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Letters

Synthesis and Activity of Substituted 4-(Indazol-3-yl)phenols as Pathway-Selective Estrogen Receptor Ligands Useful in the Treatment of Rheumatoid Arthritis

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Abstract: Pathway-selective ligands for the estrogen receptor (ER) inhibit NF-κB-mediated inflammatory gene expression causing a reduction of cytokines, chemokines, adhesion molecules, and inflammatory enzymes. SAR development of a series of 4-(indazol-3-yl)phenols has led to the identification of WAY-169916 an orally active nonsteroidal ligand with the potential use in the treatment of rheumatoid arthritis without the classical proliferative effects associated with estrogens.

Rheumatoid arthritis (RA) is characterized by chronic joint inflammation mediated by inflammatory cell infiltration into synovial tissues as well as joint destruction through matrix metalloproteinase (MMP) overexpression in articular synoviocytes and chondrocytes. The pathologic lesions of RA are driven, in part, by the production of inflammatory mediators in synoviocytes and macrophages, likely involving the transcription factor nuclear factor κB (NF-κB). NF-κB is composed of a homodimeric and heterodimeric family of the Rel family of proteins which resides in the cytoplasm in a nonactive form bound to the cytoplasmic inhibitory protein-κB (IκB). Cell activation by a variety of extracellular inflammatory cytokines induces a cascade of events that leads to degredation of the IκB/NFκB complex. Activated NF-κB then translocates to the nucleus where it binds to DNA elements resulting in the induction of pro-inflammatory genes such as TNFα, IL-6, etc.[1] Two recent discoveries indicate that inhibition of this cellular pathway provides a novel intervention point in the control of the inflammatory process. First, it was found that overproduction of IL-6 is associated with states of chronic inflammation.[2] Second, in endothelial cells it was shown that 17β-estradiol (E2) inhibits IL-1β-induced NF-κB reporter activity and IL-6 expression in an estrogen receptor (ER) dependent fashion.[3] The latter correlates with the antiinflammatory action of E2 in vivo which is confirmed in different animal models of inflammation.[4] For a review of recent advances in ER ligands as a drug class, see ref 5.

A drug discovery project was initiated to confirm that the ER/ligand complex mimics the antiinflammatory action of E2 that occurs in chronic conditions such as RA. The ultimate goal of the project was to identify a small molecule nonsteroidal ligand for ER which will mediate the antiinflammatory effect without eliciting the classical ER responses.

Compounds were screened in vitro using an assay developed in HAECT-1 cells (immortalized human aortic endothelial cells).[1] HAECT-1 cells are transfected with human ERα and a reporter gene NFκB-luciferase. These cells are then treated for 16−18 h with IL-1β and the test compound. The level of transcription of NF-κB is directly proportional to the amount of luciferase present. The ideal profile for the test compound would be an IC_{50} of NF-κB luciferace reporters of 100 nM or less with an efficacy (% E2) near 100% coupled with a lack of classical estrogenic receptor activity. Classical estro-

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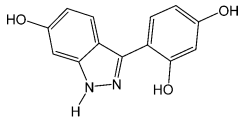
genic activity is characterized by stimulation of uterine proliferation, ER-mediated gene expression, and for our in vitro model, a lack of creatine kinase (CK) activity.

4-(Indazol-3-yl)phenols were identified as potent nonsteroidal estrogen receptor (ER) ligands that selectively inhibit NF-κB transcriptional activity but are devoid of conventional estrogenic activity. To confirm that these ligands for ER were devoid of the proliferative effects on uterine and breast tissue associated with estrogen, the compounds were tested in an uterine wet weight model in female mice in vivo. No increase in uterine wet weight after dosing for 5 days would confirm a lack of estrogen receptor activity.

The initial indazole lead **1** was identified by high throughput screening of selected corporate libraries of compounds.



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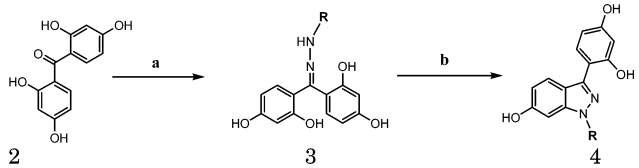
Compound **1**[7] was confirmed to have strong inhibition of the NF-κB transcription [IC_{50} = 48 nM (82% E2)] in the ER/NF-κB-luc assay and weakly active in the CK stimulation activity. Selected libraries of compounds were prepared via Schemes 1 and 2 to develop the initial SAR. The acceptable compound purity for the arrays was >85% by HPLC and exact mass. Confirmation of activity was conducted using analytically pure dry powder.

Commercially available 2,2′,4,4′-tetrahydroxybenzophenone **2** was combined with a variety of 1-substituted hydrazines in methanol to form the intermediate hydrazone **3**. Heating the intermediate to 200 °C afforded the appropriate 1-substituted indazole **4**.

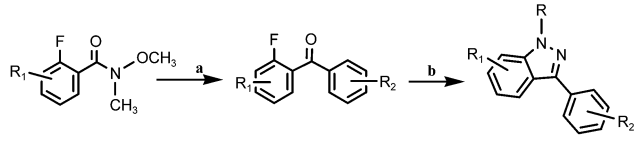
Alternatively, the Weinreb amide **5** was prepared using standard conditions from commercially available appropriately substituted 2-fluorobenzoic acids.[8] The Weinreb amide was reacted with the methoxy-bearing phenylmagnesium bromides. The resulting benzophenones **6** were reacted with the 1-substituted hydrazines in the same manner as in Scheme 1 to give the indazole **7** (R_2 = OCH_3). The free hydroxyl derivative **8** (R_2 = OH) was liberated by removal of the methyl group with excess boron tribromide in methylene chloride.[9] Shown in Table 1 are some of the most active analogues.

Scheme 1[a]



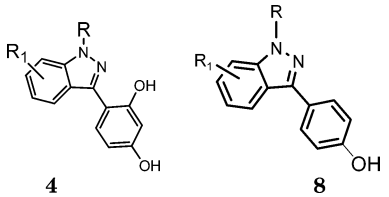
[a] (a) $RNHNH_2$, NaOAc, MeOH; (b) 200 °C, Neat.

Scheme 2[a]



[a] (a) $R_2PhMgBr$; (b) $RNHNH_2$ then BBr_3.

Table 1. Effects of 1-Substituted Analogues of Templates **4** and **8** vs E2 on NF-κB, and CK Expression in Ad5-wt-ER-Infected HAECT-1 Cells[a]

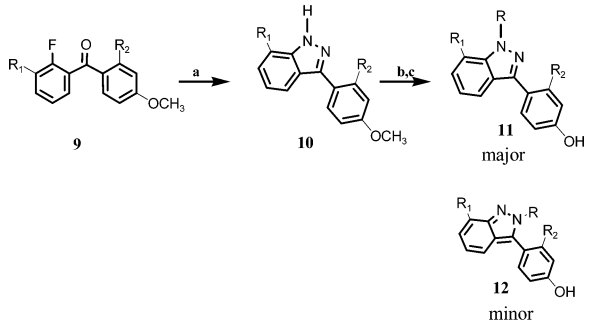


4 **8**

compd	R_1	R	ER/NFκB-luc IC_{50}, nM	%E2	$CKEC_{50}$, nM	%E2
1	6-OH	H	48	82	298	43
4a	6-OH	methyl	622	64	3715	50
4b	6-OH	ethyl	305	71	3070	97
4c	6-OH	CF_3CH_2	165	71	4790	66
4d	6-OH	*n*-propyl	62	71	1217	33
4e	6-OH	*n*-butyl	112	49	549	27
4f	6-OH	cyclohexyl	443	63	ia[b]	
4g	6-OH	4-CH_3–phenyl	40	75	149	69
4h	6-OH	3-F-phenyl	39	72	140	
4i	6-OH	3-Cl-phenyl	50	73	ia	
4j	6-OH	2,5-diCl-phenyl	15	72	ia	
4k	6-OH	2,5-diF-phenyl	31	87	ia	
8a	5-F, 6-Cl	benzyl	sd[c] 19%	33		
8b	7-Cl	benzyl	105	60	390	47
8c	7-CF_3	benzyl	196	73		
8d	6-CF_3	benzyl	ia			
8e	7-CF_3	2-CH_2CH_2OH	96	69		
8f	5-F,6-Cl	2-CH_2CH_2OH	ia			
8g	7-Cl	2-CH_2CH_2OH	410	71		
8h	7-Cl	butyl	25	90	63	55

[a] All compounds were ER dependent (only active when ER is coexpressed with NFkB-luciferase in the HAECT cells). [b] ia = inactive. [c] sd = % inhibition at single dose of 10 uM %E2 = efficacy (relative inhibition of test compound at 10 uM vs E2 at 0.1 nM).

Scheme 3[a]

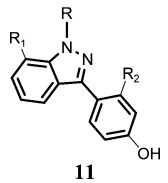


[a] (a) $NH_2NH_2{\cdot}H_2O$, DMAP, pyridine, 100 °C; (b) NaH, RX, DMF, 60 °C; (c) BBr_3.

Within the trihydroxy series **4a**−**k** the most potent analogues **4g**, **4i**, **4j**, and **4k** were evaluated in vivo for their ability to inhibit the mRNA of NF-κB target genes MHC, VCAM-1, RANTES, and TNF-α in C57BL/6 mice subjected to a high fat diet for 5 weeks.[10] All of the analogues tested in the model produced no detectable plasma levels, were inactive in the model, and were not pursued.

The compounds lacking a hydroxyl group at R_1 from Scheme 2 were used to guide future analogue development. A representative group of compounds **8a**−**h** illustrate the important positions for SAR development. Compounds **8b** and **8c** illustrate the importance of the 7 position. The only compounds from the array of 3-indazolylphenolic and nonphenolic analogues to show activity had the hydroxyl in the para-postion shown in template **8**.

Table 2. Effects of R, R_1, and R_2 Indazole Substitution vs E2 on NF-κB, and CK Expression in Ad5-wt-ER-Infected HAECT-1 Cells[a]



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compd	R_2	R_1	R	ER/NFκB-luc IC_{50}, nM	%E2	CK EC_{50}, nM	%E2
11a	H	CH_3	propyl	40	89	303	61
11b	H	CF_3	isopropyl	3	91	11	44
11c	H	Cl	propyl	52	77	138	34
11d	H	Cl	cyclohexyl	37	105	ia[b]	
11e	H	Cl	cyclopentyl	18	94	98	36
11f	H	F	cyclopentyl	18	80	330	34
11g	OH	CF_3	allyl	93	81	ia	
11h	CH_3	CF_3	allyl	59	86	ia	
11i	CH_3	CF_3	isopropyl	20	116	ia	

[a] All compounds were ER dependent (only active when ER is coexpressed with NFkB-luciferase in the HAECT cells). [b] ia = inactive, %E2 = efficacy (relative inhibition of test compound at 10 uM vs E2 at 0.1 nM.

Table 3. Effects of Ethynyl Estradiol (EE) (0.01 mg/kg/day) and Test Compound on NF-κB Target Gene Expression in C57BL/6 Mice Fed a High Fat Diet

compd	concn, mg/kg/day	RANTES, %inhib (%EE)	VCAM-1, %inhib (%EE)	TNFa, %inhib (%EE)	MHC, %inhib (%EE)
11a	10	0	0	66 (45%	29 (83%)
	5	0	49 (74%)	53 (46%)	0
11c	10	24 (55%)	42 (111%)	0	0
11f	10	45 (120%)	22 (92%)	69 (100%)	29 (110%)
	5	66 (71%)	32 (71%)	72 (100%)	39 (61%)
	2.5	80 (120%)	49 (94%)	56 (76%	31 (93%)
11h	10	0	38 (86%)	44 (115%)	0
11g	10	41 (100%)	28 (100%)	42 (93%)	38 (84%)
	5	58 (100%)	38 (94%)	42 (96%)	38 (103%)
	2.5	0	0	44 (92%)	0
11i	10	41 (100%)	59 (100%)	85 (100%)	21 (88%)

[a] The test compounds were administered po daily for 5 weeks. For signifcant inhibition, $p < 0.05$.

Thus, a more general synthesis (Scheme 3) was developed to provide a wider variety of substitution at positions R or R_2 while focusing on the 7- position (R_1) of the indazole. Thus the 2-fluorobenzophenone **9** was reacted with hydrazine hydrate to give the substituted 1-H indazole core compound **10**. The R-substitutions were obtained by initial treatment with sodium hydride in DMF followed by an alkyl halide. The reactions were typically heated to 60 °C to complete the alkylation to give **11** and **12** with ratios typically ranging from 4 to 1, respectively, which were easily separated using normal phase chromatography. A summary of the in vitro SAR from these optimized analogues are shown in Table 2.

Compounds that showed in vitro selectivity were evaluated in vivo for their ability to inhibit the mRNA

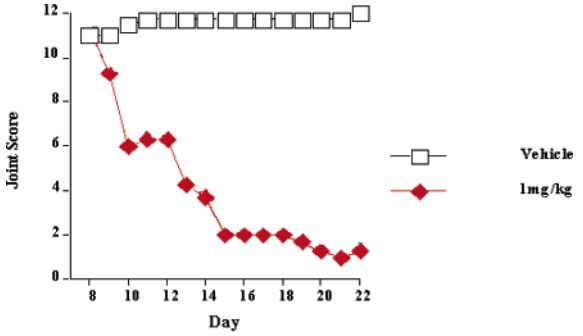

Figure 1. Dose−response of orally administered WAY-169916 in the Lewis rat model of adjuvant-induced arthritis.[11]

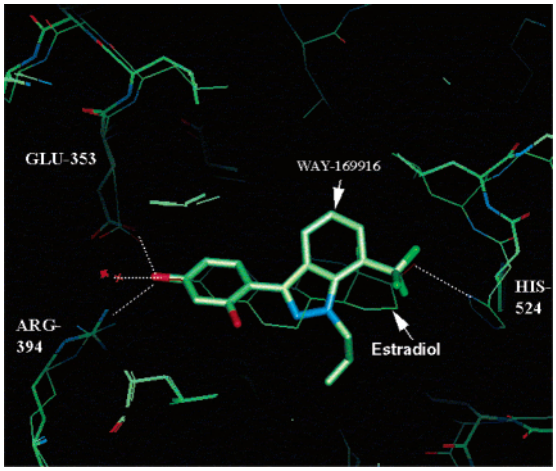

Figure 2. Comparison between ERα/WAY-169916 and ERα/estradiol binding motifs.

levels of the NF-κB target genes MHC, VCAM-1, RANTES, and TNF-α in C57BL/6 mice subjected to a high fat diet for 5 weeks.[10] Inhibition of three out of four target genes was considered orally active. A summary of the compounds is shown in Table 3.

Compound **11g** WAY-169916 demonstrated no increase in uterine wet weight versus estradiol after 5 day dosing at 10 mg/kg in C57BL/6 mice. Estradiol typically induces a 5-fold increase in uterine weight vs vehicle, indicative of classical estrogenic effects.

In a disease model for adjuvant induced arthritis (AIA),[11,12] WAY-169916 was found to be orally active at 1 mg/kg. Lewis rats were injected with Freund's Adjuvant. After the joints were inflamed, typically after 8 days, the animals were dosed each day with test compound. The activity was confirmed in the histological scoring shown in Table 4. The activity was also measured at 0.1 and 0.3 mg/kg doses and found to be dose dependent.[11]

WAY-169916 displaced [^{3}H]E_2 from ERα-ligand binding domain (LBD) protein with an IC_{50} value of 1300 nM and from ERβ-LBD with an IC_{50} value 106 nM. To

Table 4. Histological Scoring of Synovitis in the Tarsal Joints from Animals with Adjuvant-Induced Arthritis Treated Orally for 2 Weeks with WAY-169916 Beginning on Day 8 after CFA Injection. Means ± SD

dose, mg/kg po	synovial struct 0−3	fibroplasia 0−3	inflammatory cells 0−3	pannus 0−2	total score[a]
vehicle	2.92 ± 0.21	2.67 ± 0.41	2.92 ± 0.21	2.00 ± 0	10.50 ± 0.63
1	2.08 ± 0.20[b]	1.58 ± 0.38[b]	1.33 ± 0.41[b]	0.83 ± 0.98[b]	5.83 ± 1.78[b]

[a] Total score is the summation of four individual scores. [b] $p < 0.05$, statistically significant improvement in the scores over the vehicle-treated group.

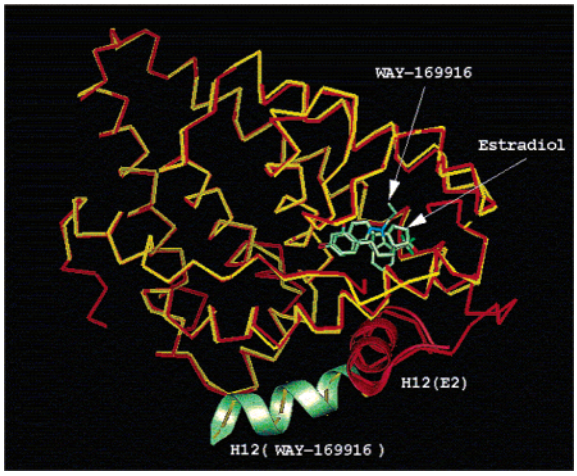

Figure 3. Comparison between WAY-169916/ERα and estradiol/ERα complex on the position of helix-12.

substantiate the interaction of WAY-169916 (**11g**) with the ERα, a cocrystallization was performed with the LBD of ERα. The compound binds in the same binding pocket as estradiol as shown in Figure 2.

The phenol of WAY-169916 interacts with the Glu-353 and Arg-394 salt bridge. The trifluoromethyl group is pointing directly to the His-524, the second hydroxyl binding position of estradiol. Of particular interest in the ERα/WAY-169916 complex is that helix 12 adopts an antagonist position (Figure 3). This supports the lack of an estrogenic agonist effect associated with estradiol. WAY-169916, however, does not have the traditional antagonist effects found in SERMS.

In summary, optimization of the SAR around the indazole ring led to the identification of WAY-169916 as a selective ligand for ER which is devoid of the traditional effects of estrogens. WAY-169916 is a potent pathway-selective inhibitor of NF-κB-induced inflammatory events. Its utility has been demonstrated in the AIA disease model for arthritis. A near complete reversal in hindpaw scores was observed as well as dramatic improvements in histological scores of synovitis and cartilage lesions in the tarsal joints.

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

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(10) Ovariectomized C57BL/6 mice (16−20 g) (Taconic) were separated into groups of eight. The mice were fed a chow diet or an atherogenic diet (15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate). EE or test compound was administered once daily by gavage in a methylcellulose/Tween vehicle (0.1 mL per mouse) for 5 weeks. At the end of the experimental period, the liver was collected and uterine wet weight was recorded.

(11) Liver total RNA was prepared by using Trizol reagent (BRL). Estrogen and compound regulation of NF-κB target genes were verified by real time RT-PCR using an ABI PRISM 7700 Sequence Detection System according to the manufacturer's protocol (Applied Biosystems). The data were analyzed using the Sequence Detector v1.7 software (Applied Biosystems) and normalized to GAPDH using the Applied Biosystems primer set.

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(13) AIA model: 12 week old ovariectomized female Lewis rats were injected with Freund's Adjuvant. After the joints were inflamed, typically after 8 days, the animals were dosed each day with test compound and monitored to determine hindpaw joint score (erythema and swelling; 0−3 score) max. = 12. Tissue was collected for histology which included synovial hyperplasia, inflammatory cell infiltration, pannus formation, and articular cartilage destruction.

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