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Letters

Estrogen Receptor Dependent Inhibitors of NF-κB Transcriptional Activation-1 Synthesis and Biological Evaluation of Substituted 2-Cyanopropanoic Acid Derivatives: Pathway Selective Inhibitors of NF-κB, a Potential Treatment for Rheumatoid Arthritis

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Abstract: Pathway selective ligands of the estrogen receptor inhibit transcriptional activation of proinflammatory genes mediated by NF-κB. Substituted 2-cyanopropanoic acid derivatives were developed leading to the discovery of WAY-204688, an orally active, pathway selective, estrogen receptor dependent anti-inflammatory agent. This propanamide was shown to be orally active in preclinical models of inflammatory diseases, such as rheumatoid arthritis, without the proliferative effect associated with traditional estrogens.

Chronic inflammation is perpetuated and amplified through a complex series of pathways fed by cytokines, chemokines, adhesion molecules, enzymes, etc. in the inflamed tissue. This response is regulated by a relatively small number of inducible genes in response to transcriptions factors such as STATs, AP-1, NF-AT, and NF-κB. NF-κB is the name given to a family of dimeric transcription factors comprising members of the Rel family. NF-κB was first characterized as a lymphoid specific protein;[1] however, it has been shown to be present, in a latent form, in the cytoplasm of virtually all cell types. Five mammalian NF-κB/Rel proteins have been cloned and characterized.[2] It is the dimeric form of these proteins that regulates many cellular functions such the immune response, response to bacterial and viral infections, cancer, chronic inflammatory disorders such as rheumatoid arthritis (RA), ulcerative colitis, and Crohn's disease as well as neurodegenerative disorders. NF-κB has been shown to be overexpressed in the inflamed synovium[3] where it amplifies the recruitment of inflammatory cells that play a role in the production of proinflammatory mediators such as IL-6 in RA synovial fibroblasts.[4] This central role of NF-κB in the inflammatory response suggested that inhibition of NF-κB driven gene transcription might be effective in the treatment of inflammatory diseases such as RA. For NF-κB to activate a gene, it must be released from its latent form by removal of the inhibitory protein IκB; this involves a series of phosphorylation and ubiquitation reactions that activate proteosomal degradation of IκB. Once NF-κB translocates into the nucleus, binding of coactivators facilitates gene transcription. Several groups have targeted the IκB and ubiquitation pathways to suppress inappropriate activation of NF-κB for the treatment of inflammatory diseases[5] and cancer.[6]

We were interested in exploiting the central role of NF-κB in inflammatory diseases such as RA, however, using a different approach. The estrogen receptor is involved in the inhibition of NF-κB transcriptional activity as demonstrated for IL-6 gene expression.[7] This data and the fact that 17β-estradiol has been shown to be effective in models of RA[8] and that RA went into remission during pregancy[9] suggested the possibility of using an estrogen receptor−ligand complex to control the up-regulation of NF-κB in chronic inflammatory states such as RA. The challenge was to develop a ligand that provided the advantages of 17β-estradiol (E2) without the classic steroid side effects.[10] To this end, human aortic endothelial cell lines (HAECT-1) were created; one cell line was cotransfected with a human estrogen receptor (ER-α or ER-β) and an NF-κB-luciferase construct, and a separate cell line was transfected with just the NF-κB-luciferase construct. This allowed the demonstration of ER dependency of the NF-κB translational inhibition

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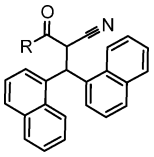
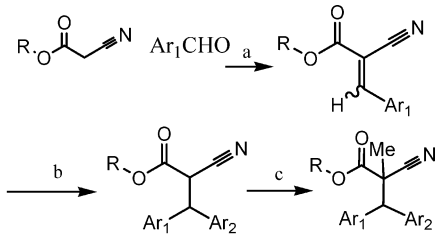
Table 1. Carboxylate SAR of NFκB and CK Expression in Ad5-wt-ER-Transfected HAECT-1 Cells[a]



		NF-κB-luc		CK	
compd	R	$IC_{50} \pm$ SE (nM)	% E2	$IC_{50} \pm$ SE (nM)	% E2
1	Oet	550 ± 122	96	IA	
2	OH	IA		IA	
3	NH_2	1900 ± 37.2	55	IA	

[a] All compounds were ER-dependent. % E2 = efficacy (relative inhibition of test compound at 10 μM vs E2 at 0.1 nM). IA = inactive.

Scheme 1. Synthesis of Propanoates[a]



Ar_1, Ar_2 = 1-Naphthyl, substituted aryl
R = Me, Et, tButyl

[a] Reagents and conditions: (a) piperazine, toluene, reflux; (b) (i) aryl Grignard in THF, room temp, (ii) HCl; (c) (i) KHMDS in THF, (ii) MeI in THF.

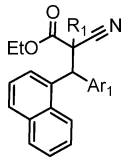
(our selectivity filter). In a typical assay, cells were treated for 16−18 h with IL-1β and the test compound; transcription was directly proportional to the amount of luciferase transcribed. Endogenous creatine kinase (CK) activity in the cells was used as a measure of classical estrogen activity in vitro.

The target profile was a compound with $IC_{50} < 100$ nM (vs NF-κB or IL-6) with an efficacy similar to that of ethinyl estradiol (EE, ≃100%) and lacking CK activity in cells transfected with the hER in vitro. Our primary in vivo assay assessed classic estrogen-like activity (i.e., undesireable side effects) by measured changes in the uterine wet weight in sexually immature female mice after 5 days of oral dosing. As a measure of oral activity, the target compound was assessed for down-regulation of four proinflammatory genes (MHC, invariant chain (MHI), VCAM-1, RANTES, and TNF-α) induced by feeding C57BL/6 mice a high-fat diet for 5 weeks. The results were measured against the effect of EE. Further in vivo characterization would be performed using preclinical models of inflammatory diseases such as collagen induced arthritis (CIA), adjuvant induced arthritis (AIA), and inflammatory bowel disease models (HLA-B27 transgenic rat model).

High-throughput screening identified 2-cyano-3,3-dinaphthalen-1-ylpropionic acid ethyl ester (**1**)[11] as an inhibitor of NF-κB transcription. Compound **1** showed $IC_{50} = 550$ nM at 96% efficacy (vs NF-κB), $IC_{50} = 185$ nM at 91% efficacy (vs IL-6), and no CK activity. The activity was ER-dependent. The related carboxylic acid (**2**) and primary amide (**3**) were less active (Table 1). The synthesis of this series, based on a Knoevenagel condensation and Michael addition sequence, was efficient and allowed rapid development of an SAR (Scheme 1).

Condensation of a cyanoacetate ester with an arylaldehyde in the presence of a base (e.g., piperidine) provided an excellent yield of the cinnamate ester; in many cases a single olefin isomer was isolated. Subsequent Michael addition of an aryl Grignard gave the bisaryl propionate in high yield. Replacement of one naphthalene with substituted aryl or heteroaryl rings improved the activity; however, this modification also gave rise to mixtures of diastereoisomers that were configurationally labile (note: initial studies were performed on the mixture of diastereoisomers). The introduction of a methyl group at C-2 improved activity in some instances (e.g., **4** → **5**) and removed the configurational lability (Table 2).

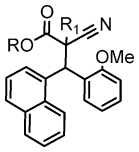
Table 2. Aryl Substitution SAR of NF-κB and CK Expression in Ad5-wt-ER-Transfected HAECT-1 Cells[a]



			NF-κB-luc		CK	
compd	R	Ar_1	$IC_{50} \pm$ SE (nM)	% E2	EC_{50} (nM)	% E2
4	Me	1-naph	206 ± 91	96	IA	
5	Me	2-NO_2Ph	268 ± 41	112	IA	
6	H	2-OMePh	139 ± 25	90	233	32
7	Me	2-OMePh	105 ± 22	110	98	33
8	H	2-naph	389 ± 2.5	76	IA	
9	H	3-OMePh	IA		IA	
10	H	4-MePh	550 ± 133	64	IA	
11	H	Ph	792 ± 19	96	IA	
12	H	2-F-Ph	19% @ 1 μM	32	NT	
13	H	4-quin	3% @ 1 μM	4	NT	
14	H	2-CF_3Ph	110 ± 0.5	106	240	24
15	H	2,4-OMe_2Ph	IA		IA	
16	H	2-SMePh	118 ± 31	102	197	24
17	Me	2-SMePh	327 ± 17	65	IA	-

[a] All compounds were ER-dependent. % E2 = efficacy (relative inhibition of test compound at 10 μM vs E2 at 0.1 nM). IA = inactive.

Table 3. Ester and α Substitution SAR of NF-κB and CK Expression in Ad5-wt-ER-Transfected HAECT-1 Cells[a]



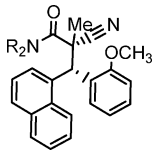
			NF-κB-luc		CK	
compd	R	R_1	$IC_{50} \pm$ SE (nM)	% E2	EC_{50} (nM)	% E2
6	Et	H	139 ± 25	81	IA	
7	Et	Me	105 ± 22	110	98	28
18	^{t}Bu	Me	119 ± 42	94	38	24

[a] All compounds were ER-dependent. % E2 = efficacy (relative inhibition of test compound at 10 μM vs E2 at 0.1 nM). IA = inactive.

Interestingly, the alkylation of many of the diaryl cyanoacetates was highly disastereoselective, providing only a single diastereomer (e.g., *RR*/*SS* in the case of **7**). While replacement of ethyl ester **7** with *tert*-butyl ester **18** resulted in an improvement in the stability in mouse plasma (32% hydrolysis in 4 h at 37 °C vs O% in 24 h at 37 °C), the in vitro profile was essentially unchanged (Table 3).

HPLC resolution of ester **18** showed the activity to reside in a single enantiomer. X-ray crystallographic analysis of an ephedrine carboxylate salt derived from **18** showed the absolute configuration of the eutomer of **18** to be *SS* ($IC_{50} = 21 \pm 12$ nM at 81% efficacy vs NF-κB with no CK activity). An efficient resolution of the carboxylic acid core of **18** (*SS*-2-cyano-3-(2-

Table 4. Effect of Amide Substituent on NF-κB and CK Expression in Ad5-wt-ER-Transfected HAECT-1 Cells[a]



			NF-κB-luc		CK	
compd	R_2	config	$IC_{50} \pm$ SE (nM)	% E2	EC_{50} (nM)	% E2
19	piperidine	*RR*/*SS*	125 ± 38	92	259	37
20	1-(3-Cl-2-CH_3Ph)-piperazine	*SS*	23 ± 7.4	99	84	41
21	4-(3-CF_3Ph)-piperidinetht	*SS*	122 ± 30	94	IA	

[a] All compounds were ER-dependenrt (activity seen only when hER is coexpressed with NF-κB-luciferase in HAECT-1 cells). IA = inactive.

Table 5. Effect of 17α-Ethinyl 17β-Estradiol (EE) (0.01 (mg/kg)/day) and Propanamides on NF-κB Mediated Gene Expression in C57BL/6 Mice on a High-Fat Diet

	% inhib (% EE)				uterine wet wt, fold increase
compd	RANTES	VCAM	MHC	TNF-α	
20	35 (45)	24 (47)	20 (41)	58 (82)	1.1
21	50 (65)	48 (94)	42 (86)	59 (83)	1
EE	58	58	64	60	4

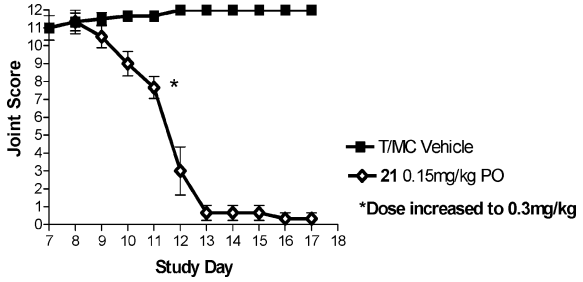


Figure 1. Mean clinical joint scores by day in animals receiving oral daily treatment with T/MC vehicle or **21** (0.3 mg/kg) in the Lewis rat AIA model.

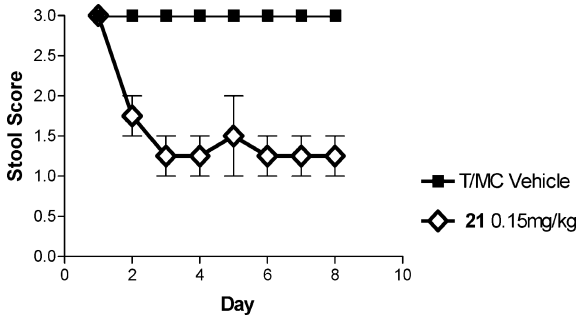


Figure 2. Mean stool scores by day in HLA-B27 transgenic rats receiving oral daily treatment with T/MC vehicle or **21** (0.15 mg/kg) in T/MC vehicle.

methoxyphenyl)-2-methyl-3-naphthalen-1-ylpropionic acid) as its quinidine salt was developed to facilitate further SAR.[12]

To improve the pharmaceutical properties of the series, we looked at several propanamides[13] (Table 4). These were prepared by activation of the carboxylic acid as the acid chloride followed by condensation with the amine. This amidation not only improved the pharmaceutical properties of the series but also significantly improved the in vitro and in vivo profiles. Compounds **20**[14] and **21** (WAY-204688, SIM 688)[15] were evaluated in vivo for the ability to inhibit four proinflammatory genes (MHC, invariant chain (MHI), VCAM-1, RANTES, and TNF-α). The effect of the test compound on induction of the gene products and on uterine wet weight was compared to that of 17α-ethinyl 17β-estradiol (EE at 10 (μg/kg)/day) in the same paradigm. The results for **20** and **21** at a dose of 5 (mg/kg)/day are shown in Table 5 (compound dosed po daily for 5 weeks; for significant inhibition, $p < 0.05$).

Further characterization of **21** was carried out in several preclinical models of inflammatory disease. In the Lewis rat adjuvant-induced arthritis model (AIA),[16] **21** was active at a dose of 0.3 (mg/kg)/day, po (Figure 1). The histology scoring from this experiment is shown in Table 6. **21** demonstrated approximately 50% improvement in synovial scoring.

In the HLA-B27 transgenic rat model of inflammatory bowel disease,[17] **21** reversed the clinical and histologic scores at a dose of 0.15 (mg/kg)/day (Figure 2). When administered to sexually immature Sprague Dawley rats, at a dose of 2 (mg/kg)/day, **21** did not stimulate uterine wet weights and did not antagonize EE's (0.06 μg/day) 4-fold increase in uterine wet of weights. Interestingly, it has been shown that NF-κB transcription can be regulated by an inhibitor that interacts with the hERα and hERβ receptors.[18,19] The interaction of **21** with ERα and ERβ was examined in vitro. Compound **21** displaces [^{3}H]E2 from the ERα ligand binding domain protein (LBD) with $IC_{50} = 2.43$ μM and from the ERβ LBD with $IC_{50} = 1.5$ μM. In vivo, **21** inhibits NF-κB induced transcriptional activation in an ERα selective fashion and shows less activity in the presence of ERβ. The difference between the binding results and the transcriptional activation results may simply reflect the differences between the conformation of the E2 bound LBDs vs the unoccupied ERα receptors or that transcriptional activation requires **21** to make unique contacts with ERα that E2 does not make.

In summary, HTS identified 2-cyano-3,3-dinaphthalen-1-ylpropionic acid ethyl ester **1** as an estrogen receptor dependent inhibitor of NF-κB transcriptional activity. SAR studies resulted in the improvement of the series profile. HPLC resolution and X-ray crystallography showed the absolute stereochemistry of the eutomer to be *SS*. Further elaboration of the structure resulted in the synthesis of **21**, an ERα ligand that is active in several preclinical models of inflammation. **21** is an ERα selective, orally active inhibitor of NF-κB transcriptional activity, which may have use in the treatment of inflammatory diseases such as rheumatoid arthritis.

Acknowledgment. The authors thank Amedeo Failli, Boyd Harrison, Robert Steffan, and Rick Winneker for helpful discussions. The authors thank Heather Harris for measuring rat uterine wet weights, Discovery Analytical Chemistry for

Table 6. Histological Scoring of Synovitis in the Tarsal Joints from Animals with Adjuvant-Induced Arthritis Treated for 10 Days with Once Daily Oral **21** (0.3 (mg/kg)/day) Beginning on Day 8 after CFA Injection (Mean ± SD)

group	synovial structure (0−3)	fibroplasia (0−3)	inflammatory cells (0−3)	pannus (0−2)	total synovitis score (0−11)
T/MC vehicle	2.92 ± 0.20	2.67 ± 0.26	2.92 ± 0.20[a,b]	2.00 ± 0.00	10.5 ± 0.55
21	1.92 ± 0.74[a]	1.08 ± 0.86[a]	0.83 ± 0.52[a]	0.33 ± 0.52[a]	4.17 ± 2.30[a]

[a] Total score is the summation of four individuals. [b] Signal is less than vehicle signal. For significant inhibition, $p < 0.05$.

spectral data, and Mark Ashwell for the evaluation and prioritization of HTS data.

Supporting Information Available: Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) A discussion of the synthesis and SAR of this series is beyond the scope of this Letter and will be the subject of a separate report.

(14) (2*S*)-3-[4-(3-Chloro-2-methylphenyl)piperazin-1-yl]-2-[(*S*)-(2-methoxyphenyl)(1- naphthyl)methyl]-2-methyl-3-oxopropanenitrile.

(15) (2*S*,3*S*)-3-(2-Methoxyphenyl)-2-methyl-3-(1-naphthyl)-2-({4-[3-(trifluoromethyl)phenyl]piperidin-1-yl}carbonyl)propanenitrile.

(16) AIA model: 12 week old ovariectomized female Lewis rats were injected with Freund's adjuvant. After 8 days, inflammation is established and the animals are dosed for 16 days with the test compound. The hindpaw joint is monitored and scored for swelling and erythema (1−3 scale; max = 12). Tissue is collected for histology scoring (synovial hyperplasia, inflammatory cell infiltration, pannus formation, and articular cartilage destruction).

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