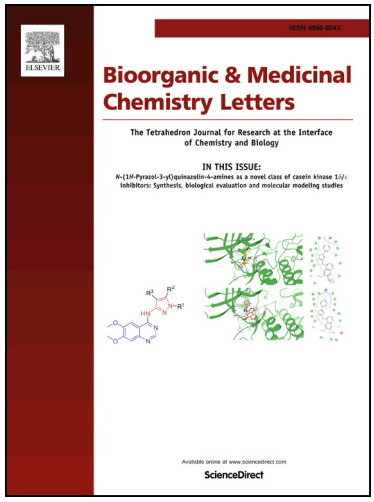
Accepted Manuscript



Discovery of 1-(4-((3-(4-methylpiperazin-1-yl)propyl)amino)benzyl)-5-(trifluoromethyl)pyridin-2(1H)-one, an orally active multi-target agent for the treatment of diabetic nephropathy

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Discovery of 1-(4-((3-(4-methylpiperazin-1-yl)propyl)amino)benzyl)-5-(trifluoromethyl)pyridin-2(1H)-one, an orally active multi-target agent for the treatment of diabetic nephropathy

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ABSTRACT:

Oxidative stress, inflammation and fibrosis can cause irreversible damage on cell structure and function of kidney and are key pathological factors in Diabetic Nephropathy (DN). Therefore, multi-target agents are urgently need for the clinical treatment of DN. Using Pirfenidone as a lead compound and based on the previous research, two novel series (5-trifluoromethyl)-2(1H)-pyridone analogs were designed and synthesized. SAR of (5-trifluoromethyl)-2(1H)-pyridone derivatives containing nitrogen heterocylic ring have been established for in vitro potency. In addition, compound 8, a novel agent that act on multiple targets of anti-DN with IC_{50} of 90uM in NIH3T3 cell lines, $t_{1/2}$ of 4.89 ± 1.33 h in male rats and LD_{50} > 2000 mg/kg in mice, has been advanced to preclinical studies as an oral treatment for DN.

Diabetic Nephropathy (DN) is considered as one of the major complications of diabetes mellitus, which is the leading cause of end-stage renal disease (ESRD) and has become a serious threat to human health. There are 285 million people in 2010 and 347 million people in 2013 with diabetes mellitus all over the world, [1]and WHO projects that diabetics will be the seventh-leading cause of death by 2030.[2] Roughly one-third of the diabetic population will develop diabetic nephropathy,[3] which is a heavy social and economic burden.[4]

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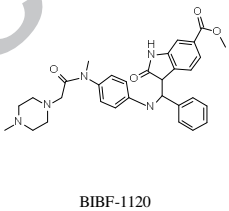
The occurrence and development of the DN is a complex process with the pathogenic nature of kidney fibrosis. Abundant evidence suggests that genetic predisposition,[5] hyperglycemia,[6-8] hyperlipidemia,[9] and microcirculatory disturbance are the major factors contributing to the initiation and progression of DN.[10] Although DN is associated with high morbidity and mortality and the prevalence of this disease is continuously increasing worldwide, there is currently no effective treatment strategy available other than symptom control. In recent years, numerous studies have shown that the development of oxidative stress,[11] inflammatory and renal fibrosis is the key to the occurrence and development of DN.[12,13] Therefore, compounds that can bring resistance to oxidative stress, inflammation, and fibrosis have been the focus of drug development studies on DN.

Reactive Oxygen Species (ROS) play an important role in DN which indicates that the direct scavenge of ROS is a potential strategy for drug discovery of anti-DN agents. Superoxide dismutase mimetic Tempol,[14] a nonspecific NOX inhibitors with IC_{50} of 0.9 uM, reduced the peroxide production in glomeruli. At the same time, due to increased myeloperoxdase, this drug failed to reduce albuminuria which is a result of the damage to the GBM. VAS-2870, a NOX specific depressor, inhibits ROS generation with an IC_{50} value of 10.6 mM in cell-free systems.[15] However, it failed in animal experiments assessing the pharmaceutical profile. Next, inflammation also plays a key role in the development and progression of DN. TLK-19705,[16] a novel CCR2 antagonist, has been tested in a group of db/db mice with dosages of 30 mg/kg/day and 10 mg/kg/day for 8 weeks by Okamoto. M' team. And the results show that the urinary ACR has improved in the animal experiment. Finally, renal fibrosis is the final common pathway of DN. IN-1130 showed efficacy in suppressing fibronectin in UUO rat kidneys by inhibiting Smad2 phosphorylation.[17] GW788388 can diminish renal fibrosis by inhibiting the proliferation of fibrosis cell.[18,19] HOE77, an inhibitor of the formation of collagen in the late stage of liver fibrosis, can cause cataracts in phase II clinical trial, and its investigation has been terminated.[20,21] The above many compounds effect in a link or target of fibrosis, which rarely passed clinical research, suggesting that single target inhibitor is not the ideal research direction of anti-DN drug discovery.

Pirfenidone (PFD), a multi-target drug, has been marketed mainly for idiopathic pulmonary fibrosis since 2008.[22] And a phase clinical trial of PFD for DN has been completed with 77 patients at San Digo Medical Center of California University.[23] Clinical effect of PFD is not satisfactory because its 5-methyl can be easily metabolized.[24] In addition, PFD showed a certain acute toxicity in animal experiments.[25] With PFD as the lead compound, our research group has carried on a series of transformation. The previous research results have been reviewed by Yi-Min Liu.[26] First, we gain Fluorofenidone（AKF-PD）by introducing fluorine in the meta position of the phenyl group of PFD.[27] AKF-PD, a “me-better” drug, show higher inhibitory activity and more alleviated fibrosis symptoms than PFD.[28] As with PDF, AKF-PD is prone to inactivation due to the metabolism of methyl groups on pyridone ring.[29] In order to prevent the metabolism of the 5-methyl group, our research group turn methyl into aromatic ring,[30] trifluoromethyl and methyl cyclization.[31,32] which greatly increase the activity of the compound. Among them, the activity of compound ZHC-102 with an IC_{50} of 290uM was obviously improved. In our early work, we found that the introduction of methylene into pyridine ring and phenyl group can improve the activity of the target compound. For the purpose of finding the target compounds with excellent activity and further exploring the structure-activity relationship, our group has developed a new series of compounds by converting the phenyl group at the 1-position of the pyridone ring to the benzyl group[31]. ZHC-159, a benzyl series compound, shows excellent activity with IC_{50} of 80uM in NIH3T3 cell lines, but it also has high toxicity.

Table 1

The anti-DN compounds in preclinical or clinical research.



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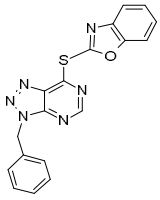
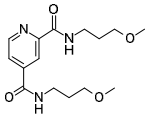
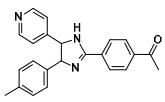


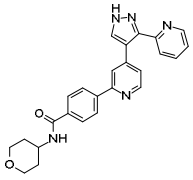
VAS-2870



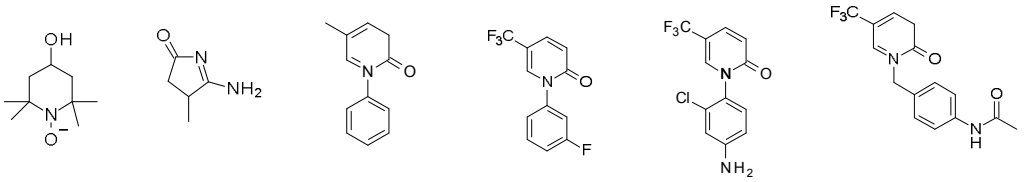
HOE-77



IN-1130



GW-788388



The purpose of this study is to develop an anti-DN drug. Although the activity of the compound has been improved by previous studies, the drugability of these compounds is not sufficient because of poor water-solubility and high toxicity. Experiments revealed that the drugability of compounds could be improved by adding the larger group to 1-N of pyridone. Herein, our research group developed two novel series of (5-trifluoromethyl)-2(1H)-pyridone derivatives containing nitrogen heterocyclic ring to improve its medical properties(see Fig.1).

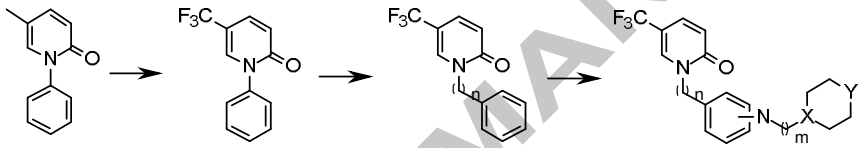
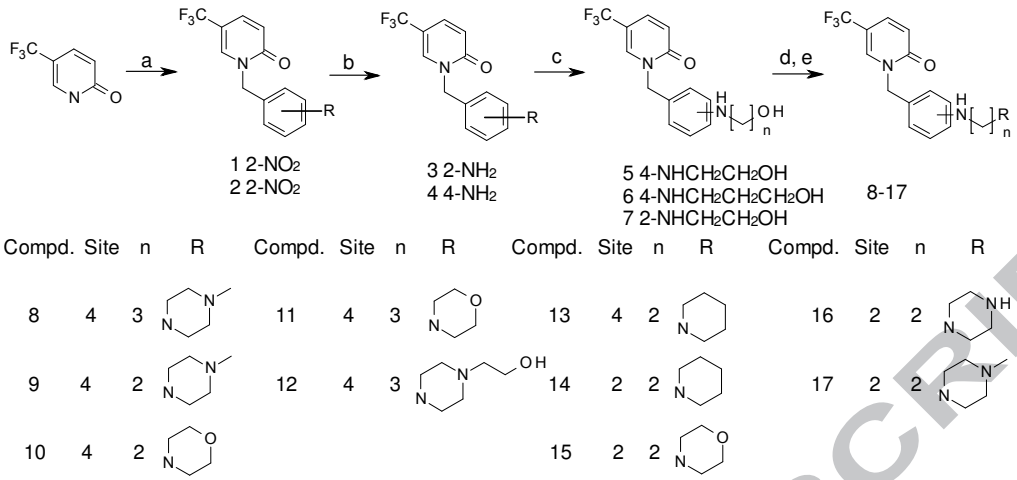


Fig. 1. The map of design route of the target compounds

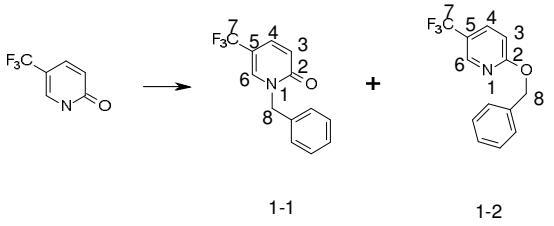
The mouse fibroblast cell line (NIH3T3 cells), a well-recognized anti-fibrosis drugs in vitro screening model,[33] was used to test the potency of the target compounds. Here we detected NIH3T3 cell proliferation by MTT assay with AKF-PD as the positive control to test the potency of the anti-fibrosis compounds and discuss the SAR on anti-fibrotic activity. Compound 8 was finally identified in the research by conducting a series of research of SAR and pharmacokinetic experiments.

An efficient synthesis of a wide range of (5-trifluoromethyl)-2(1H)-pyridone derivative was reported earlier with our effort to discover NIH3T3 inhibitor. Based on this, PFD was extended by introducing versatile nitrogen heterocyclic ring side chains. The link chain between the aromatic amino group and the heterocycle is 2 to 3 carbon atoms in length. Representative nitrogen heterocyclic ring are listed in Table 2. Chemical synthesis and characterization of key compounds, which were used for SAR determination and in vivo evaluations were described in the experimental section.



Scheme 1. Reagents and conditions:(a) K_2CO_3,DMSO,130℃， 4h;(b)Fe,HCl,50%EtOH,reflux,3h;(c)BuOH,K_2CO_3,95℃, 20h;(d) $SOCl_2$,DCM,TEA,reflux,4h;(e) Acetonitrile,reflux.

Scheme 1 illustrated the preparing process of 10 target compounds. With the intention to prevent the metabolism of 5-site methyl group of pyridone ring, we used 5-trifluoromethyl-2(1H)pyridone as the mother moiety and starting material. The compound 1 and 2 were easily prepared through coupling of 5-trifluoromethyl-2(1H)-pyridone with substituted benzyl halide which was carried out by stirring the mixture in DMF with K_2CO_3.[34] The compounds 3 and 4 were subsequently synthesized via the reduction of compounds 1 and 2 in 90-95% yield, in which Fe was used as the reducing agent in the solution of HCl/ethanol.[35] Treatment of 3 and 4 with halohydrin and K_2CO_3 in BuOH gave the key intermediate 5, 6 and 7.[36] In the experiment, we found an interesting phenomenon: The temperature had a great influence on the alkylation products. At temperatures below 95 □, the reaction hardly occurs; whereas at temperatures above 100 □, the products were mainly aminodialkylated. The compounds 8 to 17 were obtained by one-pot method from the intermediate 5 to 7 through chlorination as described in literature and then refluxing and stirring reaction with nitrogen heterocycle in acetontrile.[37,38]



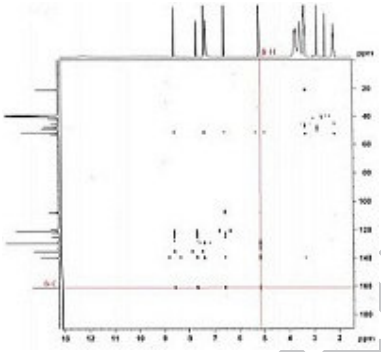
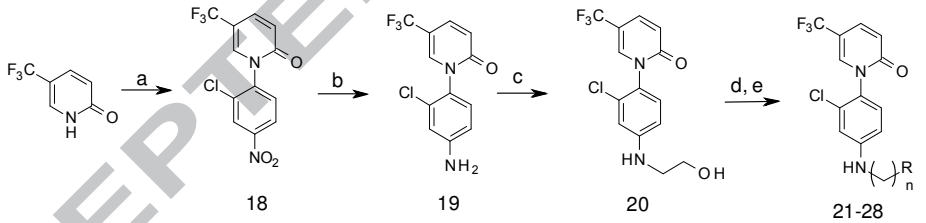


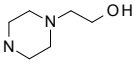
Fig. 2. The reaction route map of first step.

Fig. 3. HMBC of compound 8.

On the basis of the reaction mechanism analysis, there could be two productions of N-alkylation (1-1) and O-alkylation (1-2) in the first step (see **Fig. 2**). C^{13}-NMR and H^{1}-NMR of the two compounds are similar in theory, so the two compounds cannot be judged just by C^{13}-NMR and H^{1}-NMR. In order to further elucidate the structure, HMBC of compound 8 was determined. As all know, there is a correlation between 8-H and 6-C of 1-1 but 1-2. The HMBC result shown: 8-H (5.1597 ppm) and 6-C (139.39 ppm) had a long rang correlation (see **Fig. 3**). It indicated that compound 8 belonged to productions of N-alkylation.

Chemical structures of all others were confirmed by IR, MS, H^{1}-NMR and C^{13}-NMR.



Compd.	n	R	Compd.	n	R	Compd.	n	R	Compd.	n	R
21	3		23	3		25	2		27	2	
22	3		24	2		26	2		28	2	

Scheme 2. Reagents and conditions:(a) K_2CO_3,DMSO,130℃, 4h;(b)Fe,HCl,50%EtOH,reflux,3h;(c)BuOH,K_2CO_3,95℃, 20h; (d) $SOCl_2$,DCM, TEA,reflux,4h;(e) Acetonitrile reflux, 4h.

According to Scheme 2, 8 designed phenyl substituted compounds were synthesize d. There is a small amount of 1-(3-chloropropyl)piperidine, 4-(3-chloropropyl)morpho line and 1-(3-chloropropyl)-4-methylpiperazine in the laboratory. So the target compo unds 21, 22 and 23 were directly obtained by treatment of 19 with the above react

ants and K_2CO_3 in BuOH. The synthesis methods of the other compounds was the same with Scheme 1.

The mouse fibroblast cell line(NIH3T3 cells), which has been used as routine screening model of anti-fibrosis drugs in vitro,[39] was used to test the potency of the desired compounds. Compounds 8 to 17 and 21 to 28 were examined for their anti-proliferative activity by MTT assay using the NIH3T3 cell line with AKF-PD as the positive control. The results of these assays are presented in Table 2. The inhibition ratio was processed by Ascent Software™ and IC_{50} values were obtained from the dose-response curves. Compound 8 is currently one of the most active compounds that inhibit NIH3T3 cell lines with IC_{50} value of 90uM.

Firstly, it could be seen that the introduction of the heterocyclic side chain further enhanced the activity of the target compound. As these data illustrate in Table 2, all the desired compounds showed significantly improved inhibition activities relative to the positive control AKF-PD.

The effect of heterocycles side chains on activity was different in benzyl and phenyl series target compounds. As shown in the table 2, the activity of compound 25, 21 of phenyl series were better than compound 26, 22 and 23, 27, respectively. It can be concluded that piperidine ring was favor to morphine ring and 4-methyl piperazine ring in phenyl series; while compounds 8, 9 of benzyl series was superior to compound 10, 11 and 13, 14. So the activity of the 4-methylpiperazine derivative in the benzyl series is superior to the morphine and piperidine derivatives.

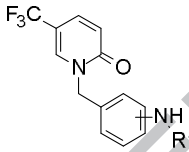
From Table 2. It could also be concluded that the length of the link chain between heterocyclic and amino group has an effect on the activity of the benzyl series compounds. However, this effect was not observed in the phenyl series. In the article the number of carbon in link chain was investigated ranging from 2 to 3，the anti-fibrosis activity of compound 13 with IC_{50} of 580uM was better than compound 14 with IC_{50} of 2550uM, while the anti-fibrosis activity of compounds 25 and 26 were roughly equal to compounds 21 and 22, respectively. The results showed that the activity of the 3 carbon atom link chains in the benzyl series is better than that of the 2 carbon atom link chains; the link chain length was 2 carbons or 3 carbons in the phenyl series made no significantly difference to inhibit NIH3T3 cell.

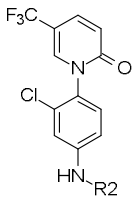
From the previous study we found that:[31] 5-trifluoromethy l-substituted derivatives are superior to 5-methyl or 5-chloro-substituted derivatives. The ideas have been extended and confirmed in this study. In the earlier studies the activity of the target compounds in the benzyl series was better than that in the phenyl series; however, this phenomenon was not reflected in this study. Our analysis showed that the activity of the target compound was greatly improved after introducing the heterocyclic side chain, which masked the difference of the activity of the two series of compounds.

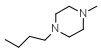
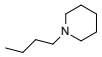
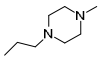
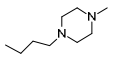
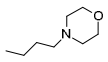
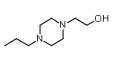
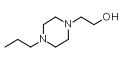
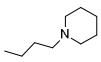
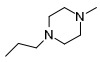
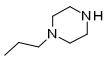
The compound to be highlighted was compound 8 with an IC_{50} value of 90uM. The improved inhibition may be attributed to the facts that compound 8 can form more hydrogen bond with the related receptors, higher solubility and excellent membrane permeability. Further studies need to be conducted on these derivatives to evaluate their potent.

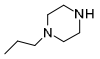
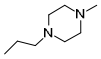
Table 2

The structures of 5- trifluoromethyl -2(*1H*)-pyridone target derivatives and their inhibitory activities against NIH3T3 cells.





Benzyl series compound	Substituent site	Substituent R_1	IC_{50} (uM)	Phenzyl series compound	R_2	IC_{50} (uM)
8	4		90	21		80
9	4		100	22		150
10	4		220	23		170
11	4		260	24		60
12	4		330	25		70
13	4		580	26		110
14	4		2550	27		200
15	2		100	28		1300

16	2		100			
17	2		340	AKF-PD	/	2750

For a follow-up study of one of the preferred compounds, the LD_{50} of compounds 8, 23, and 24 in mice were assayed. From the experimental results, we could see that compound 8 was the least toxicity with a LD_{50} more than 2000 mg/kg. Compound 8 was selected as the preferred compound for further study because of higher activity and lower toxicity.

To determine the therapeutic effect in vivo, we performed an in vivo study of compound 8 against diabetic nephropathy in the db/db mouse model. db/db mice (C57BLKs/J Leprdb/db mice) is a mutant strain from C57BLKs/J mice with leptin receptor gene mutation that causes type 2 diabetes. The disease progression in this model is very similar with the human type 2 DN, which makes it widely used as a research animal model for DN.[40-42] Angiotensin II receptor blockers (ARBs) show good kidney protection effects in a variety of experimental animal models and humans for the treatment of kidney disease. Losartan is the first anti-hypertensive drugs of AIIA class and has been used in the treatment of diabetic nephropathy.[43] So Losartan was selected as the positive control drug in the experiment. Losartan, PFD and compound 8 were selected for in vivo assessment of the anti-DN effect in db/db mice of 8 week-old. The effect of compound 8 on anti-glomerulosclerosis was superior to that of the Losartan in db/db mice ranging from 8 to 24 week-old. The blood glucose of model group was significantly higher than that of diabetes model (DBM) group ($P<0.05$). The difference was statistically significant, and the diabetic model was justified to be successful by comparing with model group. Then, 24-hour urine was collected from the model mice and urine albumin measured by ELISA. The model group 24-hour urinary albumin was significantly higher than that of the normal group, and the difference was statistically significant, indicating the success of diabetic nephropathy model. Compounds were dissolved in 0.5% CMC-Na in water by gavage administration to db/db mice once a day continuing to 24 weeks of age (except for DBM pathological control group). The dose of Losartan, PFD, and compound 8 were 20 mg/kg.d, 250 mg/kg and 40 mg/kg.d, respectively. The therapeutic effect of anti-DN was assessed by determination of serum creatinine, blood urea nitrogen, creatinine clearance ratio, urinary albumin, albumin-to–creatinine ratio,

glomerular sclerosis index, kidney weight/body weight and pathological section dying with PAS and score in treated groups compared to the control group.[44]

Table 3

The measurement of BUN, Scr, Ccr, Urinary Albumin, ACR in each experimental groups.

Groups	BUN (mmol/L)	Scr (umol/L)	Ccr (ul/min)	Urinary albumin(ug)	ACR (ug/mg)
DBM	10.31±0.31	28.25±8.25	216.96±16.96	18.746	0.19±.194
Model	11.22±1.22⋆	38.86±8.86⋆	120.77±20.77⋆⋆	244.98±44.98/⋆⋆⋆	2.72±0.729⋆⋆⋆
Losartan	11.70±1.70⋆	41.13±1.13t⋆	155.14±55.14⋆	112.32±12.32△	1.17±0.173△△
PFD	13.08±3.08⋆	41.67±1.67⋆	136.36±36.36⋆	105.85±05.85△	1.16±0.168⋆
Compd. 8	11.58±1.58⋆◆	29.83±9.83⋆◆	222.92±22.92△◆	41.96±1.962△◇	0.37±0.376△△△◇◇

Note：Compared to DBM group ⋆P>0.05,*P<0.05, ⋆⋆P<0.01 ⋆⋆⋆P<0.001; Compared to modle group,▲P>0.05,△P<0.05; △△P<0.01; △△△P<0.001; Compared to Losartan group with 8 weeks age ◆P>0.05 ◇P<0.05 ◇◇P<0.01,◇◇◇P<0.001.

The four compounds in Table 3 were assessed: Losartan, PFD and compound 8. Given equal doses after treatment, the model group serum creatinine, blood urea nitrogen, 24 hours urinary albumin, urine albumin creatinine ratio (ACR), creatinine clearance rate (Ccr) significantly decreased ($P < 0.05$) compared with DBM group. Serum creatinine and blood urea nitrogen have no significant difference between each treatment group and model group with same ages (P value of 0.05), but the amount of 24 hours urinary albumin, ACR, (except compound 8 8 weeks) reduced to some extent, and the decrease in creatinine clearance was significantly delayed.

In comparison with the DBM group, the result of urinary albumin in 24 hours and ACR of Losartan group fell by 54.15%(P=0.021), 56.87%(p=0.005), respectively, and Ccr decreased slowly by 28.45% (p=0.397). compound 8 and PFD 24-hour urinary albumin has declined by 82.87% (p=0.002) and 56.79% (p=0.023); ACR decreased by 86.22% and 57.23% （p=0.000 and 0.070）; while the decline of Ccr slowed by 84.57% and 12.9% (p=0.050 and 0.686).

The urinary albumin in 24 hours, ACR of compound 8 group dropped significantly compared with Losarton group at 8 weeks and the difference had statistical significance($p<0.005$). The rest of result had changed but with no statistical difference.

Renal tissue PAS staining slice was examined under ordinary optical microscope 400 times the microscopic. The results show that DBM mice kidney pathological control group and

DBM group had no obvious pathological changes; model group showed glomerular hypertrophy, thickening of basement membrane, broadening mesangial area, including increased mesangial cell and mesangial matrix. In contrast, the lesions in the treatment groups were significantly reduced. (see **Fig. 4**).

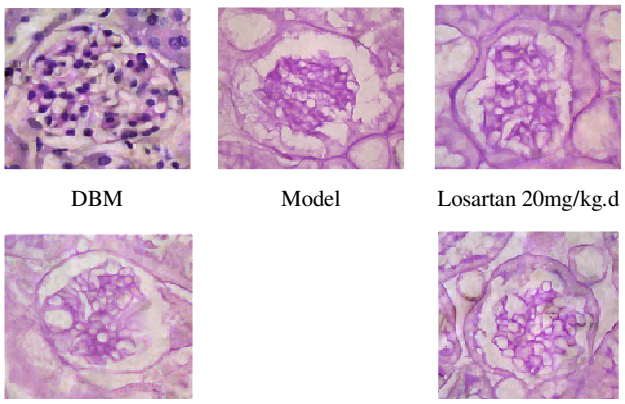


Fig. 4. PAS staining of mice kidney in each groups

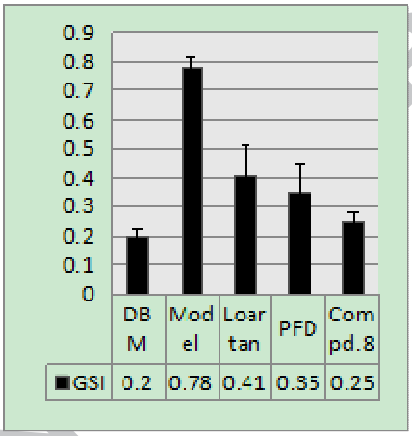


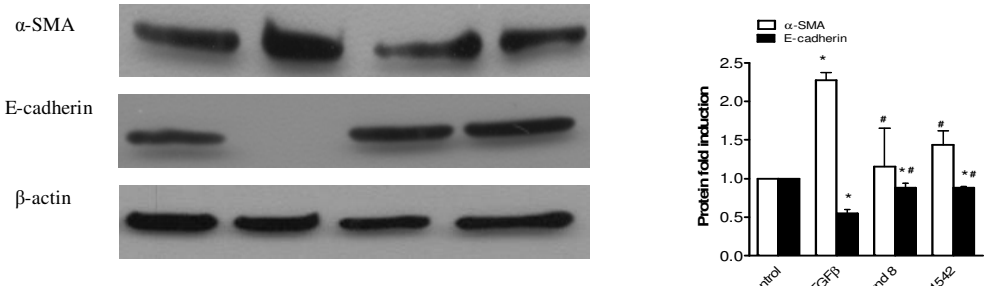
Fig. 5. GSI chart of mice in each experimental group

The glomerular sclerosis lesions were clearly visible and GSI increased significantly in renal tissue pathological examination in model group(see **Fig. 5**). The kidney tissues of all DBM pathological control group and DBM group had no serious glomerular sclerosis. Compared with the DBM group, the GSI of the model group increased significantly. And compared with the model group, GSI of each treatment group decreased obviously, the difference was statistically significant. GSI of Losartan, PFD and compound 8 group declined and fell by 47.25%($p<0.01$), 55.04%($p<0.001$) and 67.31%($p<0.001$), respectively. GSI of compound 8 group dropped significantly compared with Losartan and PFD group at 8 weeks and the difference had statistical significance($p<0.005$). The results shown that 40 mg/kg.d compound 8 had better anti-glomerular sclerosis effect than 20 mg/ kg.d Losartan and 250 mg / kg.d PFD at 8 weeks of age. It can be concluded from overall analysis that Losartan, PFD, AKF-PD and compound 8 had obviously curative effect on glomerular sclerosis. Moreover, compound 8 shows efficacy with a dosage of 40 mg/kg. D was superior to that of other groups.

In early renal fibrosis and anti-inflammatory studies, we found that compound 8 may interact with multiple targets. The reasearch about the target and mechanism of

compound 8 has been published by other researchers in our study group.[45-47] Although we observed the effects of compound 8, its mechanism of action remain unclear. Fibrosis is the central pathology of DN that manifests from the initiation to the end-stage. Since we observed that compound 8 treatment exerts curative effect on DN in mouse model and anti-proliferation effect on NIH3T3 cells, we hypothesize that the mechanism underlying such effects involves the regulation of fibrosis. To test this hypothesis, we firstly assessed the effect of compound 8 treatment on the fibrotic conversion of NRK-52E cell, a rat renal tubular epithelial cell line. Previous study has demonstrated that TGF-β1 is increased in DN and induces the expression of kidney fibrotic markers. Therefore, we used TGF-β1 to treat the cell in the presence or absence of 24.5 mol/L compound 8 or SB431542, which is a TGF-β1 inhibitor. After 24 hour treatment, we firstly examined the cell morphology change. We observed that TGF-β1 treatment induced the NRK-52E cells to be elongated and changed the spearhead shape which is a fibrotic feature. Interestingly, this cell morphology change can be blunted by treatment of both compound 8 and TGF-β1 inhibitor SB431542, suggesting similar effect of compound 8 in blocking TGF-β1 mediated fibrotic signaling.

Next, it is well-known that once undergoing fibrotic transformation, kidney cells over express α-SMA and decrease the expression of E-cadherin. And these changes are mediated by TGF-β1. To further validate if compound 8 exerts anti-fibrotic effect through TGF-β1, we determined the expression of α-SMA and E-cadherin after 48 hour treatment by TGF-β1. Intriguingly, we found that the expression of α-SMA is induced by TGF-β1 but this induction can be blocked by compound 8 treatment. On the contrary, the inhibition of E-cadherin expression by TGF-β1 can be reversed by compound 8 back to that of normal levels. These data clearly suggest that compound 8 has an anti-fibrosis effect via inhibiting the effect of TGF-β1 in inducing renal tubular epithelial cell fibrotic conversion.(see **Fig. 6.**)



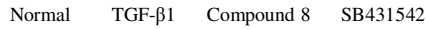


Fig. 6. Effect of on TGF-β1 induced expression of a-SMA and E-cadherin in NRK-52E cell
Note: *p＜0.05 VS normal; #p＜0.05 VS TGF-β1

Besides tubular epithelial cells, mesangial cells can also undergo fibrotic transformation with the challenge of TGF-β1 and high glucose. To determine if compound 8, PFD and AKF-PD can prevent fibrotic change of mesangial cells, we treated the mouse MES-13 cell line with TGF-β1 and different concentrations of glucose, respectively. CTGF is an important downstream component of TGF-β1 induced pro-fibrosis effect by increasing the expression of Collagen I and FN in fibroblasts. Because CTGF level is generally much lower with very singular biological effects, it serves as a more specific target for the treatment of kidney fibrosis than TGF-β1. First, we found that after 48 hour treatment with TGF-β1, both CTGF and FN increased significantly in MES-13 cells by 3.99 and 2.76 folds respectively. Interestingly, all the three compounds can dramatically decrease the levels of CTGF and FN induced by TGF-β1 with a more potent effect than TGF-β1 inhibitor SB431542, with compound 8 being the most potent one. These results strongly suggest that compound 8 is a very promising anti-fibrosis drug candidate.

Next, to assess if compound 8 can prevent the pro-fibrosis effect of high glucose, we treated the MES-13 cells with different concentration of glucose for 48 hours. High glucose is a major contributor to the pathology of DN. Mesangial cells are particularly susceptible to high glucose challenge and can easily undergo fibrotic transformation. To determine if compound 8, PFD and AKF-PD can block such pro-fibrosis effect induced by high glucose, we treated the cells with all the three compounds at different concentrations with Losartan as the positive control. After carefully assessing the expression levels of CTGF and FN, we found that in comparison to the no-treatment group, FN levels decreased by 2.42 folds, 0.80 fold, 1.78 folds and 1.23 folds respectively with the treatment of compound 8, PFD, AKF-PD and Losartan; while CTGF levels decreased 1.85 folds, 1.39 folds, 1.38 folds and 1.66 folds, respectively. These data suggest that at a concentration of 24.5 mol/L(10-5), compound 8 can bring the elevated FN and CTGF levels induced by high glucose back to normal and thus prevent the progression of pro-fibrosis change of mesangial cells and kidney as a whole, which will in turn prevent DN.

The interaction of between inflammatory response and fibrosis runs through DN.[48-50]The inflammation has a pivotal role in kidney interstitial fibrosis, and the essence of DN is renal fibrosis. Compound 8 could effectively restrain inflammatory and the proliferation of renal fibroblasts. It was reported that compound 8 could obviously inhibit TNF-α or LPS-induced production of proinflamatory cytokines from our team's previous study. This effect can be attributed to the inhibition of phosphorylation of STAT3, ERK, and NF-kb.[51]

PFD liability decreased with 5-methyl of pyridone, in particular the phase□metabolic liability as demonstrated by the short half-lives in liver microsomal assays. The elimination half-life of the tablet of PFD is 1.90±0.13 h from six rats by single-dose.[52]The main purpose of this study was to improve the metabolic stability, extend the biological half-life, and thus improve the effect of these compounds. To determine whether the pharmacokinetics of target compound 8 was improved, we performed a preliminary study on the pharmacokinetic properties of compound 8. In a preliminary study, LC-MS assay was established to detect the plasma concentration of compound 8 and its metabolites with PFD as the internal standard by WinNonlin® software. The pharmacokinetics profile of compound 8 was evaluated in male rats using LC/MS analysis with a standard curve formulation: y=0.0113x-0.0043, R^2=0.999.

As shown in Table 4, we obtained the pharmacokinetic parameters of compound 8. Compared to PFD, the elimination half-life of compound 8 was prolonged to 2.58-fold, and C_{max} and AUC_{0-t} of compound 8 increased by 1.29 times and 2.66 times, respectively. So the pharmacokinetics properties of compound 8 has made significant improvements.

Table 4

Main pharmacokinetic parameters of compound 8 and pirfenidone.

Compd.	Dose(mg/kg)	$t_{1/2}$(h)	C_{max}(ng/mL)	AUC_{0-t}(h.ng/mL)
PFD	10.0	1.90±0.13	1020.3±220.1	2722.0±731.9
Compd. 8	15.5	4.89±1.33	2032±620.4	11214.77±5375.2

By optimization of the lead compound PFD, 18 novel pyridone derivatives were designed. In the present study, we have developed methods for efficient preparation of 5-trifluoromethyl-2(1H)-(1-nitrogen heterocycle alkyl amino benzyl or phenyl) pyridone derivatives and evaluated their inhibitory activity against NIH3T3 cell proliferation. As expected, all the target derivatives showed sharply improvement in their inhibitory activity

against NIH3T3 cell lines proliferation. Based on the results of biological evaluation, preliminary SAR of pyridone derivatives could be presented for NIH3T3 cell.

As mentioned above, cellular activity of compound 8 has obviously improved effect against NIH3T3 cell line proliferation. In the follow-on test, we conducted a series of studies on the activity of compound 8, toxicity, in vivo anti-DN efficacy, PK and mechanism. As a multi-target agent, the parameters of compound 8 were tuned and balanced by modifying substituents at positions 1 and 5 of the pyridone ring. The pharmacokinetic and toxicological properties of compound 8 were superior to those of PFD, and it was better than Losartan in diabetic nephropathy. In general, compound 8 was a potent NIH3T3 inhibitor with excellent druggability properties, and had high efficacy in in vivo DN models, moderate dose proportional and wide therapeutic window and pharmaceutical properties.

Compound 8 was advanced to preclinical studies as an oral treatment for diabetic nephropathy.

ABBREVIATIONS USED

ACR, albumin-to-creatinine ratio; AUC_{0-t}, area under the curve; BuOH, n-butanol; BUN, blood urea nitrogen; $C_{max,}$ peak concentration; C^{13}-NMR, C^{13}-nuclear magnetic resonance; CCr, creatinine clerance rate; CCR2, CCchemkin receptor2; $CDCl_3$, Deuterotrichloromethane; DBM, diabetes model, DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DN, diabetic nephropathy; ECM, extracellular matrixc; ELISA, enzyme-linked immunosorbent assay; ERSD, end stage renal disease; GC-MS, Gas Chromatograph-Mass Spectrometer-computer; GLP, Good laboratory practice of drug; GSI, glomerular sclerosis index; HMBC, heteronuclear multiple bond correlation; HPLC, high performance liquid chromatography; IR, infrared radiation; LC-MS, liquid chromatography-mass spectrum; LPS, lipopolysaccharide; MTT, methylthiazolyl diphenyl tetrazolium bromide; NIH3T3, mouse embryonic fibroblast cell line; NOX, nicotinamide adenine dinucleotide phosphate oxydaes; PD, pharmacodynamics; PDGF, platelet-derived growth factor; PFD, pirfenidone; PK, pharmacokinetics ; ROS, reactive oxygen species; SAR, structure activity relationships; SCr, serum creatinin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factors-α; UUO, unilateral ureteral occlusion.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version, at DOI: xxxxx.

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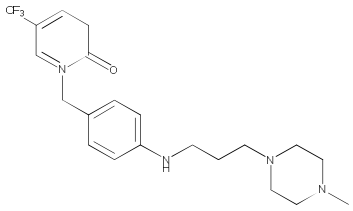
Table of Contents graphic



Compound 8 IC_{50}=90uM in NIH3T3 cell lines

$t_{1/2}$= 4.89± 1.33h in male rats

LD_{50}>2000mg/kg in mice