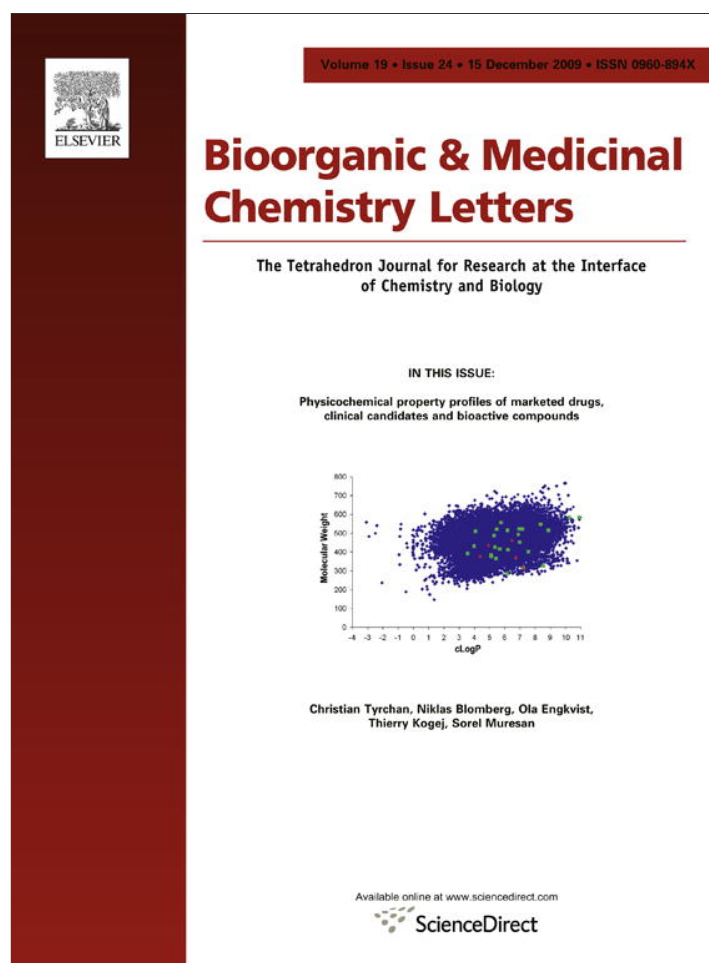


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Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Evaluation and optimization of antifibrotic activity of cinnamoyl anthranilates

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ARTICLE INFO

Article history:

Received 21 July 2009

Revised 28 September 2009

Accepted 30 September 2009

Available online 7 October 2009

Keywords:

Diabetes

Antifibrotic

Albuminuria

Transforming growth factor- β

ABSTRACT

Tranilast is an anti-inflammatory drug in use for asthma and atopic dermatitis. In studies over the last decade it has been revealed that tranilast can reduce fibrosis occurring in the kidney during diabetes, thereby delaying and/or preventing kidney dysfunction. We report a structure–activity study aimed at optimizing the antifibrotic activity of tranilast. A series of cinnamoyl anthranilates were prepared and assessed for their ability to prevent TGF- β -stimulated production of collagen in cultured renal mesangial cells. We reveal derivatives with improved potency and reduced cellular toxicity relative to tranilast. 3-Methoxy-4-propargyloxycinnamoyl anthranilate reduces albuminuria in a rat model of progressive diabetes, and thus has potential as an innovative treatment for diabetic nephropathy.

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Chronic kidney disease can be caused by diabetes and high-blood pressure and has devastating impacts on health, increasing the risk of cardiovascular mortality and morbidity, blindness and amputation. Pathological fibrosis is a hallmark of progressive renal disease and correlates closely with the loss of kidney function. Platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) and transforming growth factor- β (TGF- β) are cytokines that have been implicated in the progression of renal disease through the stimulation of fibrosis in the mesangium.¹ Inhibition of TGF- β ameliorates tubulointerstitial fibrosis in a rat model of diabetic nephropathy,^{2,3} and other models of chronic renal disease.^{4,5}

Antifibrotic drugs may provide a novel strategy for the treatment of progressive kidney disease, and indeed other disorders associated with pathological fibrosis. A recent review highlighted the fact that some 45% of all deaths in the developed world may have an underlying pathology of aberrant fibrosis.⁶ Despite this, there are no drugs in clinical use that are optimized for antifibrotic action. One promising antifibrotic agent is the cinnamoyl anthranilate tranilast. Tranilast has been used in Japan and South Korea for over 20 years to treat allergic disorders, hypertrophic scars and scleroderma. This drug has been shown to inhibit the pro-fibrotic growth factors TGF- β ,^{7,8} PDGF⁸ and CTGF.⁹ However, the recent PRESTO (prevention of restenosis with tranilast and its outcomes)

study highlighted several reversible abnormalities associated with the use of tranilast at the elevated doses required for antifibrotic action.¹⁰ These included hyperbilirubinemia, increased alanine aminotransferase (ALT)/serum glutamic pyruvic transaminase (SGPT), decline in haemoglobin, and increased serum creatinine.

In order to optimize the antifibrotic effects of tranilast, a series of cinnamoyl anthranilate derivatives were synthesized and their ability to prevent TGF- β stimulated collagen production in vitro was investigated. Modifications included saturation of the double bond connecting the two rings, and removal and/or replacement of the methoxy (positions A and B) and carboxyl groups (position C) (Fig. 1). The most active compounds were assessed for their ability to inhibit TGF- β in cultured mesangial cells at a range of concentrations and to inhibit the onset of albuminuria in a rat model of progressive diabetes.

Two general approaches were used for the synthesis of the various substituted cinnamoyl anthranilates. In the first approach a piperidine-catalyzed Knoevenagel reaction of a carboxyacetamidobenzoic acid and a benzaldehyde derivative provided the substituted cinnamoyl anthranilates. In the second approach, substituted cinnamic acids were converted to the corresponding acid chloride and treated with an aniline derivative (Scheme 1).

Benzaldehyde precursors were either obtained from commercial sources, or were synthesized by alkylation of precursor phenolic benzaldehydes with alkyl halides or alkyl tosylates (the latter derived in turn from the corresponding alcohols). 2-Carboxyacetamidobenzoic acid was prepared by reaction of anthranilic acid and Meldrum's acid. 2-Aminobenzamide was obtained by reaction

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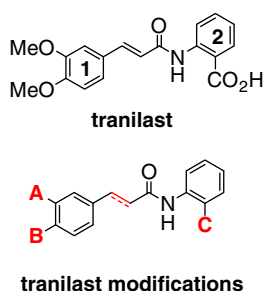
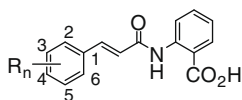


Figure 1. Structural modifications of tranilast.

of ammonia and isatoic anhydride.¹¹ The alkene of tranilast was saturated by reduction with hydrogen and palladium on carbon. Antifibrotic activity was assessed by evaluating the ability of each compound to reduce the incorporation of [³H]-proline into collagen upon TGF- β stimulation. For initial screening purposes, compounds were screened at a concentration of 100 μ M in triplicate. More active compounds were rescreened at 30 μ M in triplicate, prior to more detailed analysis.

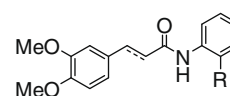
As shown in Chart 1, variation of the methoxy substituents at the 3 and 4 positions of ring 1 results in a considerable variation of biological activity. Removal of the methoxy substituents (compounds 2–4), or replacement of a single methoxy substituent with a hydroxy group (compounds 5 and 6), resulted in a reduction in activity whereas dihydroxy derivative 7 demonstrated more potent inhibition of TGF- β .

Chart 2 reveals that the carboxylic acid functional group of tranilast and the α,β -unsaturated system are requirements for activity. Removal of the carboxylic acid or replacement of the acid with a primary amide gave 8 and 9, which were both less active than tranilast. Saturated derivative 10 was inactive.



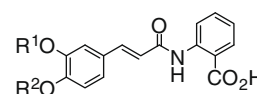
Compound number	n	R	Inhibition at 100 μ M (%)
1 (tranilast)	2	3,4-(OMe) ₂	70
2	0	-	no inhibition
3	1	4-OMe	no inhibition
4	1	3-OMe	40
5	2	3-OH, 4-OH	50
6	2	3-OH, 4-OMe	50
7	2	3-OH, 4-OH	90

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



Compound number	Double Bond	R	Inhibition at 100 μ M (%)
1 (tranilast)	Yes	CO ₂ H	70
8	Yes	H	50
9	Yes	CONH ₂	35
10	No	CO ₂ H	no inhibition

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



Number	R ¹	R ²	Inhibition at 30 μ M (%)	Inhibition at 100 μ M (%)
1 (tranilast)	Me	Me	40	70
11	Me	2-propynyl	55-70	80-100
12	Me	2-butynyl	65	90
13	Me	3-butynyl	70	100 ^b
14	Me	2-pentynyl	100	100 ^b
15	Me	4-pentynyl	75	100 ^b
16	Me	3-hexynyl	100	damaged
17	Me	5-hexynyl	80	damaged
18	Me	3-octynyl	necrotic	necrotic
19	2-propynyl	Me	60	90
20	2-butynyl	Me	75	80
21	3-butynyl	Me	65	damaged
22	2-pentynyl	Me	90	100 ^b
23	4-pentynyl	Me	80	damaged
24	3-hexynyl	Me	100	100 ^b
25	5-hexynyl	Me	95	100 ^b
26	3-octynyl	Me	necrotic	necrotic
27	Me	cyclopropyl	70	100 ^b
28	Me	cyclobutyl	60 ^a	damaged
29	Me	cyclopentyl	100	damaged
30	Me	cyclohexyl	necrotic	necrotic
31	Me	cycloheptyl	necrotic	necrotic
32	Me	cyclooctyl	necrotic	necrotic

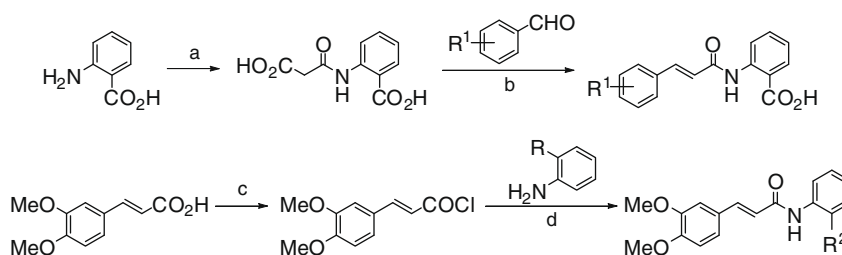
^aInhibition measured at 10 μ M.

^bSome cells were stressed and beginning to detach.

Damaged = Significant proportion of stressed cells beginning to detach.

Necrotic = Cells have completely detached resulting in cell death.

Chart 3. Inhibitory activities for compounds on TGF- β stimulated production of collagen synthesis in cultured mesangial cells.



Scheme 1. Synthesis of cinammoyl anthranilate derivatives. Reagents and conditions: (a) Meldrum's acid, toluene, reflux, 3 h; (b) piperidine, toluene, reflux, 30 min; (c) SOCl₂, cat. DMF, toluene, 50 °C, 1 h; (d) pyridine, 24 h.

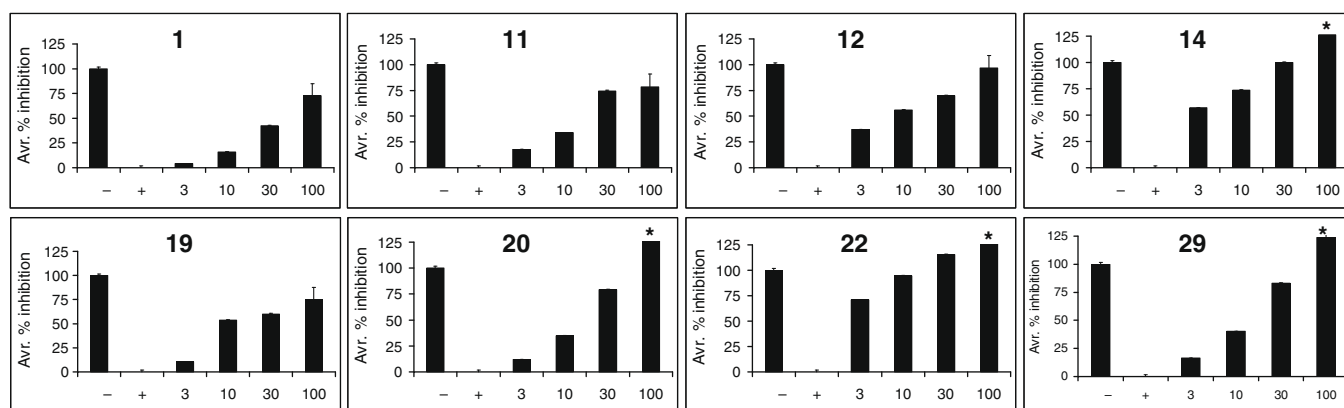


Figure 2. Dose–response histograms of inhibition of TGF- β stimulated [^3H]-proline incorporation by tranilast (**1**) and compounds **11**, **12**, **14**, **19**, **20**, **22** and **29** measured at concentrations of 3, 10, 30 and 100 μM . Inhibition values were determined in triplicate. (–) and (+) refer to the absence and presence of stimulation by the cytokine TGF- β . Error bars indicate standard error. (*) Some toxicity was observed at the indicated concentration.

The effect of alteration of the nature of the alkyl ethers on ring 1 was assessed next (Chart 3). This was of particular interest as the PRESTO trial, which evaluated the prevention of restenosis by tranilast, found that tranilast increased the risk of hyperbilirubinemia in patients with Gilbert's syndrome, which was attributed to tranilast and/or its metabolites inhibiting the phase II glucuronosyltransferase UGT1A1.^{12,13} Glucuronidation, 4-demethylation and sulfation of the 4-demethylated product are major metabolic routes of tranilast.¹⁴ Thus, modification of the alkyl groups at the 3 and 4 positions may lead to drugs that are more metabolically stable and limit side effects. To limit the number of rotatable bonds we investigated the effect of alkyloxy and cycloalkyloxy groups. As chain length increased so did inhibition of TGF- β stimulated col-

Table 1

Calculated metabolic stability parameters of test compounds based on degradation profiles in human liver microsomes.¹⁵

Compound	Microsome-predicted CL_{int} (mL/min/kg)	Microsome-predicted E_{H}
1 (Tranilast)	6.7	0.24
11	5.3	0.20
12	6.9	0.25
14	21.8	0.51
19	54.1	0.72
20	12.6	0.38
22	36.3	0.64
29	17.4	0.46

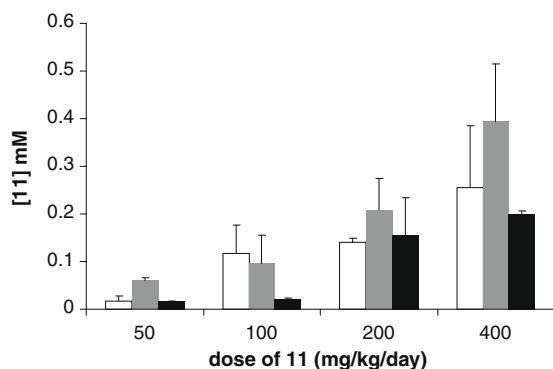


Figure 3. Oral bioavailability of **11** as assessed by blood plasma concentration determined by LC–MS. Bar colors denote plasma concentration at varying times after dosing: white, 1 h after dosing; grey, 4 h after dosing; black, 8 h after dosing.

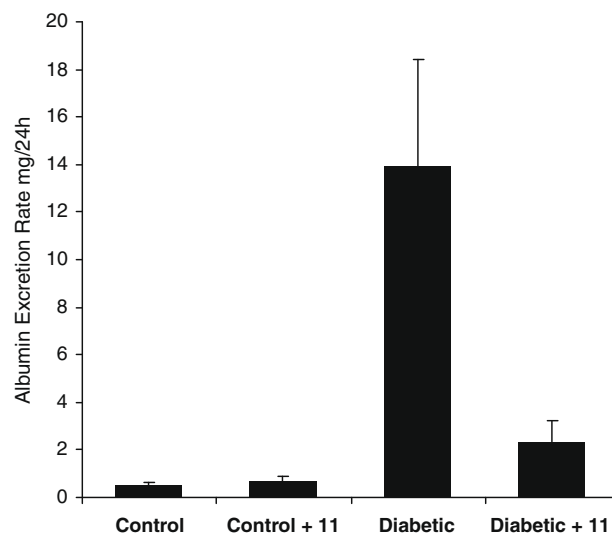


Figure 4. Albuminuria after four weeks of experimental diabetes in a streptozotocin-treated hypertensive Ren2 rat model. Treatment with **11** commenced within 24 h of streptozotocin injection.

lagen production in mesangial cells. The C_5 and C_6 derivatives **14**, **16**, **22**, **24**, **25** and **29** showed the greatest activity. However, the C_6 , C_7 and C_8 derivatives (**18**, **26**, **30**, **31** and **32**) were toxic in cellular assays and thus could not be assessed for TGF- β inhibition.

Compounds **11** and **12**, and their regioisomeric derivatives **19** and **20**, demonstrated the most promising increase in TGF- β inhibitory activity relative to tranilast without showing any cellular toxicity. Similarly, compounds **14**, **22** and **29** also showed potent inhibition of TGF- β whilst displaying only mild cellular toxicity when dosed at 100 μM , and no observed toxicity at 30 μM .

To better characterize the antifibrotic activity of this series of compounds, more detailed inhibition profiles were determined at a range of concentrations. Figure 2 shows that the TGF- β inhibitory activity of the homologous series **11**, **12** and **14** increases as the chain length increases. A similar result was seen for the regioisomeric compounds **19**, **20** and **22**. It appears that similar sized substituents possess similar activities; thus the 2-pentynyl regioisomers **14** and **22** have similar potencies.

The metabolic stability of tranilast relative to the most promising structural analogues was assessed in human liver

microsomes. Compounds were incubated with microsomes and the loss of parent compound assessed by LC–MS analysis. Microsome-predicted intrinsic clearance values are shown in Table 1. For the pair of homologous series **11**, **12** and **14**, and **19**, **20** and **22**, clearance rates increased with increasing substituent chain length. Comparison of the regioisomeric pairs: **11** and **19**; **12** and **20**; and **14** and **22**; shows that clearance rates are higher for the 3-substituted series. Given that tranilast is in clinical use, the observation of similar predicted metabolic stability for compounds **11** and **12** suggests that they are sufficiently stable to merit further investigation.

Based on the activity data and the predicted intrinsic clearance values, compound **11** was selected for preliminary bioavailability studies. Varying amounts of compound **11** were given as a single oral dose to Sprague-Dawley rats and blood plasma levels were assessed by LC–MS. Maximal blood plasma levels were obtained 4 h post oral, and significant amounts of **11** were still present 8 h after dosing (Fig. 3).

The ability to reduce albuminuria was experimentally assessed in a rat model of diabetic nephropathy (Fig. 4). Hypertensive Ren2 rats were exposed to streptozotocin to induce diabetes. After four weeks of experimental diabetes, a 24 h urine collection was assayed for albuminuria by radioimmunoassay. Untreated rats developed significant albuminuria that was attenuated by oral administration of **11** by oral gavage at 200 mg/kg/day. In contrast, tranilast attenuates albuminuria only when administered at higher doses of 400 mg/kg/day.¹⁶

In conclusion novel derivatives of the known antifibrotic, tranilast (**1**), were prepared and assayed for their ability to inhibit TGF- β in cultured mesangial cells. Several lipophilic derivatives possessed increased activity relative to **1**, especially compound **11**, which also showed excellent metabolic properties, and improved in vivo activity. Compound **11** can reduce albuminuria in a hypertensive diabetic rat model and therefore represents a promising lead in the development of potential antifibrotic drugs.

Acknowledgment

We thank the National Health and Medical Research Council for financial support. Denis Scanlon is thanked for HPLC analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.120.

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