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Potent and selective [2-imidazol-1-yl-2-(6-alkoxy-naphthalen-2-yl)-1-methyl-ethyl]-dimethyl-amines as retinoic acid metabolic blocking agents (RAMBAs)

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Abstract—A series of [2-imidazol-1-yl-2-(6-alkoxy-naphthalen-2-yl)-1-methyl-ethyl]-dimethyl-amines were designed and synthesized as CYP26 inhibitors, serving as retinoic acid metabolic blocking agents (RAMBA's).

All-*trans* retinoic acid (a*t*RA) (**1**), a naturally occurring retinoid, is biosynthesized and present in a multitude of human and mammalian tissues, and performs a crucial role in the regulation of gene expression, cellular differentiation, and proliferation of epithelial cells.[1] Retinoids have proven to be useful agents in tumor therapy and in treating skin related diseases.[2] Furthermore, all-*trans* retinoic acid has been successfully used to treat acute promyelocytic leukemia (APL) and acute myelogenous leukemia (AML), changing the prognosis of APL from a fatal leukemia to a highly curable disease.[3]

Despite the usefulness of a*t*RA and retinoid compounds in treating tumors and skin-related diseases, clinical uses of a*t*RA have been significantly hampered by the emergence of resistance, believed to be caused by the oxidative catabolism to 4-hydroxy-a*t*RA (**2**) by CYP26, an inducible cytochrome P450 enzyme (Fig. 1).[4] This tightly controlled negative feedback mechanism limits the systemic concentrations of a*t*RA and thereby limits its biological activity.[5]

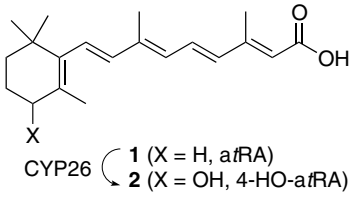

Figure 1. Metabolism of a*t*RA to 4-hydroxy-a*t*RA by CYP26.

An alternative approach of potentiating endogenous a*t*RA can be through inhibition of CYP26 and may avoid the frequency and severity of complications associated with intensive high dose a*t*RA therapy and may provide an effective means of treatment following relapse in cases where resistance emerges due to CYP26 upregulation. As a result, inhibitors of CYP26, also known as retinoic acid metabolic blocking agents (RAMBAs), have proven to be effective in blocking the catabolic effects on a*t*RA and have demonstrated an increase in endogenous a*t*RA levels.[6] Liarozole (**3**, IC_{50} = 6.0 μM) was the first generation CYP26 inhibitor (Fig. 2) and in in vitro studies, suppressed the metabolism of a*t*RA in MCF7 cells.[7] Additionally, liarozole in conjunction with exogenously administered a*t*RA, displayed growth inhibition in MCF7, T47D, and AT6.1 cells. In in vivo studies, liarozole (30 mg/kg

Keywords: CYP26; All-*trans* retinoic acid (a*t*RA); Retinoic acid metabolic blocking agent (RAMBA); CYP26; Cancer.
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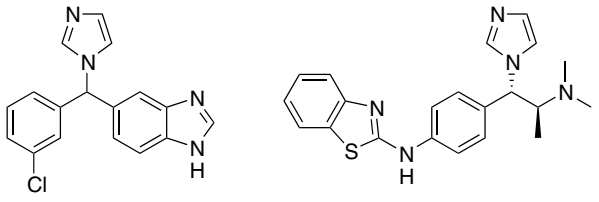
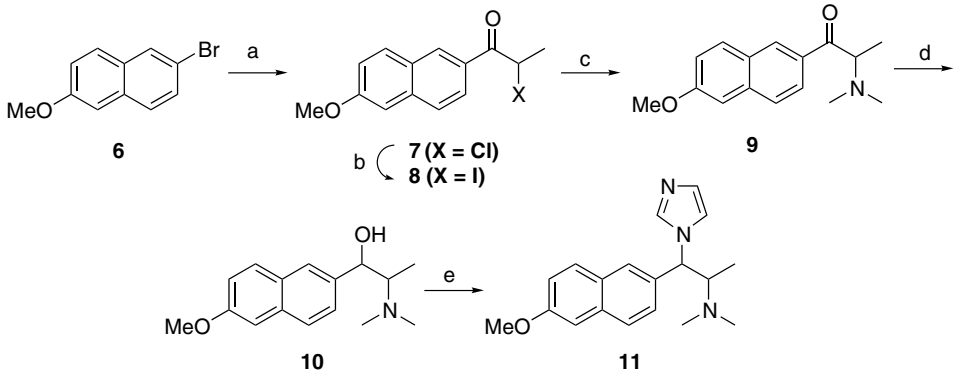


Figure 2. CYP26 inhibitors (RAMBAs).

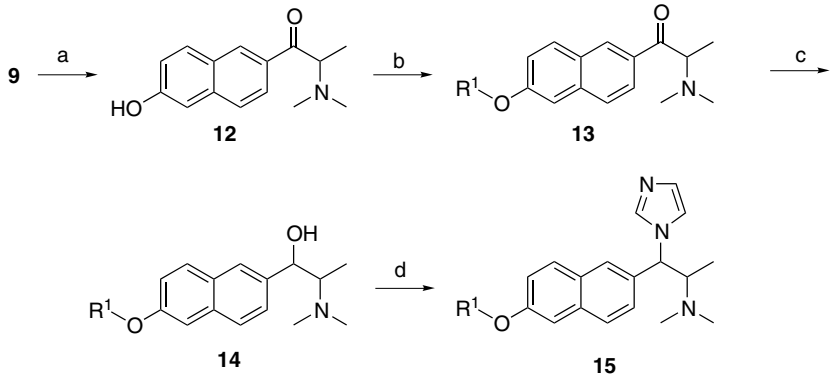
(b.i.d.)) showed 90% growth inhibition in both AT-6 androgen independent prostate adenocarcinoma (R3227 Line) and in AT-6 androgen independent prostate tumors in rats. Several other RAMBAs quickly followed liarozole, one of which being R116010 (**4**, IC_{50} in intact T47D cells = 8.7 nM) (Fig. 2) addressing the potency toward CYP26, which liarozole lacked.[8]

To explore the therapeutic potential of increasing endogenous levels of a*t*RA through small molecule CYP26 inhibitors, we prepared a novel class of highly potent and CYP26 selective naphthyl-based inhibitors, which show inhibition of proliferation of both T47D and AT6.1 cells in vitro in combination with a*t*RA.

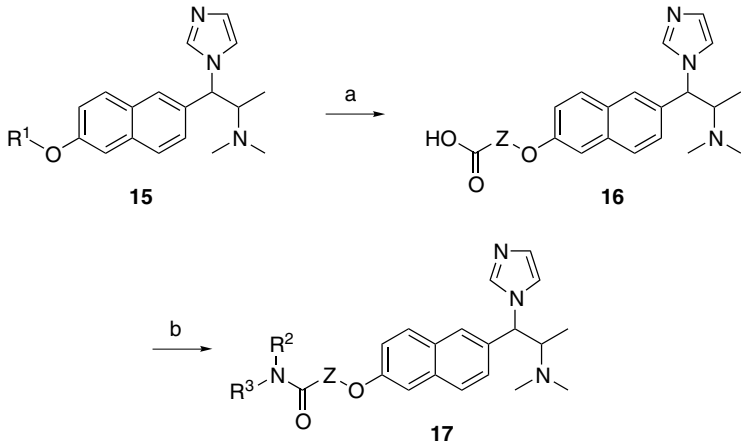
Our inhibitor design fuses the heme-binding imidazolylpropylamino moiety of R116010 (**4**) with a naphthalene core carrying a CYP26 selectivity handle at the 6-position. The 2,6-disubstituted naphthalene core, from a 2-D perspective, overlays in a complementary fashion with the conjugated olefinic moiety of the tetraenoic acid side chain of a*t*RA. Additionally, a suitable tether at the 6-position of the naphthyl core was proposed to mimic that of the a*t*RA side chain. With this design strategy in mind, the synthesis of a series of [2-imidazol-1-yl-2-(6-alkoxy-naphthalen-2-yl)-1-methyl-ethyl]-dimethylamines (**15**–**17**) as shown in Schemes 1–3 was initiated. Treatment of 2-bromo-6-methoxynaphthalene **6** with magnesium generated a Grignard species which was added to a solution of 2-chloropropionylchloride in THF cooled to −78 °C to afford α-chloromethylketone **7** (Scheme 1).[9] Conversion of **7** to the more reactive iodoketone **8** via the Finkelstein reaction (NaI, acetone) proceeded smoothly and subsequent alkylation with dimethylamine cleanly afforded dimethylaminoketone **9** (direct reaction of chloromethylketone **7** with dimethylamine did not proceed as cleanly). Reduction of aminoketone **9** with sodium borohydride afforded a 4:1 mixture of *syn*:*anti* aminoalcohol isomers whereas hydrogenation afforded the reverse, a 4:1 ratio of *anti*:-*syn* isomers (**10**). The isomers were easily separable by column chromatography and were each independently taken on to their respective imidazolyl final product by reaction with CDI in acetonitrile. The *syn*-aminoalcohol afforded the *syn*-imidazolylpropylamino product and the *anti*-aminoalcohol afforded the *anti*-imidazolylpro-



Scheme 1. Reagents and conditions: (a) (i) Mg, THF; (ii) 2-chloropropionylchloride, −78 °C; (b) NaI, acetone; (c) dimethylamine, MeOH; (d) $NaBH_4$, MeOH; (e) CDI, CH_3CN, 65 °C.



Scheme 2. Reagents and conditions: (a) HBr/AcOH, 120 °C; (b) DIAD, Ph_3P, R^1OH; (c) $NaBH_4$, MeOH; (d) CDI, CH_3CN, 65 °C.



Scheme 3. Reagents and conditions when $R^1 = Z–CO_2R^{1a}$: (a) NaOH, THF/H_2O; (b) EDC, HOBT, HNR^2R^3 or CDI, CH_3CN, then HNR^2R^3.

pylamino derivative (**11**). The regio- and stereospecific nature of the CDI reaction of aminoalcohols as well as the mechanistic rational behind the reaction was determined through model studies conducted on (1*S*,2*S*)-(+)-*N*-methylpseudoephedrine and (1*S*,2*R*)-(+)-*N*-methylephedrine and have been previously reported by our research group.[10]

In order to explore various ether tethers, a naphthol intermediate was required. Deprotection of methyl ether **9** with HBr/acetic acid afforded naphthol **12** (Scheme 2). Alkylation of **12** with *tert*-butylbromoacetate using potassium *tert*-butoxide as the base smoothly afforded ether **13**. However, these conditions with other alkylating agents afforded a mixture of both *N*- and *O*-alkylated products. We discovered that treatment of **12** under standard Mitsunobu conditions (PPh_3, DIAD, R^1OH) cleanly afforded ethers **13**. Reduction of ketone group in **13** with $NaBH_4$, afforded a 4:1 mixture of *syn*:*anti*-aminoalcohols **14** as seen previously with aminoketone **9**. Separation of the isomers and subsequent reaction with CDI afforded imidazolylpropylamino derivatives **15**. In order to mimic the side chain of a*t*RA, terminal ester moieties (compound **15** where $R^1 = Z–CO_2R^{1a}$) were hydrolyzed to their respective carboxylic acids **16** (Scheme 3). In some cases, the carboxylates **16** were converted to amides **17** via typical amide coupling procedures (EDC/HOBT/HNR^2R^3 or CDI then HNR^2R^3).

The efficacy of compounds **15**–**17**, as inhibitors of CYP26 were confirmed in a number of in vitro assays. A biochemical assay was performed using microsomal preparations from T47D cells induced to express CYP26. Enzymatic activity was measured as the conversion of the radiolabeled substrate to its metabolites, 4-OH-a*t*RA (4-hydroxy all-*trans* retinoic acid) and 4-oxo-a*t*RA (4-oxy retinoic acid) by reverse phase HPLC separation. Inhibition of CYP26 activity in the presence of variable inhibitor concentrations was used to determine IC_{50} values. Microsomal preparation from T47D cells were conducted as follows: T47D cells were grown in RPMI 1640 containing 10% FBS and 1% P/S, plated and treated 16–25 h later with 5 μM a*t*RA and allowed to incubate for an additional 48 h before cell harvest. Cells were washed twice with PBS and scraped from plates. Cells were pelleted and resuspended in homogenization buffer (0.1 M Tris–Cl, pH 7.4, 0.1 M DTT, 0.2 mM EDTA, 1.15% w/v KCl, 0.1 mM PMSF and 20% v/v glycerol). Microsomes were prepared by differential centrifugation of homogenized cells. Homogenate was spun at 17,000*g* and the supernatant spun again at 100,000*g*. The pellet was resuspended in 25 mM potassium phosphate, pH 7.4, 20% v/v glycerol and stored at −80 °C.

Enzymatic assays (HPLC biochemical CYP26 assay) were performed in a total volume of 100 μL in a reaction mixture composed of 100 mM Tris pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 2 mM NADPH, 40 nM 3H-a*t*RA, and varying concentrations of novel compound dissolved in DMSO such that the concentration in the reaction is 0.1% final, and 20–30 μg of T47D microsomes. The reactions were incubated at 37 °C for 30 min in the dark. The reaction was stopped by the addition of 125 μL of acetonitrile, mixed and spun at 10,000*g* for 10 min. The supernatant was removed and a*t*RA and metabolites were separated on a C18 Waters Spherisorb column with an in-line radiometric detector with a flow rate of 1 mL/min and detected at 350 nM. The gradient used was the mixture of 60 mM ammonium acetate, pH 5.2/30% CH_3OH, solvent A and solvent B (CH_3OH). An increasing CH_3OH gradient was run for the separation of products by increasing solvent B.

Enzymatic assays to measure the inhibition of CYP3A4 activity was determined in 100 μL volume in a 96-well black plate by the use of a fluorescence substrate (BD, Gentest). Compounds were tested at various concentrations in a reaction that contained 200 mM potassium phosphate buffer, pH 7.4, 200 mM NADPH and 50 μM 7-benzyloxy-4-(trifluoromethyl)-coumarin. The reaction was incubated at 37 °C for 45 min followed by the addition of 37 μL of 80% acetonitrile in 0.5 M Tris base to terminate the reaction. The plates were read at excitation/emission of 405/535 nm, respectively.

The novel naphthalene analogs inhibited the proliferation of breast cancer and prostate cells in vitro. Experiments were conducted on T47D breast cancer cell line

and on the AT6.1 rat prostate adenocarcinoma cell line. T47D cells were grown in RPMI 1640 containing 10% FBS and 1% P/S. Cells were plated into 96-well culture plates (2000 cells per well) in 100 μL of same medium. After attachment for 16–24 h, the vehicle (DMSO), or a*t*RA alone (at concentrations of 1 nM to 1 μM), or a*t*RA at these concentrations in combination with varying concentration of novel compound were added to triplicate wells.[11] Medium/treatments were repeated 3 days after the first treatment and measure of the decrease in cell proliferation was measured 48 h later using CellTiter-Glo^{TM} (Promega).

The method described above was also used for AT6.1 cells except that cells were plated at 1500 cells per well and treatment was performed once with measure of the decrease in cell proliferation 72 h post treatment. AT6.1 cells were grown in RPMI 1640 containing 10% FBS, 1% P/S and 250 nM dexamethasone.

As shown in Table 1, a general trend emerged where the *syn*-isomers were generally more potent than the *anti*-isomers. The imidazoyl moiety proved to be a critical element for CYP26 activity as seen by the lack of activity in the aminoalcohol precursors (**14**). Our chemistry efforts focused around the ether tether (OR^1/O–Z–CO_2H/O–Z–$CONR^2R^3$) while maintaining the dimethylaminopropylimidazole and naphthalene ring constant. We discovered that a variety of tethers and substituents were tolerated. While in some cases only a moderate degree of selectivity for CYP3A4 was noted, we discovered that a terminal carboxylate moiety in the case of compounds **16f** and **16h** afforded a larger degree of selectivity. The carboxylate moieties of the two lead compounds, **16f** and **16h**, were converted to their respective amides (**17a–f**), however, a reduction in both potency and selectivity (CYP3A4) was noted. Compounds with a biochemical potency below 50 nM were screened in a cellular assay (AT6.1 and T47D). Representative lead compounds **16f** and **16h** exhibit good cellular potency unlike the corresponding amides (Table 2).

The two lead compounds, **16f** and **16h**, were further profiled in vivo for pharmacokinetic properties. Compound **16f** had minimal bioavailability and was not pursued further. Compound **16h** displayed good PK properties (female CD-1 mouse), including bioavailability of 99%,[12] and favorable selectivity for CYP26 over CYP3A4, 1A2, 2D6, and 2C9 (Table 3).

In conclusion, a series of [2-imidazol-1-yl-2-(6-alkoxy-naphthalen-2-yl)-1-methyl-ethyl]-dimethyl-amines were designed and synthesized as CYP26 inhibitors, serving as retinoic acid metabolic blocking agents (RAMBA's). Various substituted ether moieties at C6 of the naphthyl

Table 1. CYP26 potency and CYP3A4 selectivity of naphthyl derivatives

Compound	OR^1/O–Z–CO_2H/O–Z–$CONR^2R^3$	Stereochemistry	Biochemical IC_{50}[a] (nM)	
			CYP26	CYP3A4
15a	(O)–CH_2–Ph–4-(O-*t*-Bu)	*syn*	34.0	580.0
15b	(O)–CH_2–Ph–4-(O-*t*-Bu)	*anti*	86.0	40.00
15c	(O)–CH_2Ph	Mix	50.0	200.0
15d	(O)–CH_2–Ph–4-(CH_2CO_2Me)	Mix	100	4.000
15e	(O)–CH_2–Ph–4-(OCH_2CO_2Me)	*syn*	19.0	220.0
15f	(O)–CH_2–Ph–4-(OCH_2CO_2Me)	*anti*	153	140.0
15g	(O)–CH_2–Ph–3-(CO_2Me)	*syn*	7.00	210.0
15h	(O)–CH_2–Ph–3-(CO_2Me)	*anti*	18.0	160.0
15i	(O)–$CH_2C(CH_3)_3$	*syn*	21.4	700.0
15j	(O)–$CH_2C(CH_3)_3$	*anti*	73.0	220.0
15k	(O)–CH_2–Ph–4-(CO_2Me)	*syn*	16.0	40.00
15l	(O)–CH_2–Ph–4-(CO_2Me)	*anti*	85.0	10.00
15m	(O)–CH_2–Ph–4-(CN)	*syn*	6.00	320.0
15n	(O)–CH_2–Ph–4-(CN)	*anti*	71.0	260.0
15o	(O)–CH_2–Ph–4-(NO_2)	*syn*	77.0	344.0
16a	(O)–CH_2CO_2H	Mix	335	2200
16b	(O)–CH_2–Ph–4-(CH_2CO_2H)	Mix	25.0	4.000
16c	(O)–CH_2–Ph–4-(OCH_2CO_2H)	*syn*	8.00	750.0
16d	(O)–CH_2–Ph–3-(CO_2H)	*syn*	3.50	3790
16e	(O)–CH_2–Ph–3-(CO_2H)	*anti*	6.30	1590
16f	(O)–CH_2–Ph–4-(CO_2H)	*syn*	3.30	640.0
16g	(O)–CH_2–Ph–4-(CO_2H)	*anti*	12.0	1180
16h	(O)–$CH_2C(CH_3)_2CO_2H$	*syn*	20.0	6300
16i	(O)–$CH_2C(CH_3)_2CO_2H$	*anti*	46.0	1488
17a	(O)–CH_2–Ph–3-($CONH_2$)	*syn*	48.0	481.0
17b	(O)–CH_2–Ph–3-($CONHCH_3$)	*syn*	37.0	397.0
17c	(O)–CH_2–Ph–3-($CON(CH_3)_2$)	*syn*	14.4	357.0
17d	(O)–$CH_2C(CH_3)_2CONH_2$	*syn*	47.0	1835
17e	(O)–$CH_2C(CH_3)_2$–($CONHCH_3$)	*syn*	54.0	2010
17f	(O)–$CH_2C(CH_3)_2$–($CON(CH_3)_2$)	*syn*	35.0	2032

[a] Average of $n > 2$.

Table 2. Cellular potency of lead naphthyl derivatives

Compound	OR^1/O–Z–CO_2H/O–Z–$CONR^2R^3$	Stereochemistry	Cellular IC_{50}[a] (nM)	
			AT6.1	T47D
17a	(O)–CH_2–Ph–3-($CONH_2$)	*syn*	IA[a]	IA[b]
17b	(O)–CH_2–Ph–3-($CONHCH_3$)	*syn*	250	IA[b]
17c	(O)–CH_2–Ph–3-($CON(CH_3)_2$)	*syn*	IA[a]	IA[b]
17d	(O)–$CH_2C(CH_3)_2CONH_2$	*syn*	IA[a]	250
17e	(O)–$CH_2C(CH_3)_2$–($CONHCH_3$)	*syn*	IA[a]	IA[b]
17f	(O)–$CH_2C(CH_3)_2$–($CON(CH_3)_2$)	*syn*	250	IA[a]
16f	(O)–CH_2–Ph–4-(CO_2H)	*syn*	<100	<100
16h	(O)–$CH_2C(CH_3)_2CO_2H$	*syn*	300	125

[a] Inactive at 250 nM.
[b] Inactive at 1 μM.

Table 3. Compound **16h** profile

Properties	Compound **16h**
Biochemical potency (nM)	20
T47D cell activity (nM)	125
CYP3A4 (nM)	6300
CYP1A2 (nM)	>5000
CYP2D6 (nM)	>5000
CYP2C9 (nM)	>5000
C_{max}, 5 mg/kg p.o., (μM)	6.0
AUC, 5 mg/kg p.o., (ng h/mL)	4919
T_{max}, 5 mg/kg p.o. (h)	0.5
Terminal $t_{1/2}$ (h)	2.5
C_0, 3 mg/kg i.v., (μM)	19.7
C_L, 3 mg/kg i.v. (mL/min/kg)	15
V_{ss}, 3 mg/kg i.v. (L/kg)	3.1
Oral bioavailability (F%)	99

ring were explored for potency against CYP26 and selectivity versus other CYP's including CYP3A4. The carboxylate alkyl moiety, conceived as a potential key selectivity determinant, proved to limit general CYP inhibition by these inhibitors. Utilization of structure–activity relationships ultimately led to the discovery of **16h** as a novel, potent, and selective CYP26 inhibitor with excellent pharmacokinetic and physiochemical properties. By suppressing CYP26-mediated resistance, **16h** has the potential to modulate exposure to endogenous or co-administered a*t*RA, therefore improving responses and providing a therapeutic anticancer modality.

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12. The oral PK was performed with compound **16h** as the bis-HCl salt, formulated in water and i.v. PK with the free base compound formulated in 20% hydroxypropyl-β-cyclodextrin.