Hz, CH₂); ¹³C NMR (CDCl₃, 125 MHz, *δ* ppm) 172.41, 169.64, 166.39, 158.45, 158.30, 151.30, 151.24, 147.86, 144.40, 141.29, 137.30, 133.72, 130.32, 127.32, 122.67, 121.15, 118.15, 114.85, 111.77, 111.54, 105.74, 69.99, 66.28, 66.13, 60.61, 56.04, 51.99, 49.43, 37.79, 29.71, 26.50; ESI-MS: 711.2 [M + H]⁺; HRMS calculated for C₃₃H₃₅N₄O₁₂S [M + H]⁺ 711.1972, found 711.1986.

(*E*)-4-(*Nitrooxy*)*butyl* 3-[3-methoxy-4-((((5-(4-(2-(methyl(pyridin-2-yl)amino)ethoxy)*benzyl*)-2,4-dioxo-3-thiazolidinyl)methoxy)carbonyl)oxy)phenyl]acrylate (**9b**). This title compound was obtained in 14% yield as a light yellow solid: mp 52–53 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.13 (ddd, 1H, *J* = 4.5 Hz, 2.4 Hz, 0.9 Hz, ArH), 7.62 (d, 1H, *J* = 15.9 Hz, CH=), 7.45–7.41 (m, 1H, ArH), 7.16–7.14 (m, 1H, ArH), 7.11–7.08 (m, 4H, ArH), 6.85–6.82 (m, 2H, ArH), 6.54 (ddd, 1H, *J* = 6.7 Hz, 5.4 Hz, 0.8 Hz, ArH), 6.49 (d, 1H, *J* = 8.7 Hz, ArH), 6.38 (d, 1H, *J* = 16.0 Hz, CH=), 5.67–5.62 (m, 2H, CH₂), 4.51–4.49 (m, 3H, CH₂ and CHS), 4.24 (t, 2H, *J* = 6.6 Hz, CH₂O), 4.15 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.95 (t, 2H, *J* = 5.8 Hz, CH₂N), 3.87 (s, 3H, CH₃O), 3.47 (dd, 1H, *J* = 14.2 Hz, 4.0 Hz, CH), 3.11 (s, 3H, CH₃), 3.12–3.06 (m, 1H, CH), 1.88–1.81 (m, 4H, $2 \times CH_2$); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.40, 169.62, 166.57, 158.45, 158.30, 151.29, 151.24, 147.87, 144.06, 141.24, 137.29, 133.80, 130.31, 127.32, 122.65, 121.10, 118.47, 114.85, 111.76, 111.51, 105.73, 72.68, 66.28, 66.13, 63.68, 56.04, 51.98, 49.43, 37.78, 29.70, 25.09, 23.72; ESI-MS: 725.2 [M + H]⁺; HRMS calculated for C₃₄H₃₇N₄O₁₂S [M + H]⁺ 725.2129, found 725.2117.

(*E*)-5-(*Nitrooxy*)*pentyl* 3-[3-*methoxy*-4-((((5-(4-(2-(*methyl*(*pyridin*-2-*yl*)*amino*)*ethoxy*)*benzyl*)-2,4-*dioxo*-3-*thiazolidinyl*)*methoxy*)*carbonyl*)*oxy*)*phenyl*]*acrylate* (*9c*). This title compound was obtained in 10% yield as a light yellow solid: mp 47–49 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.13–8.12 (m, 1H, ArH), 7.61 (d, 1H, *J* = 15.9 Hz, CH=), 7.42–7.440 (m, 1H, ArH), 7.15–7.13 (m, 1H, ArH), 7.09–7.07 (m, 4H, ArH), 6.84–6.81 (m, 2H, ArH), 6.52 (ddd, 1H, *J* = 6.7 Hz, 5.4 Hz, 0.8 Hz, ArH), 6.48 (d, 1H, *J* = 8.7 Hz, ArH), 6.38 (d, 1H, *J* = 16.0 Hz, CH=), 5.66–5.61 (m, 2H, CH₂), 4.49 (dd, 1H, *J* = 9.4 Hz, 4.0 Hz, CHS), 4.44 (t, 2H, *J* = 5.8 Hz, CH₂ONO₂), 4.20 (t, 2H, *J* = 6.0 Hz, CH₂O), 4.13 (t, 2H, *J* = 5.5 Hz, CH₂O), 3.94 (t, 2H, *J* = 5.8 Hz, CH₂N), 3.85 (s, 3H, CH₃O), 3.45 (dd, 1H, *J* = 14.2 Hz, 4.0 Hz, CH), 3.10 (s, 3H, CH₃), 3.11–3.05 (m, 1H, CH), 1.79–1.71 (m, 4H, 2 × CH₂), 1.54–1.50 (m, 2H, CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.41, 169.64, 166.66, 158.43, 158.31, 151.27, 151.25, 147.87, 143.84, 141.18, 137.27, 133.86, 130.32, 127.35, 122.62, 121.08, 118.68, 114.83, 111.75, 111.50, 105.71, 73.08, 66.26, 66.13, 64.14, 56.03, 51.95, 49.40, 37.74, 28.25, 26.47, 22.34; ESI-MS: 739.2 [M + H]⁺; HRMS calculated for C₃₅H₃₉N₄O₁₂S [M + H]⁺ 739.2285, found 739.2293.

(*E*)-6-(*Nitrooxy*)*hexyl* 3-[3-methoxy-4-((((5-(4-(2-(methyl(pyridin-2-yl)amino)ethoxy)*benzyl*)-2,4-dioxo-3-thiazolidinyl)methoxy)carbonyl)oxy)phenyl]acrylate (**9d**). This title compound was obtained in 12% yield as a light yellow solid: mp 42–44 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.14 (ddd, 1H, J = 4.5 Hz, 2.4 Hz, 0.9 Hz, ArH), 7.63 (d, 1H, J = 15.9 Hz, CH=), 7.46–7.43 (m, 1H, ArH), 7.17–7.14 (m, 1H, ArH), 7.13–7.09 (m, 4H, ArH), 6.86–6.84 (m, 2H, ArH), 6.55 (ddd, 1H, J = 6.7 Hz, 5.4 Hz, 0.8 Hz, ArH), 6.51 (d, 1H, J = 8.7 Hz, ArH), 6.39 (d, 1H, J = 16.0 Hz, CH=), 5.68–5.64 (m, 2H, CH₂), 4.51 (dd, 1H, J = 9.4 Hz, 4.0 Hz, CHS), 4.46 (t, 2H, J = 5.8 Hz, CH₂ONO₂), 4.22 (t, 2H, J = 6.0 Hz, CH₂O), 4.16 (t, 2H, J = 5.5 Hz, CH₂O), 3.97 (t, 2H, J = 5.8 Hz, CH₂N), 3.89 (s, 3H, CH₃O), 3.50 (dd, 1H, J = 14.2 Hz, 4.0 Hz, CH), 3.13 (s, 3H, CH₃), 3.13–3.08 (m, 1H, CH), 1.78–1.72 (m, 4H, 2 × CH₂), 1.49–1.46 (m, 4H, 2 × CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.39, 169.60, 166.76, 158.47, 151.27, 147.87, 143.77, 141.16, 137.30, 133.92, 130.30, 127.28, 122.63, 121.08, 118.78, 114.86, 111.76, 111.44, 105.73, 73.19,

66.31, 66.11, 64.43, 56.04, 52.01, 49.46, 37.85, 37.82, 28.52, 26.71, 25.60, 25.40; ESI-MS: 753.2 $[M + H]^+$; HRMS calculated for $C_{36}H_{41}N_4O_{12}S$ $[M + H]^+$ 753.2442, found 753.2431.

(*E*)-[5-(4-(2-(*Methyl(pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl]methyl 3-[3-methoxy-4-(3-(nitrooxy)propoxy)phenyl]acrylate (15a). This title compound was obtained in 23% yield as a light yellow solid: mp 55–57 °C. ¹H NMR (CDCl₃, 500 MHz, \delta ppm) 8.14–8.13 (m, 1H, ArH), 7.63 (d, 1H, <i>J* = 15.9 Hz, CH=), 7.43 (ddd, 1H, *J* = 9.1 Hz, 7.1 Hz, 2.0 Hz, ArH), 7.10–7.02 (m, 4H, ArH), 6.86–6.82 (m, 3H, ArH), 6.54 (dd, 1H, *J* = 7.0 Hz, 5.0 Hz, ArH), 6.49 (d, 1H, *J* = 8.6 Hz, ArH), 6.25 (d, 1H, *J* = 15.9 Hz, CH=), 5.67–5.62 (m, 2H, CH₂), 4.68 (t, 2H, *J* = 6.1 Hz, CH₂ONO₂), 4.49 (dd, 1H, *J* = 9.3 Hz, 3.9 Hz, CHS), 4.14–4.12 (m, 4H, 2 × OCH₂), 3.93 (t, 2H, *J* = 5.6 Hz, NCH₂), 3.87 (s, 3H, OCH₃), 3.47 (dd, 1H, *J* = 14.1 Hz, 3.8 Hz, CH), 3.13–3.06 (m, 4H, CH₃N and CH), 2.25 (p, 2H, *J* = 6.0 Hz, CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.66, 169.96, 165.41, 158.40, 158.29, 150.39, 149.73, 147.86, 146.36, 137.29, 130.34, 127.70, 127.42, 122.77, 114.79, 114.38, 113.02, 111.77, 110.38, 105.72, 69.86, 66.27, 64.82, 63.13, 55.91, 51.94, 49.41, 37.78, 26.89; ESI-MS: 667.2 [M + H]⁺; HRMS calculated for C₃₂H₃₅N₄O₁₀S [M + H]⁺ 667.2074, found 667.2085.

(*E*)-[5-(4-(2-(*Methyl*(*pyridin-2-yl*)*amino*)*ethoxy*)*benzyl*)-2,4-*dioxo-3-thiazolidinyl*]*methyl* 3-[3-*methoxy*-4-(4-(*nitrooxy*)*butoxy*)*phenyl*]*acrylate* (**15b**). This title compound was obtained in 32% yield as light yellow solid: mp 53–55 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.11 (ddd, 1H, *J* = 4.9 Hz, 1.9 Hz, 0.7 Hz, ArH), 7.61 (d, 1H, *J* = 15.9 Hz, CH=), 7.42–7.39 (m, 1H, ArH), 7.08–7.00 (m, 4H, ArH), 6.81–6.79 (m, 3H, ArH), 6.53–6.50 (m, 1H, ArH), 6.46 (d, 1H, *J* = 8.7 Hz, ArH), 6.24 (d, 1H, *J* = 15.9 Hz, CH=), 5.65–5.59 (m, 2H, CH₂), 4.51 (t, 2H, *J* = 6.2 Hz, CH₂ONO₂), 4.47 (dd, 1H, *J* = 9.3 Hz, 4.0 Hz, CHS), 4.09 (t, 2H, *J* = 6.6 Hz, CH₂O), 4.02 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.90 (t, 2H, *J* = 5.6 Hz, CH₂N), 3.84 (s, 3H, CH₃O), 3.43 (dd, 1H, *J* = 14.2 Hz, 3.9 Hz, CH), 3.07 (s, 3H, CH₃N), 3.07–3.03 (m, 1H, CH), 1.92–1.91 (m, 4H, 2 × CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.67, 170.00, 165.44, 158.35, 158.28, 150.72, 149.58, 147.86, 146.46, 137.29, 130.37, 127.45, 127.22, 122.90, 114.75, 114.09, 112.44, 111.76, 110.17, 105.72, 72.99, 68.11, 66.21, 63.08, 55.86, 51.89, 49.36, 37.74, 37.68, 25.32, 23.88; ESI-MS: 681.2 [M + H]⁺; HRMS calculated for C₃₃H₃₇N₄O₁₀S [M + H]⁺ 681.2230, found 681.2221.

(*E*)-[5-(4-(2-(Methyl(pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl]methyl 3-[3-methoxy-4-(5-(nitrooxy)pentoxy)phenyl]acrylate (**15c**). This title compound was obtained in 16% yield as a light yellow solid: mp 45–46 °C. ¹H NMR (CDCl₃, 300 MHz, δ ppm) 8.15–8.13 (m, 1H, ArH), 7.64 (d, 1H, J = 15.9 Hz, CH=), 7.47–7.41 (m, 1H, ArH), 7.11–7.03 (m, 4H, ArH), 6.85–6.81 (m, 3H, ArH), 6.56–6.48 (m, 2H, ArH), 6.25 (d, 1H, J = 15.9 Hz, CH=), 5.69–5.61 (m, 2H, CH₂), 4.51–4.44 (m, 3H, CH₂ONO₂ and CHS), 4.13 (t, 2H, J = 5.6 Hz, CH₂O), 4.04 (t, 2H, J = 6.4 Hz, CH₂O), 3.93 (t, 2H, J = 5.6 Hz, CH₂N), 3.88 (s, 3H, CH₃O), 3.47 (dd, 1H, J = 14.1 Hz, 3.8 Hz, CH), 3.13–3.05 (m, 4H, CH₃ and CH), 1.94–1.76 (m, 4H, 2 × CH₂), 1.65–1.55 (m, 2H, CH₂); ¹³C NMR (CDCl₃, 75 MHz, δ ppm) 172.67, 170.00, 165.50, 158.38, 150.87, 149.52, 147.87, 146.54, 137.30, 130.34, 127.39, 127.04, 122.94, 114.76, 113.96, 112.31, 111.75, 110.10, 105.70, 73.10, 68.43, 66.25, 63.10, 55.93, 51.95, 49.39, 37.79, 28.54, 26.53, 22.41; ESI-MS: 695.2 [M + H]⁺; HRMS calculated for C₃₄H₃₉N₄O₁₀S [M + H]⁺ 695.2387, found 695.2396.

(*E*)-[5-(4-(2-(*Methyl*(*pyridin*-2-*y*))*amino*)*ethoxy*)*benzy*])-2,4-*dioxo*-3-*thiazolidinyl*]*methyl* 3-[3-*methoxy*-4-(6-(*nitrooxy*)*hexy*]*oxy*)*pheny*]*acry*]*acry*]*ate* (**15***d*). This title compound was obtained in 36% yield as a light yellow solid: mp 39–41 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.13 (ddd, 1H, *J* = 4.9 Hz, 1.9 Hz, 0.8 Hz, ArH), 7.64 (d, 1H, *J* = 15.9 Hz, CH=), 7.43 (ddd, 1H, *J* = 8.9 Hz, 7.1 Hz, 2.0 Hz, ArH), 7.10–7.03 (m, 4H, ArH), 6.85–6.81 (m, 3H, ArH), 6.54 (ddd, 1H, *J* = 7.0 Hz, 5.0 Hz, 0.6 Hz, ArH), 6.49 (d, 1H, *J* = 8.6 Hz, ArH), 6.24 (d, 1H, *J* = 15.9 Hz, CH=), 5.67– 5.62 (m, 2H, CH₂), 4.49 (dd, 1H, *J* = 9.3 Hz, 4.0 Hz, CHS), 4.44 (t, 1H, *J* = 4.0 Hz, CH₂ONO₂), 4.13 (t, 2H, *J* = 5.8 Hz, CH₂O), 4.04 (t, 2H, *J* = 5.6 Hz, CH₂O), 3.92 (t, 2H, *J* = 5.6 Hz, CH₂N), 3.88 (s, 3H, CH₃O), 3.47 (dd, 1H, *J* = 14.2 Hz, 3.9 Hz, CH), 3.13–3.07 (m, 4H, CH₃N and CH), 1.87–1.84 (m, 2H, CH₂), 1.78–1.73 (m, 2H, CH₂), 1.54–1.46 (m, 4H, 2 × CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.67, 169.97, 165.51, 158.39, 158.28, 151.02, 149.55, 147.82, 146.57, 137.31, 130.34, 127.42, 126.97, 122.96, 114.79, 113.92, 112.34, 111.77, 110.19, 105.74, 73.23, 68.66, 66.28, 63.11, 55.97, 51.93, 49.43, 37.79, 28.81, 26.68, 25.60, 25.45; ESI-MS: 709.3 [M + H]⁺; HRMS calculated for C₃₅H₄₁N₄O₁₀S [M + H]⁺ 709.2543, found 709.2549.

(*E*)-*tert-Butyl* 3-[3-*methoxy*-4-(((5-(4-(2-(*methyl*(*pyridin*-2-*yl*)*amino*)*ethoxy*)*benzyl*)-2,4-*dioxo*-3-*thiazolidinyl*)*methoxy*)*carbonyloxy*)*phenyl*]*acrylate* (**18**). This title compound was obtained in 73% yield as an off-white solid: mp 69–71 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.14 (ddd, 1H, *J* = 5.0 Hz, 2.0 Hz, 0.9Hz, ArH), 7.53 (d, 1H, *J* = 15.9 Hz, CH=), 7.44 (ddd, 1H, *J* = 8.9 Hz, 7.1 Hz, 2.0 Hz, ArH), 7.13–7.07 (m, 5H, ArH), 6.87–6.84 (m, 2H, ArH), 6.55 (ddd, 1H, *J* = 7.1 Hz, 5.0 Hz, 0.7Hz, ArH), 6.51 (d, 1H, *J* = 8.6 Hz, ArH), 6.32 (d, 1H, *J* = 15.9 Hz, CH=), 5.68–5.64 (m, 2H, CH₂), 4.51 (dd, 1H, *J* = 9.5 Hz, 4.0 Hz, CHS), 4.16 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.97 (t, 2H, J = 5.7 Hz, CH₂N), 3.88 (s, 3H, CH₃O), 3.50 (dd, 1H, J = 14.2 Hz, 4.0 Hz, CH), 3.13 (s, 3H, CH₃N), 3.13–3.08 (m, 1H, CH), 1.54 (s, 9H, 3 × CH₃); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.39, 169.59, 166.01, 158.47, 151.29, 151.20, 147.90, 142.53, 140.92, 137.28, 134.21, 130.30, 127.29, 122.53, 120.98, 120.91, 114.86, 111.76, 111.27, 105.71, 80.74, 66.31, 66.09, 56.01, 52.00, 49.45, 37.85, 37.80, 30.94, 28.20; ESI-MS: 664.2 [M + H]⁺; HRMS calculated for C₃₄H₃₈N₃O₉S [M + H]⁺ 664.2329, found 664.2336.

(*E*)-3-[3-methoxy-4-(((5-(4-(2-(methyl(pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3thiazolidin)methoxy)carbonyloxy)phenyl]acrylic acid (**19**). This title compound was obtained in 98% yield as a white solid: mp 125–126 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.27 (d, 1H, J =5.9 Hz, ArH), 7.80 (t, 1H, J = 8.1 Hz, ArH), 7.69 (d, 1H, J = 15.9 Hz, CH=), 7.17–7.10 (m, 5H, ArH), 6.90–6.80 (m, 4H, ArH), 6.40 (d, 1H, J = 15.9 Hz, CH=), 5.67–5.62 (m, 2H, CH₂), 4.49 (dd, 1H, J = 9.1 Hz, 4.1 Hz, SCH), 4.26 (t, 2H, J = 4.8 Hz, CH₂), 4.11 (t, 2H, J = 4.8 Hz, CH₂), 3.89 (s, 3H, OCH₃), 3.47–3.43 (m, 1H, CH), 3.37 (s, 3H, NCH₃), 3.11 (dd, 1H, J = 14.3 Hz, 9.1 Hz, CH); ¹³C NMR (CDCl₃, 75 MHz, δ ppm) 172.28, 169.79, 169.42, 157.59, 152.88, 151.32, 151.08, 145.30, 142.70, 141.43, 139.35, 133.69, 130.52, 128.06, 122.60, 121.28, 118.15, 114.73, 112.02, 111.63, 110.66, 66.15, 65.66, 56.06, 51.68, 51.50, 39.51, 37.71; ESI-MS: 608.2 [M + H]⁺; HRMS calculated for C₃₀H₃₀N₃O₉S [M + H]⁺ 608.1703, found 608.1697.

 O^2 -[(E)-3-(3-methoxy-4-((5-(4-(2-(methyl(pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3thiazolidinyl)methoxy)carbonyloxy)phenyl)acryloyl]methyl 1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (21). Chloromethyl ester 20 (150 mg, 0.2 mmol) was diluted in 5 mL anhydrous tetrahydrofuran (THF) and added to the stirring slurry mixture of 74 mg (0.48 mmol) DEA/NO (27c) in 5 mL anhydrous THF in the presence of catalytic amount of 15-crown-5 under argon protection at 0 °C. The reaction was stirred for 3 h at 0 °C and poured into water (50 mL) followed by extraction with EtOAc (3 × 20 mL) and successive wash with water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried by anhydrous sodium sulfate, filtered, and evaporated in vacuum to give the crude product which was purified by column chromatography (MeOH/CH₂Cl₂ = 1:200) to yield the title compound in 53% yield as a light yellow solid: mp 67–69 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.14 (ddd, 1H, *J* = 4.5 Hz, 2.4 Hz, 0.9 Hz, ArH), 7.71 (d, 1H, *J* = 16.0 Hz, CH=), 7.46–7.42 (m, 1H, ArH), 7.17–7.09 (m, 5H, ArH), 6.86–6.84 (m, 2H, ArH), 6.55 (ddd, 1H, *J* = 6.7 Hz, 5.4 Hz, 0.8 Hz, ArH), 6.51 (d, 1H, *J* = 8.6 Hz, ArH), 6.38 (d, 1H, *J* = 16.0 Hz, CH=), 5.96 (s, 2H, CH₂), 5.68–5.63 (m, 2H, CH₂), 4.51 (dd, 1H, *J* = 9.4 Hz, 4.0 Hz, CHS), 4.16 (t, 2H, J = 5.7 Hz, CH₂O), 3.97 (t, 2H, J = 5.8 Hz, CH₂N), 3.89 (s, 3H, CH₃O), 3.49 (dd, 1H, J = 14.2 Hz, 4.0 Hz, CH), 3.22 (q, 4H, J = 7.1 Hz, 2 × NCH₂), 3.13 (s, 3H, CH₃N), 3.14–3.08 (m, 1H, CH) , 1.12 (t, 6H, J = 7.1 Hz, 2 × CH₃); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.37, 169.57, 164.80, 158.46, 158.29, 151.32, 151.18, 147.88, 145.59, 141.47, 137.28, 133.52, 130.29, 127.26, 122.71, 121.30, 117.50, 114.86, 111.76, 111.60, 105.71, 87.59, 66.32, 66.13, 56.06, 52.01, 49.45, 48.13, 37.85, 37.81, 11.48; ESI-MS: 753.3 [M + H]⁺; HRMS calculated for C₃₅H₄₁N₆O₁₁S [M + H]⁺ 753.2554, found 753.2563.

tert-Butyl [(5-(4-(2-(*methyl*(*pyridin-2-yl*)*amino*)*ethoxy*)*benzyl*)-2,4-*dioxo-3-thiazolidinyl*) *methyl*]*succinate* (**24**). This title compound was obtained in 52% yield as a light yellow solid: mp 53–55 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.14 (ddd, 1H, *J* = 4.9 Hz, 2.0 Hz, 0.9 Hz, ArH), 7.44 (t, 1H, *J* = 8.3 Hz, ArH), 7.11–7.08 (m, 2H, ArH), 6.85–6.82 (m, 2H, ArH), 6.54 (ddd, 1H, *J* = 7.1 Hz, 5.0 Hz, 0.8 Hz, ArH), 6.51 (dt, 1H, *J* = 8.6 Hz, 0.8 Hz, ArH), 5.55–5.50 (m, 2H, CH₂), 4.46 (dd, 1H, *J* = 9.4 Hz, 3.9Hz, CHS), 4.16 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.97 (t, 2H, *J* = 5.7 Hz, CH₂N), 3.46 (dd, 1H, *J* = 14.2Hz, 3.9 Hz, CH), 3.13 (s, 3H, CH₃N), 3.08 (dd, 1H, *J* = 14.2 Hz, 9.4 Hz, CH), 2.58–2.50 (m, 4H, 2 × CH₂CO), 1.44 (s, 9H, 3 × CH₃); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.51, 171.05, 171.03, 169.77, 162.52, 158.40, 158.29, 147.85, 137.29, 130.32, 127.39, 114.76, 111.75, 105.72, 80.93, 66.30, 63.20, 51.88, 49.45, 37.80, 37.75, 36.46, 31.42, 30.01, 28.89, 28.03; ESI-MS: 544.2 [M + H]⁺, 566.2 [M + Na]⁺; HRMS calculated for C₂₇H₃₄N₃O₇S [M + H]⁺ 544.2177, found 544.2127, C₂₇H₃₃N₃O₇NaS [M + H]⁺ 566.1937, found 566.1931.

4-[(5-(4-(2-(*Methyl*(*pyridin-2-yl*)*amino*)*ethoxy*)*benzyl*)-2,4-*dioxo-3-thiazolidinyl*)*methoxy*]-4oxobutanoic acid (**25**). This title compound was obtained in 97% yield as a white solid: mp 118– 119 °C. ¹H NMR ((CD₃)₂CO, 500 MHz, δ ppm) 8.23 (ddd, 1H, J = 6.1 Hz, 1.8 Hz, 0.7 Hz, ArH), 7.94 (ddd, 1H, J = 9.0 Hz, 7.0 Hz, 1.8 Hz, ArH), 7.26 (d, 1H, J = 9.2 Hz, ArH), 7.19–7.17 (m, 2H, ArH), 6.92–6.87 (m, 3H, ArH), 5.53–5.45 (m, 2H, CH₂), 4.85 (dd, 1H, J = 8.1 Hz, 4.4 Hz , CHS), 4.31 (t, 2H, J = 5.3 Hz, CH₂O), 4.16 (t, 2H, J = 5.3 Hz, CH₂N), 3.42–3.38 (m, 1H, CH), 3.40 (s, 3H, CH₃N), 3.24–3.20 (m, 1H, CH), 2.58–2.53 (m, 4H, 2 × CH₂CO); ¹³C NMR ((CD₃)₂CO, 125 MHz, δ ppm) 173.34, 171.45, 170.46, 158.57, 154.70, 142.66, 140.07, 131.42, 128.96, 115.04, 112.53, 111.62, 66.10, 63.64, 51.83, 51.32, 38.70, 37.25, 29.09, 28.75; ESI-MS: 488.1 [M + H]⁺; HRMS calculated for C₂₃H₂₆N₃O₇S [M + H]⁺ 488.1491, found 488.1499.

(E)-2-Methoxy-4-[3-(3-(nitrooxy)propoxy)-3-oxoprop-1-en-1-yl]phenyl [(5-(4-(2-(methyl (pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl)methyl]succinate (26a). This title compound was obtained in 47% yield as a light yellow solid: mp 48-49 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.15 (ddd, 1H, J = 5.0 Hz, 2.0 Hz, 0.8 Hz, ArH), 7.65 (d, 1H, J = 16.0 Hz, CH=), 7.46–7.43 (m, 1H, ArH), 7.13–7.11 (m, 1H, ArH), 7.09–7.05 (m, 4H, ArH), 6.84–6.81 (m, 2H, ArH), 6.57 (dd, 1H, J = 6.6 Hz, 5.0 Hz, ArH), 6.54 (d, 1H, J = 8.6 Hz, ArH), 6.39 (d, 1H, J = 15.9 Hz, CH=), 5.59–5.54 (m, 2H, CH₂), 4.60 (t, 2H, J = 6.3 Hz, CH₂ONO₂), 4.44 (dd, 1H, J = 9.4 Hz, 3.9 Hz, CHS), 4.32 (t, 2H, J = 6.1 Hz, CH₂O), 4.16 (t, 2H, J = 5.7 Hz, CH₂O), 3.97 (t, 2H, J = 5.7 Hz, CH₂N), 3.86 (s, 3H, CH₃O), 3.44 (dd, 1H, J = 14.2 Hz, 3.9 Hz, CH), 3.13 (s, 3H, CH₃N), 3.05 (dd, 1H, J = 14.2 Hz, 9.4 Hz, CH), 2.92 (t, 2H, J = 6.9 Hz, CH₂CO), 2.74 (t, 2H, J = 7.1 Hz, CH₂CO), 2.15 (p, 2H, J = 6.3 Hz, CH₂); ¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.48, 170.53, 169.83, 169.78, 166.47, 158.40, 151.37, 144.68, 141.45, 133.23, 130.33, 127.35, 123.31, 121.27, 117.70, 114.76, 111.77, 111.32, 69.95, 66.31, 63.23, 60.54, 55.97, 51.90, 49.47, 37.75, 28.78, 28.69, 26.55; ESI-MS: 767.2 $[M + H]^+$; HRMS calculated for $C_{36}H_{39}N_4O_{13}S$ $[M + H]^+$; HRMS calculated fo H]⁺ 767.2234, found 767.2219.

(*E*)-2-*Methoxy*-4-[3-(4-(nitrooxy)butoxy)-3-oxoprop-1-en-1-yl]phenyl [(5-(4-(2-(methyl (pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl)methyl]succinate (**26b**). This title compound was obtained in 36% yield as a light yellow solid: mp 45–47 °C. ¹H NMR (CDCl₃, 300 MHz, δ ppm) 8.14 (d, 1H, *J* = 4.9 Hz, ArH), 7.62 (d, 1H, *J* = 16.0 Hz, CH=), 7.44 (t, 1H, *J* = 8.1 Hz, ArH), 7.12–7.05 (m, 5H, ArH), 6.82 (d, 2H, *J* = 8.1 Hz, ArH), 6.56–6.50 (m, 2H, ArH), 6.36 (d, 1H, *J* = 16.0 Hz, CH=), 5.58–5.53 (m, 2H, CH₂), 4.51 (t, 2H, *J* = 5.8 Hz, CH₂ONO₂), 4.43 (dd, 1H, *J* = 9.3 Hz, 3.8 Hz, CHS), 4.24 (t, 2H, *J* = 5.8 Hz, CH₂), 4.16 (t, 2H, *J* = 5.8 Hz, CH₂), 3.96 (t, 2H, *J* = 5.8 Hz, CH₂), 3.84 (s, 3H, OCH₃), 3.42 (dd, 1H, *J* = 14.0 Hz, 3.7 Hz, CH), 3.13 (s, 3H, NCH₃), 3.05 (dd, 1H, *J* = 14.0 Hz, 6.2 Hz, CH₂); ¹³C NMR (CDCl₃, 75 MHz, δ ppm) 172.45, 170.47, 169.77, 169.70, 166.59, 158.39, 158.20, 151.38, 147.67, 144.27, 141.42, 137.35, 133.31, 130.30, 127.39, 123.26, 121.18, 118.05, 114.77, 111.75, 111.37, 105.79, 72.62, 66.30, 63.58, 63.21, 55.95, 51.81, 49.48, 37.75, 37.68, 28.77, 28.68, 25.09, 23.74; ESI-MS: 781.2 [M + H]⁺; HRMS calculated for C₃₇H₄₁N₄O₁₃S [M + H]⁺ 781.2391, found 781.2403.

(E)-2-Methoxy-4-[3-(5-(nitrooxy)pentoxy)-3-oxoprop-1-en-1-yl]phenyl [(5-(4-(2-(methyl (pyridin-2-yl)amino)ethoxy)benzyl)-2,4-dioxo-3-thiazolidinyl)methyl]succinate (26c). This title

compound was obtained in 46% yield as a light yellow solid: mp 41–43 °C. ¹H NMR (CDCl₃, 500 MHz, *δ* ppm) 8.14 (ddd, 1H, *J* = 4.9 Hz, 2.0 Hz, 0.8 Hz, ArH), 7.62 (d, 1H, *J* = 16.0 Hz, CH=), 7.43 (t, 1H, *J* = 8.1 Hz, ArH), 7.11–7.05 (m, 5H, ArH), 6.82 (d, 2H, *J* = 8.7 Hz, ArH), 6.55–6.50 (m, 2H, ArH), 6.37 (d, 1H, *J* = 16.0 Hz, CH=), 5.58–5.53 (m, 2H, CH₂), 4.47–4.42 (m, 3H, CHS and CH₂ONO₂), 4.21 (t, 2H, *J* = 6.6 Hz, CH₂O), 4.15 (t, 2H, *J* = 5.7 Hz, CH₂O), 3.95 (t, 2H, *J* = 5.6 Hz, CH₂N), 3.84 (s, 3H, CH₃O), 3.42 (dd, 1H, *J* = 14.2 Hz, 3.9 Hz, CH), 3.12 (s, 3H, CH₃), 3.04 (dd, 1H, *J* = 14.2 Hz, 9.4 Hz, CH), 2.90 (t, 2H, *J* = 6.9 Hz, CH₂CO), 2.73 (t, 2H, *J* = 7.1 Hz, CH₂CO), 1.80–1.73 (m, 4H, 2 × CH₂), 1.54–1.53 (m, 2H, CH₂); ¹³C NMR (CDCl₃, 125 MHz, *δ* ppm) 172.45, 170.47, 169.78, 169.70, 158.39, 158.28, 151.37, 147.78, 144.07, 141.37, 137.27, 133.38, 130.30, 127.38, 123.24, 121.16, 118.26, 114.77, 111.74, 111.35, 105.72, 72.99, 66.29, 64.03, 63.20, 55.95, 51.80, 49.44, 37.71, 28.76, 28.68, 28.26, 26.49, 22.33; ESI-MS: 795.3 [M + H]⁺; HRMS calculated for C₃₈H₄₃N₄O₁₃S [M + H]⁺ 795.2547, found 795.2562.

(*E*)-2-*Methoxy*-4-[3-(6-(*nitrooxy*)*hexyloxy*)-3-*oxoprop*-1-*en*-1-*y*][*phenyl* [(5-(4-(2-(*methyl* (*pyridin*-2-*y*])*amino*)*ethoxy*)*benzy*])-2,4-*dioxo*-3-*thiazolidiny*]*methyl*]*succinate* (**26d**). This title compound was obtained in 35% yield as a light yellow solid: mp 36–38 °C. ¹H NMR (CDCl₃, 500 MHz, δ ppm) 8.15 (ddd, 1H, *J* = 4.9 Hz, 1.9 Hz, 0.8 Hz, ArH), 7.63 (d, 1H, *J* = 15.9 Hz, CH=), 7.45 (t, 1H, *J* = 8.3 Hz, ArH), 7.13–7.06 (m, 5H, ArH), 6.84–6.82 (m, 2H, ArH), 6.56–6.51 (m, 5H, ArH), 6.38 (d, 1H, *J* = 16.0 Hz, CH=), 5.59–5.54 (m, 2H, CH₂), 4.48–4.43 (m, 3H, CHS and CH₂ONO₂), 4.21 (t, 2H, *J* = 5.8 Hz, CH₂O), 4.16 (t, 2H, *J* = 5.6 Hz, CH₂O), 3.97 (t, 2H, *J* = 5.6 Hz, CH₂N), 3.85 (s, 3H, CH₃O), 3.44 (dd, 1H, *J* = 14.2 Hz, 3.9 Hz, CH), 3.14 (s, 3H, NCH₃), 3.08–3.03 (m, 1H, CH), 2.91 (t, 2H, *J* = 6.9 Hz, CH₂CO), 2.74 (t, 2H, *J* = 7.1 Hz, CH₂CO), 1.78–1.68 (m, 4H, 2 × CH₂), 1.49–1.46 (m, 4H, 2 × CH₂);¹³C NMR (CDCl₃, 125 MHz, δ ppm) 172.48, 169.85, 166.83, 158.40, 151.33, 144.02, 141.29, 133.43, 130.33, 127.48, 123.25, 121.21, 119.80, 118.35, 114.76, 111.76, 111.24, 73.19, 66.31, 64.38, 63.23, 55.96, 51.90, 37.75, 33.99, 28.78, 28.69, 28.54, 26.72, 25.62, 25.41; ESI-MS: 809.3 [M + H]⁺; HRMS calculated for C₃₉H₄₅N₄O₁₃S [M + H]⁺ 809.2704, found 809.2720.

4.2 Cell culture.

Human non-tumor hepatic L-02 cells were cultured in DMEM medium supplemented with 10% FBS, 50 U/mL penicillin and 50 μ g/mL streptomycin while and Murine preadipocyte 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% bovine calf serum (basal medium, Life Technologies, Grand Island, NY) in a humidified atmosphere of 5% CO₂ at 37 °C. In addition, primary inner medullary collecting duct (IMCD) cells were prepared from the kidneys of SD rats, as previously reported [77]. The IMCD cells were cultured in collagen-coated dishes (0.4 μ m pore size) in phenol-free DMEM-Ham's/F-12 medium containing 100 IU/mL penicillin, 100 μ g/mL streptomycin, 1% non-essential amino acids and 10% FBS.

4.3 Animals.

All animal studies were carried out at the Laboratory Animal Center of China Pharmaceutical University (Nanjing, China) according to the animal protection and care guidelines of China. The experimental protocols were approved by the Ethics Committee of the China Pharmaceutical University. *Male* diabetic db/db mice (BKS.Cg-Dock7m +/+ Leprdb/J strain) and control C57BL/6 at 6 weeks of age as well as Sprague-Dawley (SD) rat (200–250 g) were purchased from the Model Animal Research Center of Nanjing University and housed in a specific pathogen-free facility at the consistent temperature of 22-23 °C with a 12-h cycle of light-dark and free access to food and water.

4.4 Glucose uptake assay.

The impact of individual compounds on glucose uptake was examined [41]. L-02 cells were cultured into 96-well plates and treated with 1 μ M dexamethasone for 3 h, followed by changing to serum-free medium containing 5.5 mmol/L glucose in triplicate in the presence of 10 μ M each compound for 48 h. During the last 24-h culture, the cells were stimulated with 1 nM insulin and the medium in each well was collected for glucose measurement by glucose oxidase assay using

a specific kits (Nanjing Jiancheng Bioengineering Institute). The glucose uptake (%) was calculated as follows: (5.5 mmol/L - the concentration of glucose in the medium after 48 h treatment)/ $5.5 \text{ mmol/L} \times 100\%$.

4.5 Adipocytes differentiation.

The differentiation of 3T3-L1 pre-adipocytes was induced as described in the documentation of chemically-induced differentiation by the American Type Culture Collection (ATCC). Briefly, 3T3-L1 preadipocytes (20,000 cells/well) were cultured in basal medium in 24-well plates up to 100% confluency and continually cultured for another 48 h. The cells were stimulated for differentiation in DMEM containing 10% FBS, 1.0 μ M dexamethasone, 0.5 mM methylisobutylxanthine (IBMX) and 1.0 μ g/mL insulin for 48 h, followed maintaining the cells in DMEM containing 10% FBS and 1.0 μ g/mL insulin for 13-15 days.

To evaluate the effect of Rosi and **21** on adipocyte differentiation, 3T3-L1 pre-adipocytes were induced for differentiation and treated with vehicle DMSO (0.1%, v/v), Rosi (10 μ M) or **21** (10 μ M) every other day up to day 14 post induction. The control cells were treated with vehicle alone without inducing differentiation. The cells were washed twice with warm KRP buffer (127 mM NaCl, 4.7 mM KCl, 0.9 mM MgSO₄, 10 mM NaPO₄, and 0.9 mM CaCl₂) and stained with AdipoRed (30 μ L per well, LONZA) in the dark for 15 min. The fluorescent signals were photoimaged under a confocal microscope (Zeiss LSM, Germany). The fluorescent signals in individual wells were measured in a microplate reader using an excitation/emission (Ex/Em) at 485/572 nm.

4.6 Induction of insulin resistance and 2-NBDG uptake assay.

The mature murine adipocytes on day 8 post induction were treated with TNF- α (4 ng/mL, Roche Applied Science, Indianapolis, IN, USA) in 24-well plates daily for 4 days to induce insulin resistance.[48] The cells were pre-treated in triplicate with vehicle or individual compounds at 10 μ M for 48 h, and challenged with 100 μ M 2-NBDG at 37 °C for 30 min in the dark. After being washed three times with KRP buffer, the fluorescent signals in individual wells were measured in a microplate reader with an Ex/Em at 485/515 nm.

4.7 Evaluation of intracellular NO and ROS.

The insulin resistant mature adipocytes were cultured in 24-well plates and treated with 10 μ M Rosi, ionic DEA/NO or 3 or 10 μ M compound **21** in the presence or absence of 20 μ M C-PTIO for varying time periods. The control cells received the vehicle DMSO (0.1% v:v) or NaOH (10 μ M). The cells were washed twice with pre-warmed KRP buffer, and treated with 10 μ M DAF-FA-2DA or DCFH-DA for 30 min at 37 °C. The fluorescent signals in individual wells were measured in a microplate reader with an Ex/Em at 485/515 nm or 488/525 nm, respectively.

4.8 Evaluations of GSH, SOD, and MDA levels.

The insulin resistant mature adipocytes were treated in triplicate with indicated compounds for 48 h. The levels of GSH, SOD and MDA in the cell lysates of cultured individual wells of cells were determined using specific kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions.

4.9 Stability studies.

The stability of **21** in the gastrointestinal fluids that were prepared as 1% pepsin in water (pH 1.5) and 1% trypsin and 0.68% KH₂PO₄ in water (pH 6.8) [78] was determined. Briefly, the compound 21 (100 mM) in DMSO was mixed in triplicate with the gastric or intestinal fluid at 1:1000 and incubated at 37 °C for 0, 2, 4, 6, 16, or 24 h, followed by addition of triple volumes of acetonitrile to terminate the reaction. The mixtures were centrifuged at 12,000 g for 5 min and the contents of **21** in the supernatant samples (10 μ L each) were analyzed by HPLC (Shimadzu DGU-20A3R) on Shimadzu-GL WondaSil C18-WR column. The mobile phase was acetonitrilewater (60/40, v/v) at a flow rate of 1.0 mL/min with the detection wavelength at 254 nm.

The stability of **21** was also determined in human liver microsomes (RILD, Shanghai, China; 10 mg proteins/mL). Briefly, 0.5 μ L of **21** (100 mM in DMSO) was mixed in triplicate with 399.5 μ L of phosphate buffer (PB, 0.1 M, pH 7.4) containing 3.3 mM MgCl₂ and 25 μ L human liver microsomes and incubated at 37 °C for 5 min. The individual mixtures were added with 75

 μ L of 1.3 mM NADPH in PB and incubated with gently shaking at 37 °C for 0, 0.5, 1, 2, and 6 h, followed by addition of triple volumes of acetonitrile and centrifugation. The contents of 21 in the supernatant samples (10 μ L each) were analyzed by HPLC. The mobile phase A (MP A) was ammonium acetate buffer (pH 3.5) and mobile phase B (MP B) was acetonitrile. The MP A and MP B with a simple gradient program (0 min, MP A : MP B = 65:35; 15 min, MP A : MP B = 20:80) was delivered at a flow rate of 1.0 mL/min with the detection wavelength at 254 nm.

In vitro hydrolysis of **21** in SD Rat Plasma. **21** (1 μ L, 100 mM in DMSO) was mixed in triplicate with 999 μ L of SD rat plasma and incubated in a warm-shaking bath at 37 °C for 0, 0.5, 2, 4, 7, 16, or 24 h, followed by addition of triple volumes of acetonitrile to terminate the reaction. After being centrifuged, individual supernatant samples (10 μ L each) were analyzed by HPLC, as described above.

4.10 AQP2 transfection and treatment.

The IMCD cells on day 5 post preparation were cultured in 24-well plates overnight and transfected with 4 ng plasmid pcDNA-AQP2 (Shanghai Genechem) using Lipofectamine TM 2000 in serum-free DMEM-Ham's/F-12 for 5 h. The cells were cultured in DMEM-Ham's/F-12 containing 10% FBS for 48 h. Furthermore, the transfected cells were treated vehicle (0.1% DMSO), forskolin (100 μ M), Rosi (10 μ M or 50 μ M) and **21** (10 μ M or 50 μ M), respectively, for 30 min.

4.11 Immunofluorescence analysis.

The cells were fixed by 4% paraformaldehyde, permeabilized with 1% TritonX-100, and treated with 3% H_2O_2 in methanol for 15 min. After being washed, the cells were probed with FITC-anti-AQP2 (1:200, Abcam, Cambridge, UK). The distribution of AQP2 in IMCD cells was characterized by the confocal microscopy (Zeiss LSM, Germany). The digital images were collected and analyzed using the Zeiss Aim Image Examiner program.

4.12 Western blotting.

The different groups of IMCD cells were washed with the biotinylation buffer and treated with 3 mg/ml cell-impermeant biotinamidohexanoic acid 3-sulfo-N-hydroxy- succinimide ester (Sigma-Aldrich, St. Louis, MO) for 60 min at 4°C. After being washed, the cells were lyzed in lysis buffer containing 1% NP-40, followed by centrifugation. The lysates were reacted with streptavidin beads at 4 °C overnight. The concentrations of biotinylated proteins and total lysate proteins were measured by BCA assay using the BCA kit. The biotinylated protein and total lysate protein samples from individual groups of cells (30 µg/lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free dry milk in TBST and probed with mouse anti-AQP2 overnight at 4 °C. After being washed, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG and visualized using the enhanced chemiluminescence reagent. The relative levels of AQP2 expression were determined by densitometric scanning using the Image Pro Plus 6.0 software.

4.13 In vivo experiment.

The anti-diabetic activity of **21** was evaluated in diabetic db/db mice. The mice were randomized and treated with Rosi (27 μ mol/kg, ~10 mg/kg) and **21** (13.5 or 27 μ mol/kg, ~10 or ~20 mg/kg) in 0.5% CMC-Na solvent by gavage daily for 18 consecutive days. The control and model groups of mice received equal volume of 0.5% CMC-Na solvent (n=8 per group). Their body weights, food and water consumption were measured daily. The levels of fasting blood glucose of individual mice were measured every three days using the Omron HEA-232 glucometer. The mice were subjected to oral glucose tolerance test (OGTT) on day 12. Briefly, the mice were fasted overnight and administrated with 2 g/kg glucose by gavage. The levels of blood glucose in individual mice were measured at 0, 15, 30, 45, 60, 90, and 120 min post glucose challenge. On day 19, their peripheral blood samples were collected and the mice were sacrificed. The serum samples were prepared by centrifugation.

Acute Toxicity. Both genders of ICR mice (7 weeks, SLACCAS) were randomized and treated with a single dose of Rosi at 500, 1000, 2000, 3000, 4000, or 5000 mg/kg, or with **21** at 2000, 4000, and 5000 mg/kg by gavage, respectively (n = 10 per group). The animals were observed for abnormal behaviors and mortality up to 14 days post treatment.

4.14 Statistical analysis.

Data are expressed as mean \pm SD or SEM. The difference among the groups was analyzed by two-way ANOVA and the difference between groups was analyzed by Student t-test using the SPSS window 21. A P-value of <0.05 was considered statistically significant.

Associated content

Supporting information.

Synthetic routes and characterization of compound **27**; OGTT curves; in vitro metabolic stability of **21** in simulated gastrointestinal fluids and human liver microsomes; BW change curves; mean BW gain on day 18; anti-platelet aggregation data of Rosi and **21**; the inhibitory ratios of **21** and Rosi on hERG channel at indicated concentrations; HPLC assessment of compound purities; ¹H NMR, ¹³C NMR and HRMS spectra of target compounds; molecular formula strings (CSV).

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Notes

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Abbreviations used

AQP2, aquaporin-2; FDA, U. S. Food and Drug Administration; n-Bu₄NHSO₄, ntetrabutylammonium hydrogen sulfate; DMF, *N*,*N*-dimethylformamide; DCC, *N*,*N*dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; HPLC, high performance liquid chromatography; TEA, triethylamine; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; SD, standard deviation; TNF- α , tumor necrosis factor- α ; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; CMC-Na, sodium carboxyl methyl cellulose; TMS, tetramethylsilane.

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Highlights

- 21 exhibited better effects on improving glucose tolerance than rosiglitazone (Rosi), which was associated with the NO production, antioxidant and anti-inflammatory activities.
- 21 displayed relatively high stability in the simulated gastrointestinal environments and human liver microsomes.
- 21 exhibited improved safety profiles in terms of cardiovascular protection, body weight gain, hematocrit change, effect on hERG channel, and acute toxicity in vivo relative to Rosi.
- 21, unlike Rosi, had little stimulatory effect on the membrane translocation of aquaporin-2 (AQP2) in kidney collecting duct epithelial cells.