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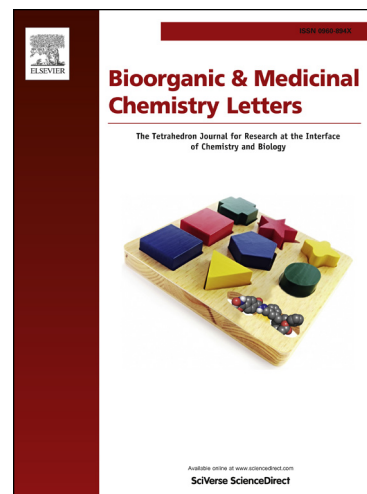
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3',4'-Bis-difluoromethoxycinnamoylanthranilate (FT061): An orally-active antifibrotic agent that reduces albuminuria in a rat model of progressive diabetic nephropathy

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Abstract—Cinnamoylanthranilates including tranilast have been identified as promising antifibrotics that can reduce fibrosis occurring in the kidney during diabetes, thereby delaying and/or preventing kidney dysfunction. Structure-activity relationships aimed at improving potency and metabolic stability have led to the discovery of FT061. This compound, which bears a bis-difluoromethoxy catechol, attenuates TGF- β -stimulated production of collagen in cultured renal mesangial cells (approx 50% at 3 μ M). When dosed orally at 20 mg/kg to male Sprague Dawley rats, FT061 exhibited a high bioavailability (73%), C_{\max} of 200 μ M and T_{\max} of 150 min, and a half-life of 5.4 h. FT061 reduced albuminuria when orally dosed in rats at 200 mg kg/day in a late intervention study of a rat model of progressive diabetic nephropathy.

Keywords: chronic kidney disease; T1D; juvenile diabetes; fluorination

Diabetic nephropathy (DN) develops in approximately 30% of subjects with type 1 diabetes. Control of hypertension, blood sugar levels, and albuminuria through the use of ACE inhibitors are key strategies in attenuating the progressive decline in renal function in patients with DN.¹ While tight glycaemic and blood pressure control are important in delaying the progression of nephropathy there is a need to treat the underlying fibrosis that leads to end organ failure.² Pro-fibrotic growth factors including platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and transforming growth factor β (TGF- β) have been identified as cytokines that stimulate the deposition of collagen in the kidney.³⁻⁵

It has been estimated that some 45% of all deaths in the developed world may have an underlying pathology of aberrant fibrosis.⁶ However, this mode of action has only recently been targeted explicitly in the clinic, with the approval of perfenidone for idiopathic pulmonary fibrosis.⁷ Tranilast (**1**), a cinnamoylanthranilate, has been used in Japan and South Korea for over 20 years to treat allergic disorders, hypertrophic scars and scleroderma (Figure 1A). Tranilast inhibits pro-fibrotic growth factors including TGF- β ,^{8,9} PDGF,⁹ and CTGF,¹⁰ and thus is an antifibrotic agent. Structure-activity relationships of cinnamoylanthranilates have led to the development of several analogues including FT011 (**2**) and FT023 (**3**).¹¹ FT011 prevents collagen synthesis in TGF- β and PDGF-BB stimulated mesangial cells in vitro.¹² In an advanced model of DN, the streptozotocin-diabetic Ren-2 rat, FT011 reduced albuminuria, glomerulosclerosis and tubulointerstitial fibrosis.¹² Reflecting the ability of FT011 to inhibit the action of locally-active growth factors involved in fibrosis, FT011 also attenuates cardiac remodelling and dysfunction in experimental diabetic cardiomyopathy.¹³ The related compound FT023 significantly attenuated the increased heart weight:bodyweight ratio in diabetic Ren-2 rats, and attenuated diastolic dysfunction.¹⁴ Further, FT011 treatment attenuates Armani–Ebstein lesions (a key morphological feature of DN) in a rat model of diabetes.¹⁵ Recently, FT011 has entered into clinical development for the treatment of DN.

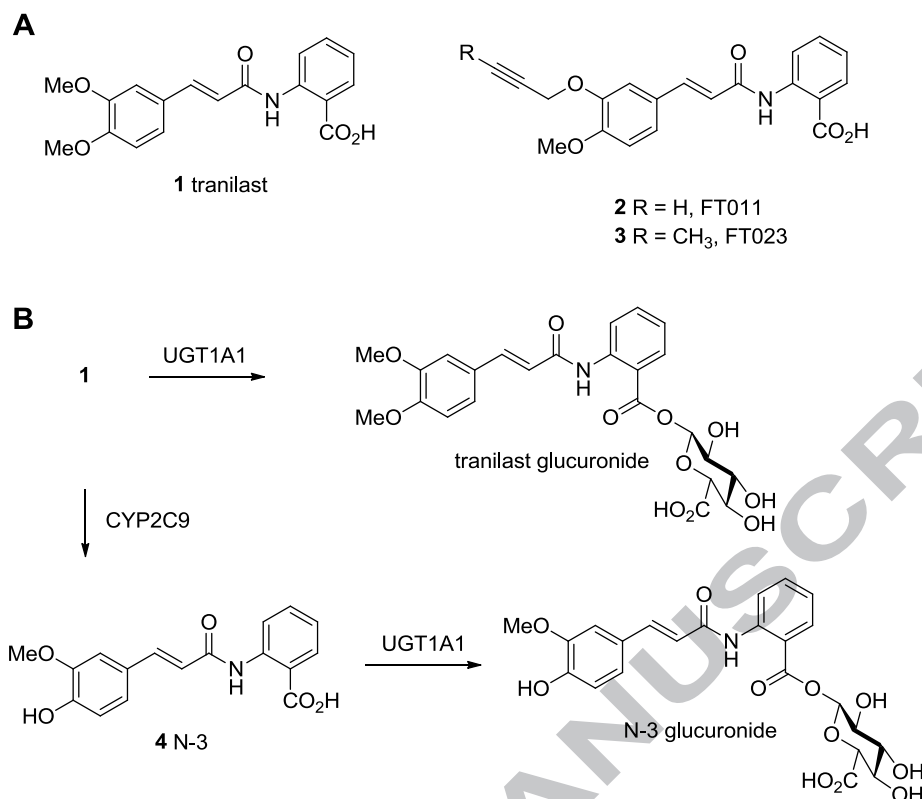
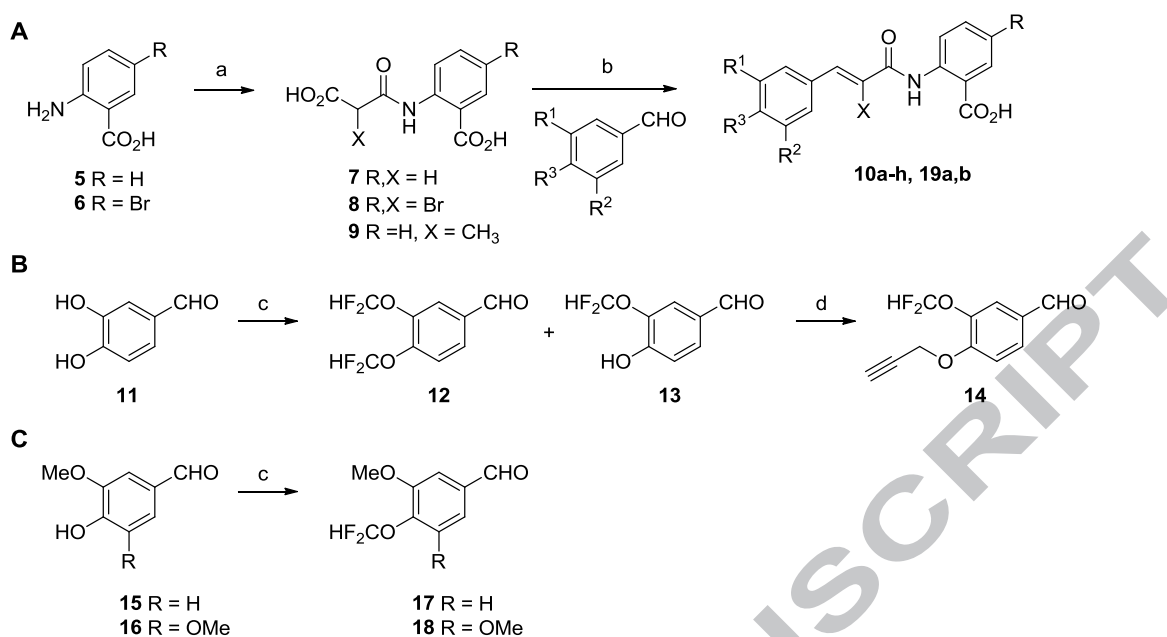


Figure 1. A) Tranilast and analogues; B) Major metabolic pathways of tranilast in humans.

The PRESTO (prevention of restenosis with tranilast and its outcomes) study sought to use tranilast to prevent adverse clinical events including re-stenosis for patients undergoing stent-based and stent-free coronary revascularization.¹⁶ At the highest doses of tranilast, a range of reversible abnormalities were identified including hyperbilirubinemia, increased alanine aminotransferase (ALT)/serum glutamic pyruvic transaminase (SGPT), decline in haemoglobin, and increased serum creatinine.¹⁷ The molecular bases of these issues are incompletely understood; however, it appears that hyperbilirubinemia is especially prevalent within patients with Gilbert's syndrome, in which carriers express lower than normal levels of UGT1A1.^{18, 19} Tranilast undergoes Phase I metabolism to the demethylated derivative N-3 (4), and both tranilast and N-3 undergo phase II glucuronidation catalyzed by UGT1A1 (Figure 1B).²⁰ Consequently, tranilast-induced hyperbilirubinemia appears to result from inhibition of bilirubin-metabolism by glucuronosyltransferase UGT1A1 through competition by tranilast and N-3, with N-3 representing a more effective substrate and thus a more effective UGT1A1 inhibitor than tranilast.²⁰ The goal of this work was to identify derivatives of tranilast modified to increase potency and prevent the formation of the hyperbilirubinemia-promoting metabolite N-3. Herein we report the realization of these aims through the development of the antifibrotic agent **10c** (FT061).

Our initial synthetic efforts were directed at modifying the catechol portion of the tranilast structure. Previous work leading to the development of FT011 and FT023 revealed key structure-activity relationships, namely that (i) increasing the size of substituents on the catechol oxygens enhances antifibrotic activity; (ii) conversion of the carboxylic acid to an amide or its removal results in reduction of antifibrotic activity; and (iii) saturation of the double bond leads to a complete loss of antifibrotic activity.¹¹ Difluoromethoxy and trifluoromethoxy groups have previously been highlighted as metabolically-stable replacements for methoxy groups.²¹⁻²⁴ We therefore investigated the effect of the introduction of fluoroalkyl groups into the tranilast structure, while maintaining the acid and double bond functionalities.

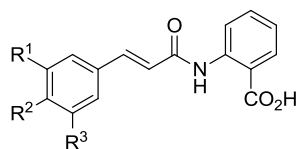
Compounds were synthesized by two main routes. Direct amidation of anthranilic acids **5** and **6** with Meldrum's acid or 2,2,5-trimethyl-1,3-dioxane-4,6-dione afforded the carboxyamidobenzoic acids **7**, **8** and **9**. Knoevenagel reaction of **7**, **8** or **9** and various benzaldehydes provided the substituted cinnamoyl anthranilates **10a-h** and **19a,b** (Scheme 1A).²⁵ The benzaldehydes were either commercially available, or were obtained by difluoromethylation of **11**, **15** or **16** by K_2CO_3 and methyl chlorodifluoroacetate in DMF,²⁶ or better, sodium chlorodifluoroacetate and K_2CO_3 in DMF/ H_2O ,²⁷ affording **12**, **13**, **17** and **18** (Schemes 1B,C). Propargylation of **13** using propargyl bromide/ K_2CO_3 afforded **14**.



Scheme 1. Synthesis of cinammoylanthranilate derivatives. *Reagents and conditions:* a) Meldrum's acid or 2,2,5-trimethyl-1,3-dioxane-4,6-dione, toluene, reflux, 3 h; b) piperidine, toluene, reflux, 30 min; c) ClF₂CO₂Me, K₂CO₃, DMF or ClF₂CO₂Na, K₂CO₃, DMF/H₂O; d) propargyl bromide, K₂CO₃, CH₃CN, 16 h.

Antifibrotic activity was assessed in cultured mesangial cells by evaluating the ability of each compound to reduce the incorporation of [³H]-proline into collagen upon TGF-β stimulation.^{11, 12, 28} For initial screening purposes, compounds were studied at concentrations of 10 and 30 μM in triplicate. Chart 1 reveals that replacement of the 4-methoxy groups of tranilast (**1**) with a 4-difluoromethoxy group (**10a**) resulted in a significant enhancement of activity. Introduction of a 5-methoxy substituent to afford **10b** gave an enhancement of activity over **10a**. An even greater effect was seen for replacement of both methoxy groups with difluoromethoxy groups (**10c**, FT061). Joining the two groups as a difluoromethylene substituent (**10d**) resulted in a complete loss of activity. Extension of these results to incorporate a 2-propynyl substituent, present in FT011,¹¹ gave an even more potent derivative **10e**, indicating that previously established structure-activity results apply to this series.¹¹ The structure activity relationships established in this set of compounds extends to a trifluoromethyl group (**10f**). Additional potent antifibrotic agents were identified through incorporation of 4-tetrafluoroethoxy (**10g**) or 3-fluoro-4-trifluoromethoxy (**10f**) groups, although significant cellular toxicity was observed at higher doses of these two compounds.

Chart 1. Inhibitory activities for cinnamoylanthranilates on TGF- β stimulated production of collagen synthesis in cultured mesangial cells.



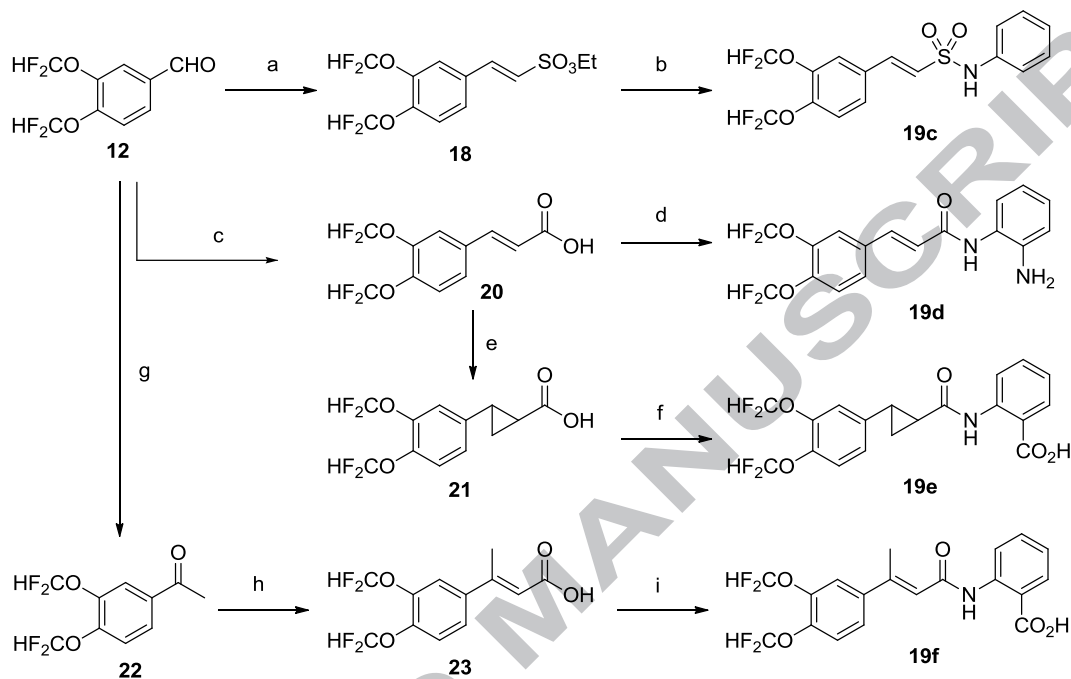
Compound number	R ¹	R ²	R ³	Inhibition (%)	
				at 10 μ M	at 30 μ M
1 (tranilast)	OMe	OMe	H	15	40
2 (FT011)	OMe	2-propynyl	H	20-50	55-70
10a	MeO	F ₂ HCO	H	20	70
10b	MeO	F ₂ HCO	MeO	24	80
10c (FT061)	F ₂ HCO	F ₂ HCO	H	60	100
10d	-OCF ₂ O-		H	-	0
10e	2-propynyl	F ₂ HCO	H	73	100
10f	F ₃ CO	MeO	H	92	100
10g	H	HF ₂ CCF ₂ O	H	84	damaged ^a
10h	F	F ₃ CO	H	55	damaged ^a

^a damaged = significant proportion of stressed cells are beginning to detach.

Among this series of compounds, FT061 (**10c**) was highlighted owing to the expectation of being metabolically inert at both positions of the catechol, and through possessing limited cellular toxicity in the cell based screen. Additional SAR investigations of FT061 were performed investigating more significant modifications of the cinnamoylanthranilate core (Scheme 2). **12** was converted to the vinylsulfonate **18** by Horner-Wadsworth-Emmons extension with (EtO)₂POCH₂SO₃Et, and thence the sulfonamide **19c**. Malonate extension of **12** to the cinnamate **20**, conversion to the acid chloride and finally reaction with phenylenediamine afforded **19d**. Cyclopropanation of **20** with CH₂N₂ and Pd(OAc)₂ afforded **21**, which was converted to **19e** via the acid chloride. MeMgCl addition to **12** followed by PCC oxidation gave acetophenone **22**, Horner-Emmons-Wadsworth extension gave **23**, and finally **19f**, by HBTU coupling with anthranilic acid.

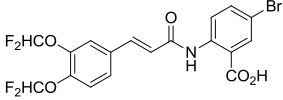
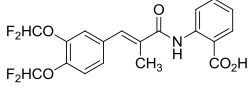
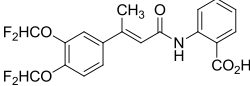
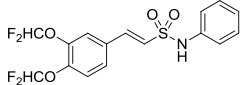
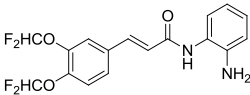
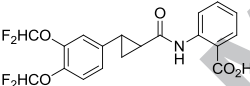
Chart 2 reveals that introduction of a substituent on the B ring (**19a**), or methyl substitution of the α - (**19b**) or β -positions (**19f**) of the double bond, resulted in an enhancement of activity, although

this was offset by increased cellular toxicity. Replacement of the amide linkage with a sulfonamide linkage and removal of the carboxylate (**19c**) resulted in a complete loss of activity. Conversion of the carboxylic acid group to an aniline (**19d**) also yielded an increase in activity. Interestingly, the cyclopropyl derivative **19e** also possessed significant ability to inhibit collagen synthesis.



Scheme 2. Preparation of FT061 analogues. *Reagents and conditions:* a) $(\text{EtO})_2\text{POCH}_2\text{SO}_3\text{Et}$, BuLi, THF, $-78\text{ }^\circ\text{C}$; b) (i) Bu_4NI , acetone, 16 h, (ii) Ph_3P , SO_2Cl_2 , 16 h, (iii) aniline, pyridine, 4 h; c) malonic acid, piperidine, pyridine, $120\text{ }^\circ\text{C}$, 16 h; d) (i) $(\text{COCl})_2$, cat. DMF, CH_2Cl_2 , 1 h (ii) 1,2-phenylenediamine, pyridine, CH_2Cl_2 , 16 h; e) (i) H_2SO_4 , MeOH, 16 h, (ii) CH_2N_2 , $\text{Pd}(\text{OAc})_2$, CH_2Cl_2 , (iii) NaOH, MeOH, 16 h; f) (i) $(\text{COCl})_2$, DMF, CH_2Cl_2 , 2 h, (ii) anthranilic acid, pyridine, 16 h; g) (i) MeMgCl , THF, 2 h, (ii) PCC, CH_2Cl_2 , 16 h; h) (i) $(\text{EtO})_2\text{POCH}_2\text{CO}_2\text{Et}$, NaH, THF, 16 h, (ii) 1 M aq NaOH, EtOH, 16 h; i) HBTU, Et_3N , CH_3CN , 72 h.

Chart 2. Inhibitory activities for compounds on TGF- β stimulated production of collagen synthesis in cultured mesangial cells.

Compound number	Structure	Inhibition (%) at	
		10 μ M	30 μ M
19a		100	damaged ^a
19b		100	damaged ^a
19f		100	damaged ^a
19c		0	damaged ^a
19d		57	79
19e		70	90

^aDamaged = Significant proportion of stressed cells beginning to detach.

To better characterize the antifibrotic activity of the most promising compounds, more detailed inhibition profiles were determined for FT061 (**10c**), **10a** and **19e** at a range of concentrations. Figure 2 reveals that **10a** has only a minor improvement in antifibrotic activity relative to tranilast, however both FT061 and **19e** were significantly more active.

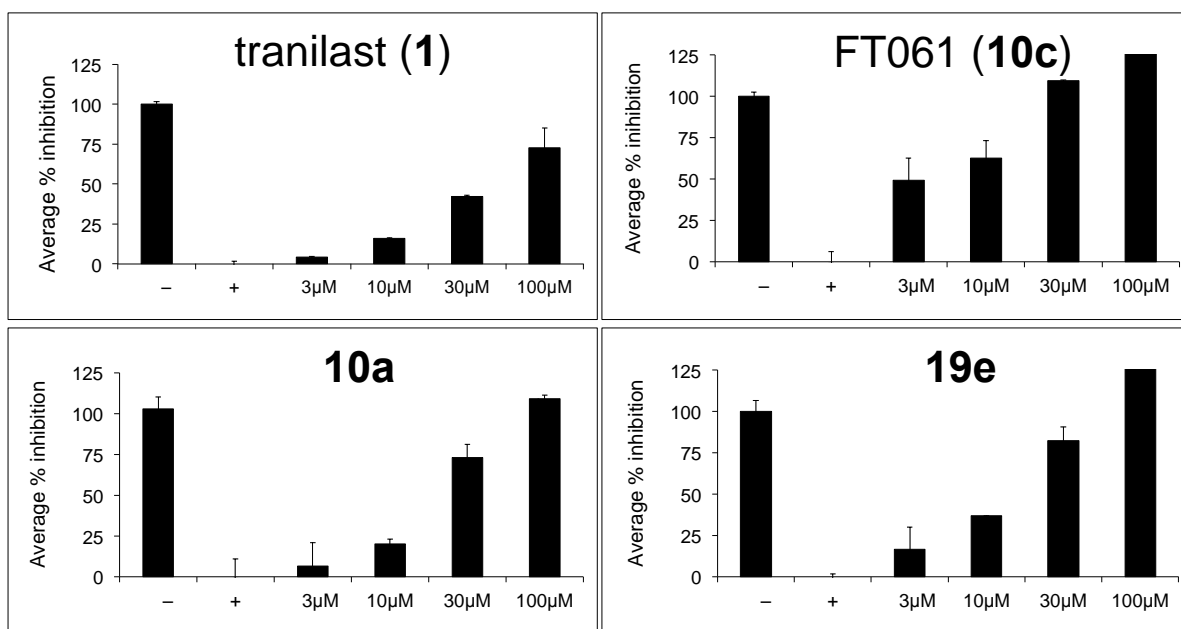


Figure 2. Dose-response histograms of inhibition of TGF- β stimulated [3 H]-proline incorporation in mesangial cells by tranilast (**1**), FT061 (**10c**), **10a**, and **19e** measured at concentrations of 3, 10, 30 and 100 μ M. Inhibition values were determined in triplicate. (+) and (-) refer to the absence and presence of stimulation by the cytokine TGF- β . Error bars indicate standard error.

The major routes of tranilast metabolism in humans involve direct glucuronidation, and demethylation of O4 followed by glucuronidation or sulfation.^{20, 29} The susceptibility of FT061 to CYP450-mediated metabolism was examined in human liver microsomes in the presence of NADPH. While minimal loss of FT061 over the 60 min incubation was indicative of good metabolic stability, the presence of a putative +16 product was detected, consistent with monooxygenation. Metabolite identification of urine and plasma samples collected during pharmacokinetic studies of FT061 (vide infra) confirmed susceptibility of FT061 to glucuronidation.

The *in vivo* pharmacokinetic profile of FT061 was compared with that of tranilast in Sprague Dawley (SD) rats following intravenous (IV) and oral administration, and the results are shown in Tables S1 and S2 (see Supporting Information) and Figure 3. Relative to tranilast, the IV pharmacokinetics of FT061 were characterised by a longer half-life, lower plasma clearance and lower volume of distribution. The differences in plasma clearance and volume of distribution of the two compounds most likely reflect differences in the plasma protein binding since the unbound intrinsic clearance and unbound volume of distribution between the two compounds are similar (see Supplementary material). Tranilast and FT061 both exhibited high oral bioavailability; however, the

plasma exposure (AUC) and maximum plasma concentration (C_{max}) following oral administration of FT061 were 4- and 1.7-fold higher respectively, than the values observed for tranilast.

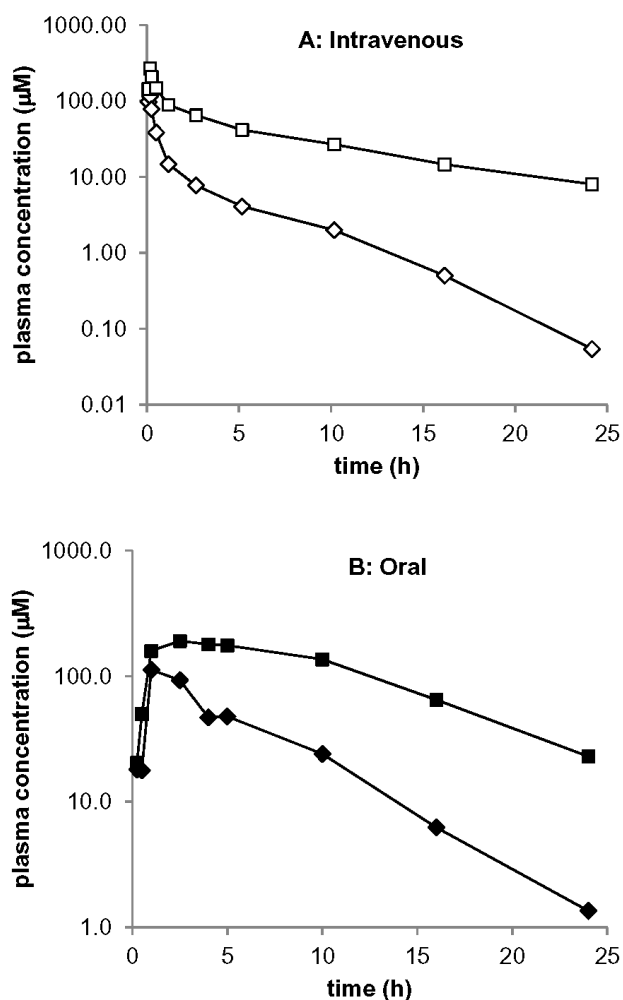


Figure 3. Plasma concentrations following administration of tranilast (diamonds) or FT061 (squares) to male SD rats. A) Intravenous administration of a 5 mg/kg solution. B) Oral administration of 20 mg/kg suspension. Levels of drug were quantified in blood plasma by LC-MS. For full details see Supporting Information.

To assess the renal and liver toxicity of FT061, Sprague-Dawley rats were treated with FT061 at doses of 50, 100, 200, 400 and 1000 mg/kg/d by gavage twice daily for two weeks. At the end of the study serum was collected to assess liver and renal function. Plasma levels of creatinine, urate, ALT and bilirubin from treated rats were similar to control rats (see Supporting Information).

The ability of FT061 to reduce albuminuria was assessed in a rat model of DN¹² (Figure 4).

Hypertensive (m-Ren2)²⁷ rats were exposed to streptozotocin, a drug that is cytotoxic towards pancreatic islet cells, to induce diabetes. Eight weeks post-streptozotocin treatment, animals were treated with 200 mg/kg/d FT061 by oral gavage for eight weeks. At 16 weeks, a 24 h urine collection was assayed for albuminuria by radioimmunoassay. Untreated rats developed significant albuminuria that was attenuated by oral administration of FT061. In contrast, tranilast attenuates albuminuria only when administered at higher doses of 400 mg/kg/day.³⁰

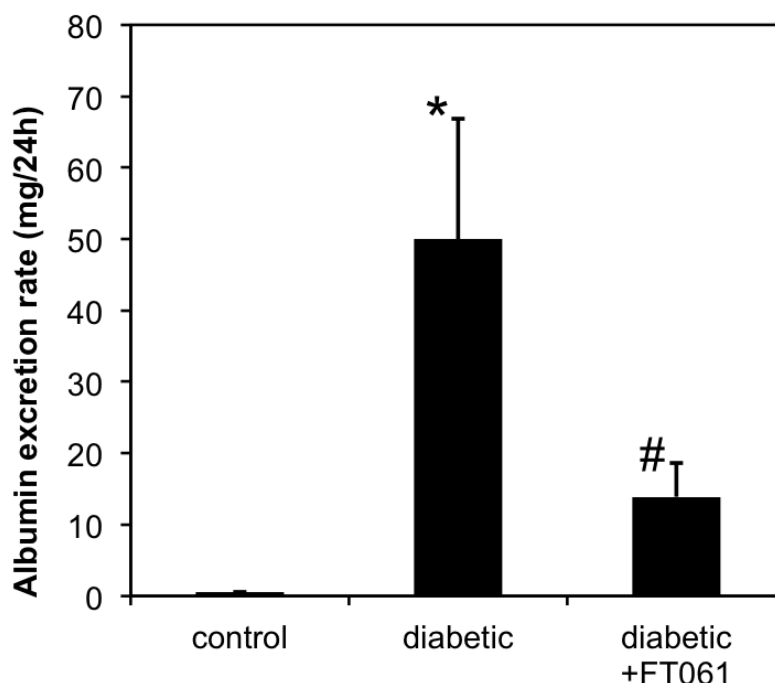


Figure 4. FT061 reduces albuminuria in a late intervention study. Albumin excretion rate (AER) was measured after 16 weeks of experimental diabetes in a streptozotocin-treated hypertensive (m-Ren2)²⁷ rat model. Treatment with FT061 commenced 8 weeks after streptozotocin injection, and continued for 8 weeks. Data are expressed as geometric mean \pm SEM. * $p < 0.01$ when compared to controls, # $p < 0.05$ when compared to diabetic rat.

In conclusion a structure-activity study undertaken to identify tranilast derivatives with altered metabolism led to the discovery of FT061. FT061 is an orally-active and metabolically-stable antifibrotic agent that attenuates albuminuria in a rat model of progressive diabetes. FT061 features replacement of the metabolically labile methyl groups of the catechol of tranilast with bis-difluoromethoxy groups. This work extends previous structure-activity relationship studies¹¹ exploring the antifibrotic properties of tranilast to delineate further structural contributors to the antifibrotic

properties of this class. FT061 is straightforward to synthesize, is a potent antifibrotic agent, appears well-tolerated in rats, and is resistant to deactivating metabolism and thus deserves further study as an agent to ameliorate the pathological effects of fibrosis.

Acknowledgments

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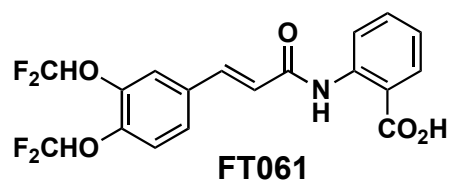
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- Inhibits TGF- β stimulated collagen biosynthesis
- Improved bioavailability versus tranilast
- Reduces albuminuria in streptozocin-treated hypertensive rat

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