Characterization of the Antiallergic Drugs 3-[2-(2-Phenylethyl) benzoimidazole-4-yl]-3-hydroxypropanoic Acid and Ethyl 3-Hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate as Full Aryl Hydrocarbon Receptor Agonists

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates most of the toxic effects of numerous chlorinated (e.g., TCDD) and nonchlorinated polycyclic aromatic compounds (e.g., benzo[*a*]pyrene). Studies in AhR null mice suggested that this receptor may also play a role in the modulation of immune responses. Recently, two drugs, namely, M50354 and M50367 (ethyl ester derivative of M50354), were described as AhR ligands with high efficacy toward reducing atopic allergic symptoms in an AhR-dependent manner by skewing T helper cell differentiation toward a T_H1 phenotype [Negishi et al. (2005) *J. Immunol. 175* (11), 7348–7356]. Surprisingly, these drugs were shown to have minimal activity toward inducing classical dioxin responsive element-driven AhR-mediated CYP1A1 transcription. We synthesized and reevaluated the ability of these drugs to regulate AhR activity. In contrast to previously published data, both M50354 and M50367 were found to be potent inducers of several AhR target genes, namely, CYP1A1, CYP1B1, and UGT1A2. M50367 was a more effective agonist than M50354, perhaps accounting for its higher bioavailability in vivo. However, M50354 was capable of displacing an AhR-specific radioligand more effectively than M50367. This is consistent with M50354 being the active metabolite of M50367. In conclusion, two selective inhibitors of T_H2 differentiation are full AhR agonists.

Introduction

Atopy is an inherited tendency to develop chronic allergic responses that manifest themselves in the form of asthma, eczema, and anaphylaxis, among other predominantly T lymphocyte helper type 2 (T_H2)[1]-driven disorders. The etiologies of these allergic conditions have not been fully established, although polymorphisms in genetic loci coding for proteins involved in immune system function are often reported (*1–4*). Biological parameters such as increased IgE production, eosinophilia, GATA-3 expression, increases in IL-4, IL-5, IL-10, and tumor necrosis factor-α cytokine production are among a complex array of markers for T_H2-driven immune responses. Accordingly, immune system disorders are often classified into T lymphocyte helper type 1 (T_H1)- and T_H2-biased responses based on the expression profiles of numerous cellular markers and cytokines of $CD4^+$ T helper cells. However, while this Mosmann paradigm (*5*) has proven useful and simplifies how immunological diseases are analyzed and classified, its application to other immune cells, namely, $CD8^+$ lymphocytes, is still occasionally challenged by complex signaling and phenotypic manifestations that cannot be fit into the T_H1-T_H2 paradigm (*6, 7*). Regardless, T_H2-driven conditions such as the acute relapse symptoms of atopic asthma and airway hyperresponsiveness are commonly treated with steroid drugs, such as the glucocorticoid receptor agonists dexamethasone and prednisone (*8*). Unfortunately, these drugs have global immunosuppressive properties toward both T_H1- and T_H2-driven clinical conditions (*9, 10*) and can also become ineffective during their prolonged use (*11*). As a result, the search for drugs capable of specifically preserving the T_H1-driven innate immune system response, while skewing naïve T_H differentiation away from T_H2 phenotypes, may hold promise in the treatment of atopic allergic diseases and similar T_H2-driven conditions.

In 1999, Kato and colleagues published the identification of a novel benzoimidazole-derived drug ethyl 3-hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate (M50367) (Figure 1), capable of significantly reducing disease scores of experimentally induced asthma and airway hyperresponsiveness in mice (*12*). This compound was shown to inhibit important signal transduction pathways, leading to airway hyperresponsiveness and asthma, by blocking IL-4 (e.g., important for IgE class switching) and IL-5 production (e.g., important for the homing of eosinophils at inflammation sites), respectively (*12*). M50367 was also capable of enhancing production of the T_H1 cytokine INF-γ in bronchoalveolar lavage fluid and cultured spleenocytes

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[1] Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B[*a*]P, benzo[*a*]pyrene; β-NF, β-naphthoflavone; α-NF, α-naphthoflavone; 3-MC, 3-methylcholanthrene; M50354, 3-[2-(2-phenylethyl)benzoimidazole-4-yl]-3-hydroxypropanoic acid; M50367, ethyl 3-hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate; XAP2, hepatitis B virus X-associated protein 2 also known as ARA9 and AIP; ARNT, AhR nuclear translocator; hsp90, 90 kDa heat shock protein; TSDS-PAGE, tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HOP, hsp90/hsp70 organizing protein; PBS, phosphate-buffered saline; CYP1A1, cytochrome P450 family I subfamily A isoform 1; T_H1 or T_H2, T lymphocyte helper type 1 and type 2, respectively; NF-κB, nuclear factor-κB; DRE, dioxin responsive element.

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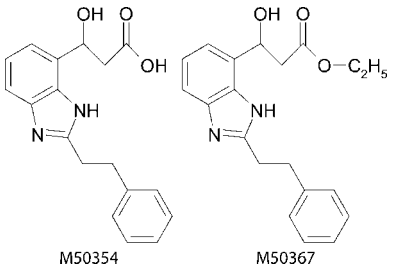


Figure 1. Structures of M50354 and M50367.

and had no effect on IL-2 expression (e.g., important for T_H1-driven responses). Consequently, and in contrast to prednisone, M50367 does not suppress the innate immune system but it is active in ameliorating both OVA and DNP ascaris-induced asthma and airway hyperresponsiveness in mice (*12*). Furthermore, no weight loss in mice or toxicity to cultured spleenocytes could be observed in vitro, which is in contrast to some of the side effects exerted by prednisone. Later studies suggested that, following an oral administration of M50367, the compound is rapidly metabolized to yield 3-[2-(2-phenylethyl)benzoimidazole-4-yl]-3-hydroxypropanoic acid (M50354), which was coined the "active metabolite" of M50367 (*13*). The target cells for M50354 and M50367 were subsequently suggested to be differentiating naïve T_H cells, since none of the drugs affected mature T_H1 or T_H2 cell functions (*13*). The mechanisms by which M50367 and M50354 elicited these immunomodulatory activities remained partially unknown until they were identified as ligands for the aryl hydrocarbon receptor (AhR), a receptor whose protein expression levels are also modulated during the differentiation program of naïve T_H cells (*14*).

The AhR is a basic helix–loop–helix and PAS domain transcription factor and an orphan receptor that mediates most of the toxic effects elicited by several halogenated polycyclic aromatic compounds (HPAHs) and nonhalogenated polycyclic aromatic compounds (PAHs) (*15*). In its unliganded state, the AhR is found in the cytoplasmic compartment primarily as a tetrameric complex composed of two molecules of 90 kDa heat shock protein (hsp90) and the immunophilin-like protein, hepatitis B virus X-associated protein 2 (XAP2) (*16–18*). After the association of a ligand with the AhR, the tetrameric complex translocates into the nucleus where the AhR heterodimerizes with AhR nuclear translocator (ARNT) and dissociates from the hsp90 dimer and XAP2. The transformed AhR-ARNT heterodimer then binds xenobiotic responsive elements with the consensus sequence 5′-TNGCGTGA-3′ and upregulates target gene expression (*19*). Activation of the AhR leads primarily to the upregulation of phase I, II, and III metabolism gene products, which are important for the clearance and/or activation of various endogenous and exogenous substances. However, chronic exposure to high-affinity AhR ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) may lead to toxic end points such as immune system suppression, hydronephrosis, and porphyric disorders (*20–23*). The mechanism by which the AhR is responsible for these conditions remains largely unknown, but it has been associated with the expression of some of its target genes such as cytochrome P450 family I subfamily A isoform 1 (CYP1A1) (*24*). Moreover, repression of the nuclear factor-κ B (NF-κB) and AP1 by the AhR signaling pathway has also been documented (*25–28*). Even though suspected to exist, no endogenous high-affinity ligand has been identified for the AhR that would make clear its role in normal physiologic homeostasis or a potential role in the immune system. Yet, the AhR can bind numerous synthetic and naturally occurring substances that serve as agonists or antagonists such as indole-3-carbinol (*29*), bilirubin (*30*), curcumin (*31*), quercetin (*32*), resveratrol (*33*), galangin (*34*), the synthetic compound α-naphthoflavone (α-NF) (*35*), as well as suspected arachidonic acid metabolites (*36*).

One of the key observations linking the AhR with normal immune system function came from the generation of AhR knockout mice ($AhR^{-/-}$). The spleen and lymph nodes of $AhR^{-/-}$ mice display a reduced presence, or perhaps recruitment, of T lymphocytes during the first 10 weeks after birth (*37*). Consequently, some of these mice succumb early to opportunistic infections (*37*). Others have challenged this evidence as being limited to the particular strain of mouse utilized since other $AhR^{-/-}$ mice display normal antigen-mediated immune responses (*38*). However, a recent study on $AhR^{-/-}$ mice revealed that these mice exhibit abnormally elevated expression of both T_H1 and T_H2 cytokines in OVA-sensitized AhR-null mice (*14*). Therefore, although the mechanisms have not been fully established, evidence suggests that the AhR could still play a balancing role in the immune system.

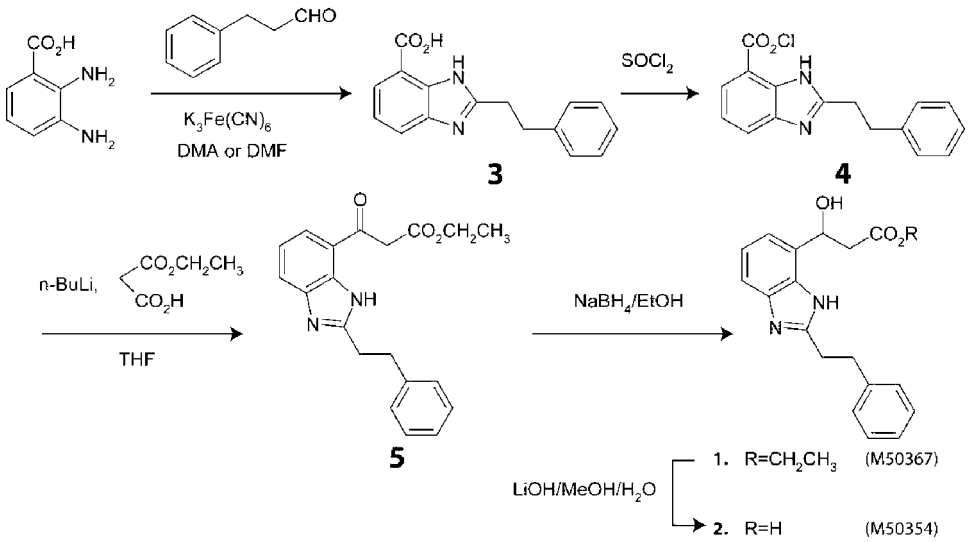
When M50367 and M50354 were identified as AhR ligands (*14*), it was stated that these drugs could only partially induce CYP1A1 activity when compared to other established AhR ligands. In addition, these compounds appeared to exhibit unique AhR-dependent gene expression profiles. These results were rather surprising and captured our interest due to a lack of planarity in these molecules, a characteristic that is typical of most high-affinity AhR ligands (e.g., TCDD). Analogous to the effects seen for selective modulators of the steroid receptors and their role in the modulation of immunological responses (discussed in ref *39*), the M50367 and M50354 compounds were suggested to be selective AhR modulators, driving receptor-dependent immunological changes without significant induction of CYP1A1 or other dioxin responsive element (DRE)-driven genes (*14*). In this study, the synthesis and further characterization of M50367 and M50354 within the AhR signal transduction pathway are reported. In contrast to published reports, we demonstrate that both M50367 and M50354 behave as transient but full AhR agonists, at previously indicated therapeutically relevant doses (*12, 13*). The transient but significant nature of M50354 and M50367 AhR-driven gene expression may have accounted for the underestimation of this response, due to the methods and time frame chosen for analyses by previous authors (*14*). The implications of these findings, on the potential use of these drugs in the treatment of atopic allergic diseases, are further discussed.

Materials and Methods

Chemistry. Synthesis of M50354 and M50367. All solvents and reagents were used as purchased, without further purification, unless noted otherwise. Anhydrous tetrahydrofuran (THF) was prepared by distillation from sodium and benzophenone. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are expressed in ppm with respect to TMS (tetramethylsilane) as the internal standard. Column chromatography was performed on a silica gel 60 Å 70–230 mesh or flash on 230–320 mesh. For thin-layer chromatography, aluminum plates precoated with silica gel 60 F_{254} (0.2 mm) were used.

2-(3-Phenylethyl)benzimidazole-4-carboxylic Acid. To a solution of 2,3-diaminobenzoic acid (7.8 g, 51.3 mmol) in 120 mL of dimethylacetamide (11 g, 82.08 mmol, 1.6 equiv), 3-phenylpropionaldehyde was added slowly with stirring followed by potassium ferricyanide (33 g, 0.1 mol, 2 equiv). The mixture was heated and stirred at 60 °C under N_2 for 12 h. The deep blue solid was filtered out and washed with ethyl acetate, washings were combined with

Scheme 1. Synthesis of M50354 and M50367



filtrate, and the solvents were removed in vacuo. The residue was purified by silica gel flash column chromatography using $CHCl_3$ with an increasing amount of EtOH (2–20%) to give 11.5 g (40%) of acid intermediate **3** (see Scheme 1) as an off-white solid; mp 220 °C (sample was recrystallized from $CHCl_3$:MeOH). ^{1}H NMR (acetone-d_6): δ 3.21–3.25 (m, 2H), 3.32–3.36 (m, 2H), 7.15–7.19 (m, 1H), 7.27–7.35 (m, 5H), 7.86 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 8.0 Hz, 1H).

Ethyl 3-Oxo-3-[2-(*β*-phenylethyl)-1*H*-benzimidazole-4-yl]propionate. A mixture of acid **3** (4 g, 15.8 mmol) and thionyl chloride (80 mL) was refluxed for 3 h. Benzene (250 mL) was then added, and the solid material was filtered off. It was washed twice with both benzene and Et_2O and dried in vacuo to yield 4.1 g of acid chloride **4** as a white powder. To a solution of monoethyl malonate (2.6 g, 20 mmol, 2.5 equiv) in anhydrous THF (45 mL) under N_2 was added a few milligrams of 2,2′-bipyridyl as an indicator. The solution was cooled to −70 °C, and *n*-BuLi (2.5 M in hexanes, 16 mL, 40 mmol) was added dropwise allowing the temperature to rise to −5 °C at the end of the addition. The mixture was maintained at this temperature for 10 min observing the persistence of pink indicator color. It was then recooled to −65 °C, and the acid chloride **4** suspension (2 g, 8.0 mmol) in THF (20 mL) was added dropwise over 5 min. After 1.5 h at −65 °C, the mixture was diluted with Et_2O (90 mL) and acidified to pH 2–3 with ∼60 mL of 1 N HCl, and the phases were separated. The water phase was alkalized to pH 8 with 10 N NaOH and extracted either, and the combined extracts were dried over $MgSO_4$ and evaporated. The crude product was purified by flash column chromatography (gradient: hexanes/ethyl acetate from 5 to 50% v/v) to give 1.4 g of pure β-ketoester **5** as a white solid. The workup of organic (Et_2O) phase yielded an additional 0.4 g of **5**; combined yield, 1.8 g (72%); mp 100–101 °C. ^{1}H NMR ($CDCl_3$): δ 1.27 (t, J = 7.0 Hz, 3H), 3.22–3.25 (m, 2H), 3.28–3.32 (m, 2H), 4.11 (s, 2H), 4.26 (q, J = 7.0 Hz, 2H), 7.24–7.28 (m, 3H), 7.32–7.36 (m, 3H), 7.74 (d, J = 7.5 Hz, 1H), 8.01 (d, J = 8.0 Hz, 1H).

Ethyl 3-Hydroxy-3-[2-(*β*-phenylethyl)-1*H*-benzimidazole-4-yl]propionate. To a solution of ketoester **5** (0.95 g, 2.83 mmol) in 20 mL of 95% EtOH was added dropwise a solution of sodium borohydride (44 mg, 1.16 mmol) in EtOH (5 mL) at room temperature. The progress of the reaction was monitored by TLC (hexanes/ethyl acetate 1:1). After 1.5 h, the next portion of $NaBH_4$ (10 mg, 0.264) in EtOH (1.5 mL) was added and a half of this amount was added again after 1.5 h. The reaction was continued for another 1 h, after which time no starting material was detected (TLC). The solution was poured into 70 mL of water, acidified to pH 5–5.5 with 25% AcOH, extracted with ethyl acetate, and dried ($MgSO_4$). The extract was stripped of solvent to leave a glassy solid, which, after flash chromatography (hexanes/ethyl acetate, gradient from 10 to 30% of ethyl acetate), gave 0.82 (86%) of pure **1** as a white solid; mp 123–124 °C. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, J = 7.0 Hz, 3H), 2.87 (d, J = 6.5 Hz, 2H), 3.17–3.21 (m, 2H), 3.23–3.27 (m, 2H), 4.24 (q, J = 7.0 Hz, 2H), 5.50 (t, J = 6.5 Hz, 1H), 7.02 (d, J = 7.5 Hz, 1H), 7.19 (dd, J = 8.0 and 7.5 Hz, 1H), 7.24–7.34 (m, 6H), 7.56 (br d, J = 7.0 Hz, 1H).

The enantiomers of **1** were separated by HPLC using Pirkle Covalent (S,S) Whelk-O1 column 250 mm × 4.6 mm #786101 (Regis Technologies Inc.). Hexanes/ethanol (92:8) + 8 mM ammonium acetate were used as a mobile phase in isocratic mode (flow rate, 1.5 mL/min.). Baseline separation was achieved, and fractions corresponding to the peaks at 22 and 24 min, respectively, were collected. Each of them contained single enantiomers as yet chiroptically unassigned.

3-Hydroxy-3-[2-(*β*-phenylethyl)-1*H*-benzimidazole-4-yl]propionic Acid. To a solution of **1** (0.2 g, 0.591 mmol) in MeOH (3 mL) was added 0.2 N LiOH in 3:1 MeOH/H_2O (6 mL). The reaction progress was monitored by TLC (4:1 ethyl acetate/MeOH). After 40 min of stirring at room temperature, 50 mL of water was added and the pH was brought to 5–5.5 with 25% AcOH. The mixture was extracted with ethyl acetate, the extract was dried ($MgSO_4$), and the solvent was removed to give 0.12 g (66%) of **2** as a white solid; mp 110–112 °C. ^{1}H NMR (acetone-d_6): δ 2.78 (dd, J = 15.6 and 9.2 Hz, 1H, $-CH_2-$), 2.94 (dd, J = 15.6 and 3.3 Hz, 1H, $-CH_2-$), 3.17–3.21 (m, 2H), 3.23–3.26 (m, 2H), 5.58 (dd, J = 9.0 and 3,5 Hz), 7.11–7.21 (m, 3H), 7.25–7.30 (m, 4H), 7.39 (d, J = 8.0 Hz, 1H).

Biology. Antibodies. The primary antibodies utilized included mouse anti-AhR monoclonal IgG_1 (RPT1) (Affinity BioReagents), rabbit anti-XAP2 IgG (*40*), rabbit anti-CYP1A1 IgG (H-70) (Santa Cruz), and mouse anti-HOP (hsp90/hsp70 organizing protein) IgG (F5) (David Toft, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN), which were used to detect the AhR, XAP2, CYP1A1, and HOP proteins, respectively.

Cell Culture. Cells were maintained in modified Eagle's α-minimum essential medium (Sigma, St. Louis, MO), supplemented with 1000 units/mL penicillin, 100 μg/mL streptomycin (Sigma), and 7% fetal bovine serum (HyClone, Logan, UT) and kept in a humidified incubator at 37 °C and 5% CO_2. The growth medium was replaced every 2 days.

Cell Culture Treatments To Determine AhR Transcriptional Activity. A thousand-fold working stocks of TCDD (10 μM, 0.5 μM, etc.), benzo[*a*]pyrene (B[*a*]P) (10 mM), M50354 (10 mM), and M50367 (10 mM) were prepared in DMSO (Sigma). Unless otherwise noted in figure legends, cells were dosed by adding each compound directly into the cell culture plates, mixed, and incubated for the stated period in the figures and legends. The amount of DMSO never exceeded 0.1%. Notice that TCDD and B[*a*]P are very toxic substances (*41*). In addition, toxicological information for M50354 and M50367 has yet to be established. Therefore, these

compounds, including the solvents and reactants utilized to synthesize them, should be treated as potentially harmful and must be disposed according to toxicological waste disposal guidelines established by your institution.

Western Blots. Proteins were resolved by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (TSDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA) using standard procedures. Membranes were preincubated with blocking buffer [10 mM monobasic sodium phosphate mono hydrate, 150 mM sodium chloride, 0.5% (v/v) Tween 20 (Sigma), and 3% (m/v) bovine serum albumin fraction V (EMD Chemicals Inc., San Diego, CA)] for 1 h. Primary antibodies were diluted in wash buffer [10 mM monobasic sodium phosphate monohydrate, 150 mM sodium chloride, 0.5% (v/v) Tween 20 (Sigma), and 0.1% (m/v) bovine serum albumin fraction V (EMD Chemicals Inc.)] and incubated for an additional 1 h at room temperature. Blots were washed three times in a 30 min period. A 1 h incubation with biotin-conjugated secondary antibodies was followed by a 15 min incubation with [^{125}I]streptavidin. Blots were washed, and proteins were visualized by autoradiography. The relative protein levels were determined by phosphor image analysis.

Real-Time PCR Analysis. Total mRNA was isolated using Sigma's TRI Reagent mRNA isolation reagent (Sigma) and amplified using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA). The levels of CYP1A1, CYP1B1, and UGT1A2 mRNA were assessed by real-time qPCR using the MyIQ single-color PCR detection system (BioRad, Hercules, CA) and the iQ SYBR Green supermix (BioRad).

Luciferase Reporter Gene Experiment. The previously established DRE-driven reporter cell lines HG40/6 (*42*) and H1L1.1c2 (*43*), derived from the Hep-G2 and Hepa-1c1c7 cell lines, respectively, were utilized to monitor AhR transcriptional activity. Two days before treatments with ligands, cells were fed with complete medium containing no antibiotics. Ligands were added at the specified concentrations for the given period as stated in the figure legends. After treatments, the luciferase activity was measured using Promega's (Madison, WI) Luciferase Assay System following manufacturer's instructions.

Determination of the CYP1A1 and CYP1B1 Activity Directly in Cells. The combined CYP1A1 and CYP1B1 activity in Hepa-1c1c7 cells was measured using Promega's P450-Glo microsomal assay with luciferin-CEE, a substrate primarily metabolized by CYP1A1, CYP1B1, and CYP3A7 and, to a much lesser extent, by CYP1A2 (<7% CYP1A1) and even lower by other cytochrome P450s. Briefly, cells grown on 12 well plates were dosed with the appropriate CYP1A1-inducing agent (e.g., TCDD) for 6 and/or 24 h. The growth medium was then discarded, and 500 μL of fresh serum-free DMEM containing 8 μL of luciferin-CEE was added to each well and incubated for an additional 3 h. The metabolism of luciferin-CEE was then determined according to the manufacturer's instructions using a Turner TD-20e luminometer (Turner BioSystems, Sunnyvale, CA).

Competition Binding Experiments. Hepa-1c1c7 cytosolic extracts were prepared in MENGM buffer [16.2 mM 3-(*N*-morpholino)propanesulfonic acid sodium salt, 10 mM free acid 3-(*N*-morpholino)propanesulfonic acid, 0.02% (m/v) sodium azide, 10% (m/v) glycerol, 4 mM EDTA, and 20 mM molybdate]. Briefly, a 150 μL of cytosol at 2 mg/mL was transferred to 12 mm × 75 mm borosilicate glass tubes. Samples were preincubated with cold competitor for 10 min at room temperature prior to the addition of radioligand. A total of 0.13 pmol of 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin (photoaffinity ligand) was added per sample and incubated at room temperature for 30 min in the presence or absence of competitor. The samples were finally exposed to 15 W UV lamps (>302 nm) at 8 cm for 4 min. Sixty microliters of each supernatant (120 μg of total protein) were then combined with 5× Laemli buffer containing β-mercaptoethanol, heated at 95 °C for 5 min, and analyzed by TSDS-PAGE. Resolved proteins were then transferred to PVDF membranes, and autoradiographs were generated. The protein bands were quantified via phosphor image analysis.

Results

Chemistry. Synthesis of M50367 and M50354. During the course of this study, the antiallergic agent M50367 [ethyl 3-hydroxy-3-[2-(β-phenylethyl)-1*H*-benzimidazole-4-yl]propionate (**1**)] and its active metabolite M50354 [3-hydroxy-3-[2-(β-phenylethyl)-1*H*-benzimidazole-4-yl]propionate (**2**)] (Figure 1) were synthesized. The synthetic strategy is outlined in Scheme 1. In the initial step, suitably substituted 2,4-benzimidazole derivative was synthesized, adopting a published method with modification (*44*). Oxidative cyclization of 2,3-diaminobenzoic acid and 3-phenylpropionaldehyde utilizing potassium ferricyanide as an oxidant gave 2-(3-phenylethyl)benzimidazole-4-carboxylic acid (**3**) with moderate yield. Acid chloride **4** prepared by standard procedure using thionyl chloride was without further purification converted to β-ketoester **5** (*45*) by condensation with monethyl malonate. Finally, reduction of **5** with $NaBH_4$ in ethanol furnished the desired ester **1**, which upon hydrolysis (*46*) gave metabolite **2**. In an attempt to obtain pure enantiomers, **1** was derivatized with auxiliary chiral reagent (*N*,*N*-dimethyl-L-phenylalanine), thus creating diastereomeric mixture of amino esters. The diastereomers were separated on flash silica column and characterized by ^{1}H NMR spectroscopy. However, efforts to restore original secondary hydroxyl functionality from amino ester protection were unsuccessful. Secondary reactions might have occurred leading to other products, the nature of which was not investigated. Required separation and purification of unmodified enantiomers of **1** were finally achieved by enantioselective HPLC on Whelk-O1 chiral stationary phase column.

Biology. M50367 and M50354 Exhibit Significant AhR Agonistic Activity. The recently published work of Negishi and colleagues suggested that two potentially useful antiasthmatic drugs, namely, M50354 and M50367 (Figure 1), had immunomodulatory activities that were dependent on the functional expression of the AhR (*14*). These substances were identified as AhR ligands in competition ligand-binding experiments, although their ability to induce classic AhR-dependent CYP1A1 gene expression was considerably lower when compared to relatively potent AhR ligands [e.g., β-naphthoflavone (β-NF) and 3-methylcholanthrene (3-MC)]. To further assess the ability of M50354 and M50367 to activate the AhR, the Hepa-1c1c7-derived mouse cell line H1L1.1c2 that stably expresses a DRE-driven luciferase reporter gene was utilized to determine the extent of AhR activation by M50354 and M50367 vs the prototypical high-affinity AhR ligand TCDD. In contrast to previous observations (*14*), this assay revealed that both M50354 and M50367 could induce significant activity (Figure 2A). In addition, consistent with the higher in vivo effects of M50367 (*12, 13*), the M50367 compound was more active than M50354 in this gene reporter test.

However, because reporter gene data alone may not completely recapitulate the induction of an endogenous AhR target gene, Promega's P450-Glo Luciferin-CEE assay was utilized. Although this assay detects the combined activity of CYP1A1, CYP1B1, and CYP3A7, the *CYP3A7* gene is not a direct target of the activated AhR. Therefore, in our assay, the ligand-treated samples predominantly represent the combined activity of CYP1A1 and CYP1B1. Accordingly, both M50367 and M50354 were capable of inducing a significant level of CYP1A1 and CYP1B1 catalytic activity, especially when compared to a saturating dose of TCDD (Figure 2B). Consistent with the reporter data, M50367 could induce microsomal enzymatic activity to a higher degree than M50354.

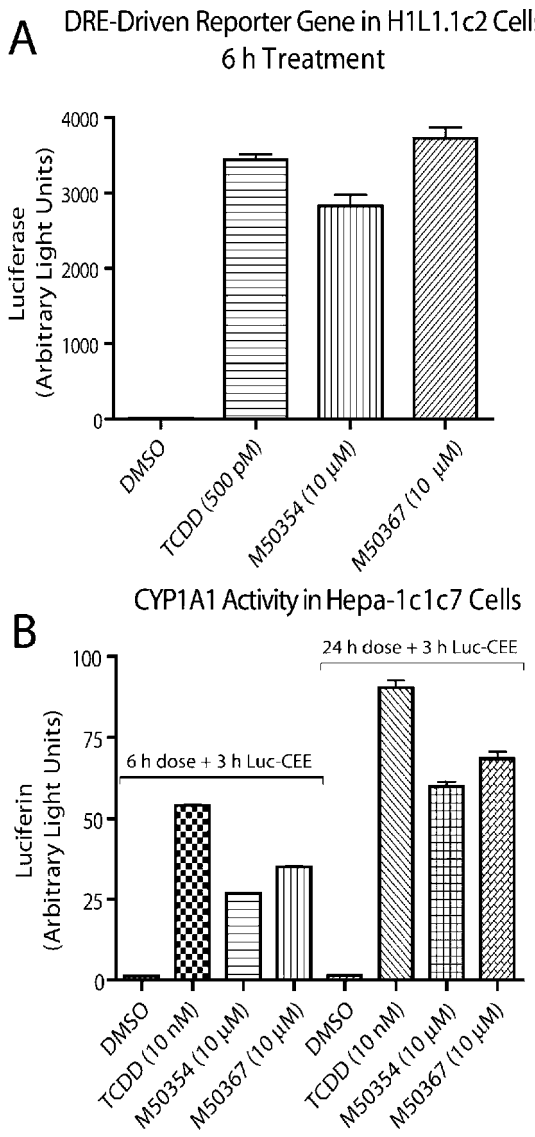


Figure 2. M50354- and M50367-mediated AhR transcriptional activity. (A) H1L1.1c2 cells were treated with vehicle (DMSO), TCDD (500 pM), M50354 (10 μM), or M50367 (10 μM) for 6 h. The vehicle concentration never exceeded 0.1% in all treatments. Cells were harvested, and the luciferase enzymatic activity was measured. (B) Hepa-1c1c7 cells were treated with TCDD (10 nM), M50354 (10 μM), or M50367 (10 μM) for 6 or 24 h. The CYP1A1 enzymatic activity was measured using Promega's P450-Glo Assay with Luciferin-CEE as a CYP1A1 and CYP1B1 substrate, according to the manufacturer's instructions. For both A and B, all samples were run in triplicate, and the results are representative of three independent experiments.

Characterization of M50367 Enantiomers. The approach utilized for the synthesis of both M50354 and M50367 compounds results in the formation of a racemic mixture of stereoisomers. M50367 and M50354 enantiomers could be readily detected via the appropriate HPLC analysis migrating as two equimolar peaks. For simplicity, we will refer to each enantiomer of M50367 as the 22 min peak 1 (P1) and 24 min peak 2 (P2). A method was designed for the separation of M50367 enantiomers, given that it was important to determine whether there was a stereospecific requirement for each M50367 enantiomer to function as an AhR agonist or even possibly as an antagonist. Because M50367 displayed higher activity than M50354 in our luciferin-CEE-based CYP1A1 assays and the indication that M50367 has a higher therapeutic effect in vivo (e.g., mice) (*14*), we pursued the characterization of both M50367 enantiomers individually. Consistent with the results obtained utilizing the racemic mixture of M50367 (Figure 2), each M50367 enantiomer at 10 μM could individually induce similar reporter activity in H1L1.1c2 cells in a time−course reporter experiment (Figure 3A). However, the extent of induction was less than a saturating dose of TCDD. Regardless, as predicted from the H1L1.1c2 reporter cell line results (Figure 2) and Negishi's report (*14*), the induction of reporter activity approached near basal expression by 24 h, while TCDD-induced reporter activity remained relatively high (Figure 3A). Both enantiomers were also tested in the human cell line HG40/6 that stably expresses a DRE-driven luciferase reporter gene. Interestingly, in the HG40/6 reporter line, there was a modest increase of reporter activity displayed by the P2 enantiomer (Figure 3B). This higher reporter activity seen for P2 in HG40/6 cells was also consistent with higher CYP1A1 mRNA levels in the same cell line (Figure 3C). Given the absence of this effect in the H1L1.1c2 cell line, it was important to test whether such differences could be recapitulated on an endogenous AhR target gene and in other cells. Hence, CYP1A1 mRNA expression was also analyzed via real-time PCR in the wild-type murine Hepa-1c1c7 and the human keratinocyte-derived cell line HaCaT. Just as in the reporter cell line H1L1.1c2 (Figure 3A), these experiments revealed no differences in CYP1A1 mRNA induction by the M50367 enantiomers (Figure 3D,E). Furthermore, while neither M50354 nor M50367 could activate the reporter gene comparably to a saturating dose of TCDD in HG40/6 and H1L1.1c2 cells (Figure 3A,B), both M50367 enantiomers could readily induce CYP1A1 mRNA expression to levels comparable to a saturating dose of TCDD in Hepa-1 and HaCaT cells at 4 h post-treatment (Figure 3D,E). Notably, when either of the M50367 enantiomers was administered at 1 μM, they approached the activity of a saturating dose of TCDD in Hepa-1c1c7 cells and to a lesser degree in HaCaT cells. At 10 μM, both P1 and P2 induced CYP1A1 mRNA levels to the same level as a saturating dose of TCDD at 4 h.

Having established that both M50367 enantiomers could readily activate CYP1A1 gene expression, we examined their ability to modulate expression of some known AhR target genes such as CYP1B1, UGT1A2, and the recently identified AhR regulated gene epiregulin[2] (*47*) in Hepa-1c1c7 and/or HaCaT cells. Consistent with M50367's AhR full agonistic activity on CYP1A1 expression, at 10 μM, both enantiomers could readily activate other AhR target genes to levels comparable of a saturating dose of TCDD (Figure 4) in a 4 h dose. Interestingly, the concentration of M50367 enantiomers required to elicit maximal induction of these genes varied between cell lines and in a gene-specific manner.

M50367 and M50354 Can Compete with 2-Azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin for Binding to the AhR in a Dose-Dependent Manner. The ability of M50354 and M50367 compounds to displace [^{3}H]M50354 were initially provided in Negishi's report (*14*), although the experiment was limited to one saturating concentration and provided no information on the relative affinity of M50354 and M50367 for the AhR. Competition-binding experiments were carried with the AhR photoaffinity radioligand 2-azido-3-[^{125}I]-iodo-7,8-dibromodibenzo-*p*-dioxin to determine the relative affinity of the M50354 and M50367 compounds for the AhR and to clearly demonstrate that these compounds are direct AhR ligands. Therefore, cytosolic extracts from Hepa-1c1c7 cells were utilized for competition ligand-binding experiments with the photoaffinity ligand. These results indicated that the M50354 was

[2] Unpublished authors' observations.

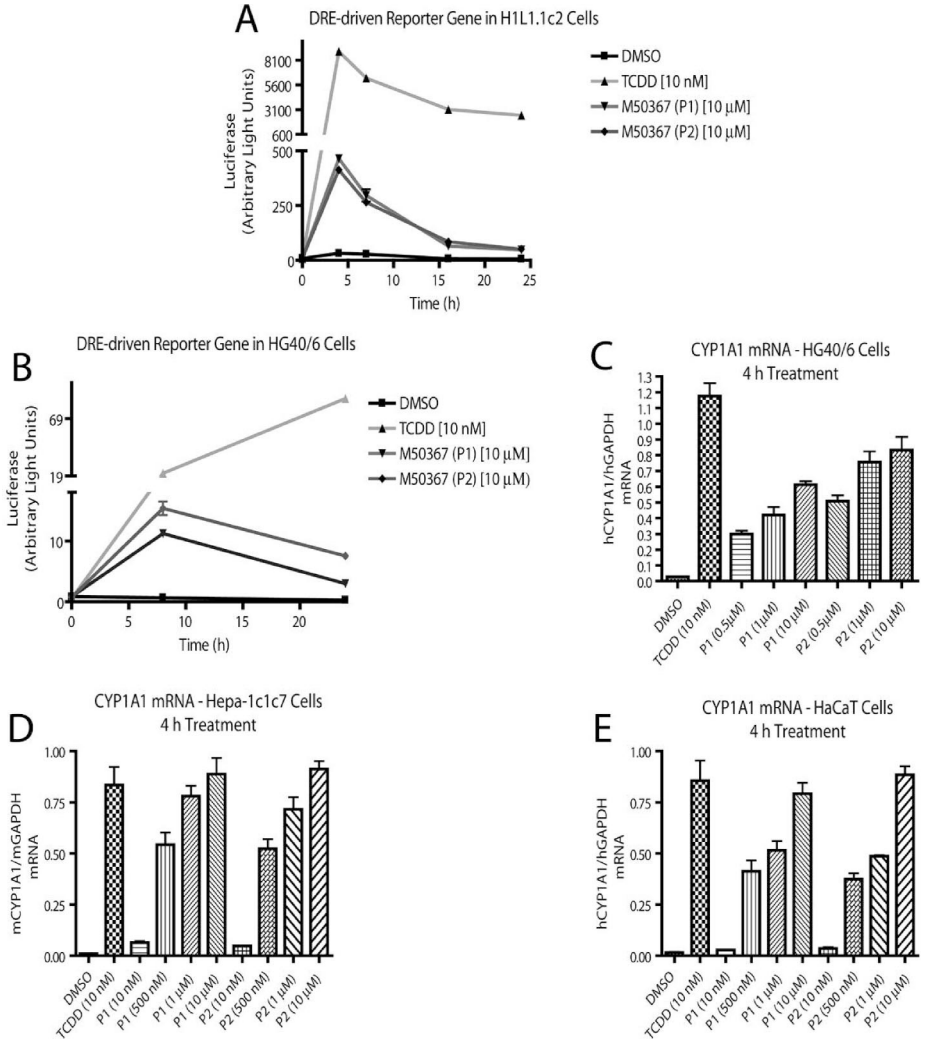


Figure 3. Characterization of M50367 enantiomers. (A) DRE-driven luciferase reporter gene experiments were performed as in Figure 2A, except that cells were treated at the concentrations stated in the figure and samples were analyzed at the 0, 4, 8, 16, and 24 h time points. The culture medium was replaced with fresh medium containing each compound at the stated concentrations. (B) Performed as in panel A, except samples were collected at 0, 8, and 24 h. (C−E) Following the treatment cells with each compound at the indicated concentrations and a 4 h incubation period, mRNA was isolated using the Trireagent (Sigma) RNA isolation method and analyzed by RT-PCR analysis. The quantities of mRNA were normalized to GAPDH mRNA values. All experimental samples were run in triplicate, and the results are representative of three isolated experiments.

capable of displacing the radioligand to a higher degree than M50367 at 46 vs 24.1% at 10 μM and 90.4 vs 74.5% at 100 μM, respectively (Figure 5). As expected, β-NF and α-NF could readily displace the radioligand. As a negative control, an equal dose with the estrogen receptor ligand 17β-estradiol was unable to significantly displace the radioligand at 100 μM. Finally, M50367 and M50354 could compete specifically with the radioligand and thus are bonafide AhR ligands.

M50354 and M50367-Promoted AhR Protein Turnover and Their Effect on the Temporal Expression of CYP1A1 Protein. A key aspect of AhR activation by high affinity ligands is the rapid loss of AhR protein to proteasome-mediated degradation (*48, 49*). To look for potential differences between these two drugs vs known AhR ligands, their ability to induce AhR protein degradation was examined. Comparably to TCDD and B[*a*]P, M50367 was capable of causing a high degree of AhR protein turnover after a 6 h incubation period in Hepa-1c1c7 cells (Figure 6). The M50354 compound was also capable of inducing AhR degradation, although to a lesser extent than M50367. In summary, the consistent ranking order for each agent in causing AhR protein degradation within a 6 h period was TCDD > M50367 > B[*a*]P > M50354. However, the level of CYP1A1 protein expression was comparable in the TCDD, M50367, and B[*a*]P experimental samples at 6 h, with lower but significant levels induced by M50354.

Having established that M50367 and M50354 can cause a high degree of AhR protein turnover, we wanted to determine whether CYP1A1 protein upregulation was transient in nature. Therefore, Hepa-1c1c7 cells were pulsed with each compound for a total of 6 and 36 h. However, the 6 h treatment group (group A), just like the 36 h (group B), was also harvested at the 36 h time point and protein levels were analyzed. As expected, the level of CYP1A1 expression seen with TCDD at 36 h for group A was comparable to group B, perhaps accounting for its nonmetabolizable nature and high affinity for the AhR. For group A, measurable differences in CYP1A1 protein expression could only be observed for TCDD, B[*a*]P, and M50367. Thus, even after the M50354-dependent induction of CYP1A1 seen in our 6 h experiment (Figure 6), the levels of CYP1A1 expression had returned to basal levels by 36 h in group A (Figure 7). Surprisingly, the level of CYP1A1 expression remained relatively high for M50367 for group B and unexpectedly even above B[*a*]P. Finally, an approximately 2-fold CYP1A1 protein expression was seen for M50354 in group B.

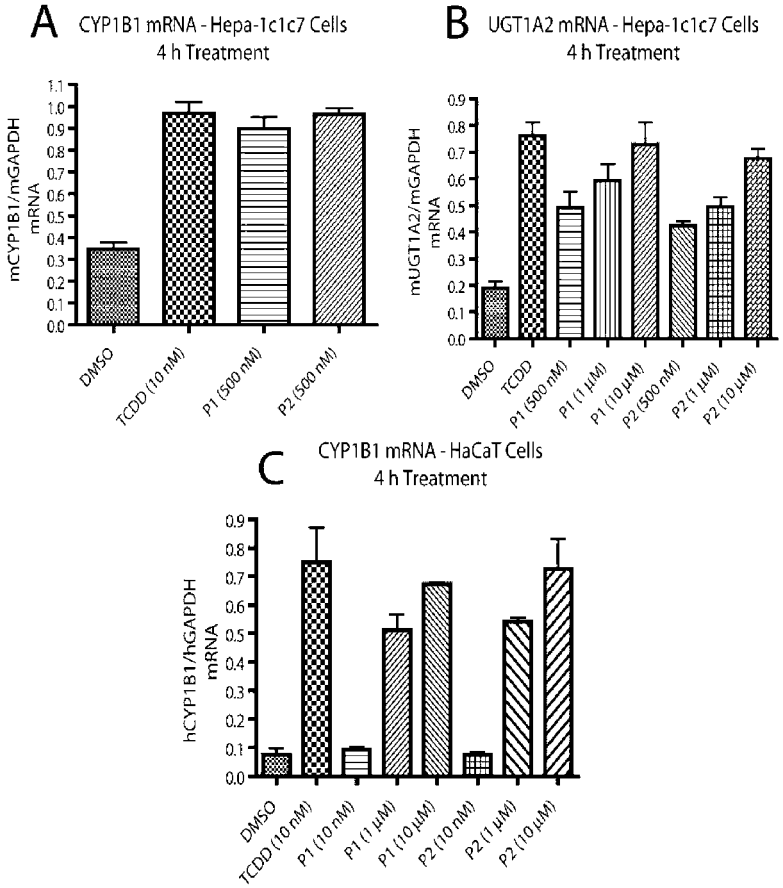


Figure 4. M50367 activates other AhR-regulated genes. For panels A−C, the mRNA levels were determined as in Figure 3C−E. All experimental samples were run in triplicate, and results are representative of two isolated experiments.

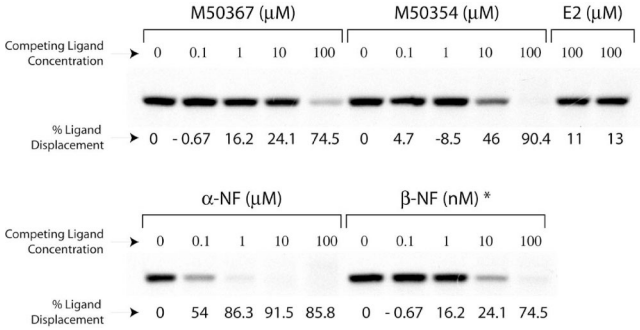


Figure 5. M50354 and M50367 are direct ligands for the AhR. Hepa-1c1c7 cytosol was preincubated with each competing ligand at the indicated concentrations for 10 min at 25 °C followed by a 30 min incubation with the photoaffinity radioligand also at 25 °C. Samples were then exposed to 15 W UV lamps (>302 nm) at a distance of 8 cm for 4 min. Sixty microliters of each supernatant (120 μg of protein) were then combined with 5× SDS sample buffer supplemented with β-mercaptoethanol and heated at 95 °C for 5 min. Proteins were resolved by TSDS-PAGE and transferred to PVDF membranes, and autoradiographs were generated. The protein bands were quantified via phosphor image analysis and visualized by autoradiography. *Notice the 1000-fold lower concentration for β-NF.

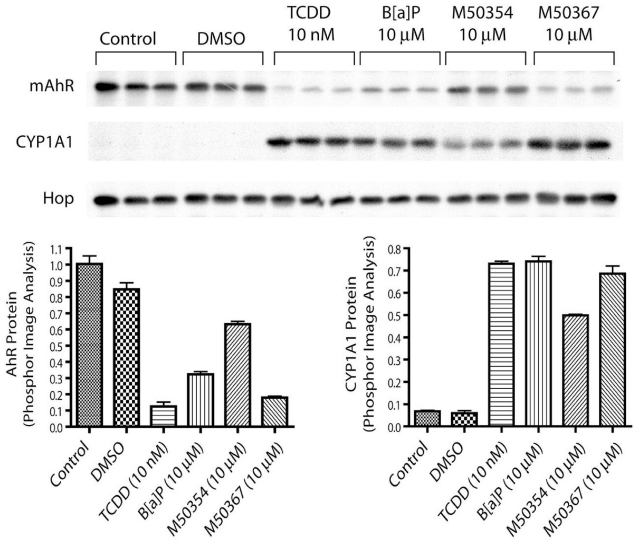


Figure 6. M50354 and M50367 induce AhR protein turnover. Following a 6 h treatment with TCDD (10 nM), B[*a*]P (10 μM), M50354 (10 μM), or M50367 (10 μM), whole cell extracts were prepared in RIPA buffer, and proteins were resolved by TSDS-PAGE and blotted into PVDF membranes. Primary antibodies mouse monoclonal IgG_1 RPT1 (Affinity BioReagents), rabbit IgG H-70 (Santa Cruz), and mouse IgG F5 (kind gift from David Toft) were used to identify the AhR, CYP1A1, and HOP proteins, respectively. To quantitatively detect proteins, these were labeled with biotin-conjugated secondary antibodies and [^{125}I]streptavidin. The relative protein levels were determined by phosphor image analysis and normalized to HOP protein levels, which were not affected by treatments. Proteins were visualized by autoradiography.

Discussion

The antiasthmatic properties of the drug M50367 and its active metabolite M50354 were revealed in a study designed to identify novel substances capable of reducing allergic disease scores in a mouse model of atopic asthma and airway hyperresponsiveness (*12, 13*). It was established that these compounds were capable of promoting a shift in T-helper cell balance toward a T_H1 phenotype, while promoting a reduction in IgE production and eosinophil infiltration at sites of inflammation.

The AhR became a prime suspect in this M50354/M50367-elicited T_H1 shift, given the ability of both compounds to induce

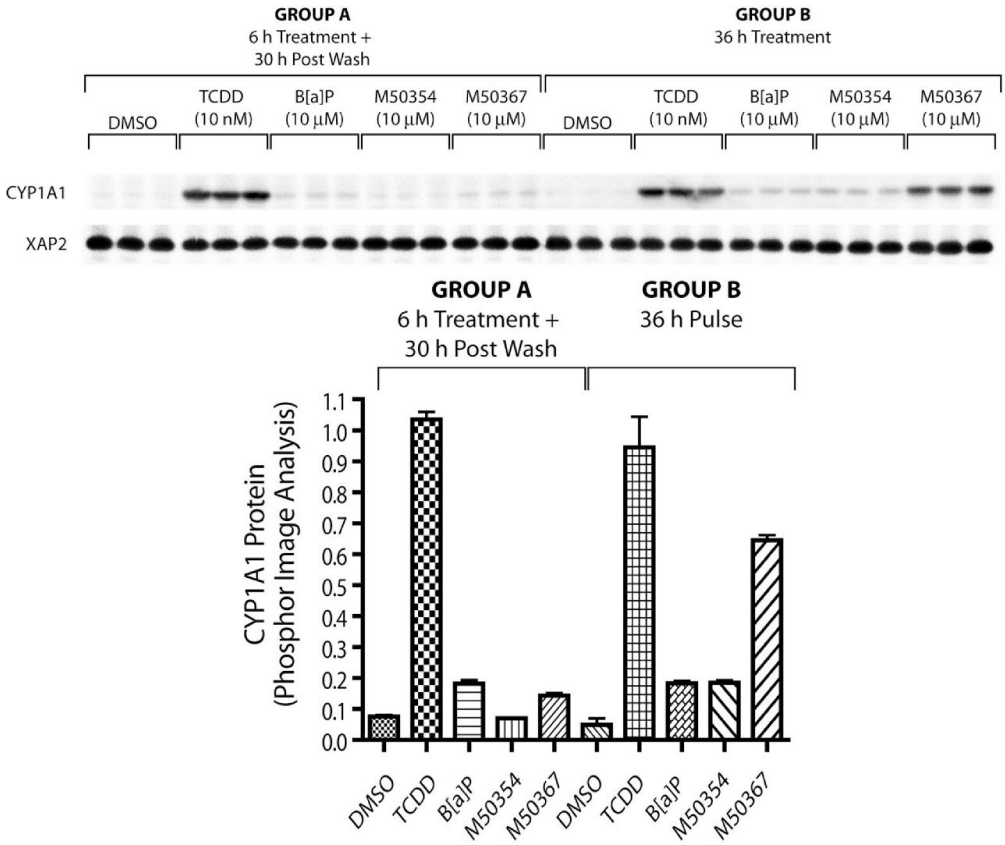


Figure 7. CYP1A1-induced activity after a short and long exposure to M50354 and M50367. Hepa-1c1c7 cells were pulsed with TCDD (10 nM), B[*a*]P (10 μM), M50354 (10 μM), or M50367 (10 μM) for a total of 6 or 36 h. The 6 h samples (group A) were washed with PBS three times, and fresh medium was administered and continued to incubate for the remaining 30 h period. The quantification of CYP1A1 and XAP2 protein expression was determined after 36 h, following the same protocol as in Figure 6, except that CYP1A1 values were normalized to XAP2 protein levels, which were also not affected by the treatments.

CYP1A1, a gene directly regulated by the AhR (*50*), and the absence of a therapeutic effect in AhR-null mice (*14*). Paradoxically, these drugs were coined as mild CYP1A1 inducers (*14*). However, as shown in Figure 2, these two substances were potent inducers of CYP1A1 activity in Hepa-1c1c7 cells, well beyond what was appreciable through reporter assays. Nevertheless, these discrepancies are reconciled from our DRE-driven reporter gene model results (Figure 3A,B), in which a time−course experiment in HG40/6 and H1L1.1c2 cells with M50367 enantiomers revealed that reporter gene expression had returned to near basal levels by 24 h, while induction by TCDD remained highly elevated (Figure 3A,B). If this time point in a reporter gene experimental approach was considered alone, as in a previous report (*14*), the ability of M50367/M50354 to activate the AhR would be clearly underestimated. Furthermore, our survey of other AhR target genes such as CYP1B1, UGT1A2 (Figure 4), and epiregulin,[2] further illustrated that all were readily activated by either of the M50367 enantiomers. These results are all consistent with the notion of M50367 as a full AhR agonist at the dose and time frames examined, while in comparison, M50354 exhibited similar activity (Figure 2B). Given that M50354 was coined the active metabolite (*13*), these minor differences are likely the result of M50367 having higher bioavailability in cells as stated previously (*12, 13*). An interesting aspect of these results is the lower reporter gene expression (Figure 4) and higher CYP1A1 activity (Figure 2B) observed by 24 h. However, these discrepancies could be related to differences in the half-life of the firefly luciferase and CYP1A1 proteins. As a result, induced metabolic enzymes (e.g., CYP1A1) would remain in the cell well beyond the initial rise and fall of AhR transcriptional activity as seen in reporter experiments, which is also consistent with the combined results shown in Figures 6 and 7.

Interestingly, while no difference could be established between the M50367 enantiomers in their ability to induce CYP1A1 and CYP1B1 mRNA expression in Hepa-1c1c7 and HaCaT cells (Figures 3 and 4), the evaluation of the Hep-G2-derived HG40/6 reporter cell line revealed that P2 was modestly more active than P1 in both reporter and real-time PCR experiments (Figure 3B,C). While the biological significance or importance of this result remains to be further established, it indicates that variations in the cell-specific magnitude of responses are theoretically possible for each enantiomer. This is consistent with the absence of this effect in HaCaT cells, which are also of human origin (Figure 3E). The possibility that the P1 enantiomer could be metabolized more readily than the P2 in a cell-specific manner is a feasible scenario. Future studies may further assess whether such differences can be observed in a tissue-specific manner in vivo.

Competition-binding experiments established that M50354 could displace the photoaffinity ligand more readily than M50367 (Figure 5). This result is consistent with previous work suggesting that M50354 was the active metabolite of M50367 (*14*). Negishi's conclusion was based on the observation that shortly after an oral dose with M50367, the compound M50367 could not be detected in plasma but only as its metabolite M50354 (*13*). It is important to note that while M50354 displaced the photoaffinity radioligand more readily than M50367 (Figure 5), this is not sufficient evidence to suggest that M50354 is the "only" active metabolite. Clearly, α-NF, a partial AhR antagonist and agonist, can displace the radioligand more readily than either M50354 or M50367; yet, both M50354

and M50367 were more active at inducing CYP1A1 and reporter activity than α-NF at 10 μM in 6 h treatments.[2] In addition, M50367 had more profound and long-lasting effects in cells than M50354 (Figure 7). Perhaps differences in the ability of these compounds to transverse the plasma membrane and activate the AhR may account for the reduced activity of M50354 in our experiments when compared to M50367 (Figure 2). However, it is also probable that M50367 is simply not hydrolyzed into M50354 as readily in cells as it is in vivo (*13*). The slow hydrolysis of M50367 into M50354 in cell culture would then account for extended activation of the AhR, as seen with the induction of CYP1A1 in Figure 7. These issues can certainly be addressed in future metabolic studies. It was also evident that at the concentrations of 1 and 10 μM, the level of radioligand displaced was not as high as expected, especially since, at these concentrations, a high level of AhR-driven gene expression could be detected in cell culture experiments. However, the explanation for such discrepancies remains unclear and will be addressed in future studies.

Another aspect that we examined was whether these substances can cause AhR protein turnover. This was important since the reported actions of M50354 and M50367 could have AhR-dependent immunomodulatory functions without affecting AhR protein levels. However, consistent with the ability of high-affinity AhR ligands to induce degradation of the AhR (*49, 51*), treatment with either M50367 or M50354 could induce AhR turnover (Figure 6). Interestingly, the higher turnover observed between each ligand tested did not correlate with the level of CYP1A1 inducibility. For example, whereas B[*a*]P could induce AhR degradation to a lesser degree than M50367 in the time frame examined, the level of CYP1A1 protein induction was still comparable. This result suggests that AhR degradation and transcriptional activity are differentially regulated.

As seen in Figure 7, the analysis of CYP1A1 expression after a 6 h pulse or 36 h pulse with each compound revealed that M50367 had greater potency and lasting effects than M50354 on the expression of CYP1A1 protein, when analyzed at the 36 h time point. While M50354 and M50367 could both induce CYP1A1 activity to a similar degree in a 6 h dose analysis (Figure 2B), noticeably after 36 h, M50367 had a profound effect on CYP1A1 protein expression (Figure 7). Given that chronic expression of CYP1A1 and other AhR target genes has been implicated in carcinogenesis (*52*), perhaps our results would indicate that M50354 may be a more favorable candidate to use in vivo. However, the therapeutic index for these substances has yet to be determined.

A potential caveat for the use of these drugs (M50354 and M50367) in the treatment of human atopic allergic diseases is warranted by the data presented in this report. The chronic activation of CYP1A1 and other AhR target genes by TCDD and several polycyclic aromatic hydrocarbons have often been associated with several toxic end-points (*53, 54*). Accordingly, the upregulation of AhR target genes by M50354 or M50367 would be of concern given the chronic nature of atopic allergic diseases and the potential need for continuous administration of these drugs. However, it is possible that the transient nature of AhR transcriptional activity elicited by M50354 and M50367, possibly due to their rapid metabolism, may not result in a toxic end-point as seen with persistent AhR activation by nonmetabolizable ligands like TCDD (there is an excellent discussion about this issue in ref *55*). Furthermore, although high-affinity AhR ligands exert pleiotropic immunosuppressive effects on the production of various antibody isotypes and cytokines (*56*), M50367 preferentially affected T_H2-specific cytokine and antibody production (*14*). Therefore, our report does not necessarily negate the potential use of these drugs or derivatives for the treatment of atopic allergic diseases. Because these drugs are able to fully induce AhR-mediated microsomal activity in our tests (Figures 2B, 6, and 7), their structures may still allow the AhR to achieve an unusual conformation that selectively modulates specific signaling cascade pathways of the immune system. Alternatively, the metabolism of these drugs by AhR-induced metabolic enzymes may activate them into modulators of the immune system. It is currently unclear whether M50354 and M50367 could also have antagonistic effects on the enzymatic activity of metabolic enzymes as seen with imidazole-derived antimycotic drugs, which could also play a role in its therapeutic effect (*57–59*). Estrogen signaling has also been implicated in the regulation of the T_H cell balance (*60*). Because the activated AhR has been shown to interact with the estrogen receptor (ER) and attenuate its transcriptional output (*61–64*), perhaps the immunomodulatory mechanisms of M50354 or M50367 could also be associated with AhR-dependent modulation of the ER pathway. Finally, *GATA-3* gene downregulation was among the effects exerted by M50354 and M50367 and was the presumed explanation for favoring T_H1 cytokine production (*14*). Future studies can address whether the AhR can tether to the promoter of *GATA-3* and transrepress its expression, although the repression mechanism could still be mediated through an alteration in gene expression of other factors involved in *GATA-3* regulation.

We have established that both M50354 and M50367 are potent but relatively transient inducers of direct AhR target genes, which is in contrast to a previous publication (*14*). Given the suitability of M50354 for a diverse series of chemical modifications, this compound may be an invaluable tool for quantitative structure–activity relationship (QSAR) studies. With specific measurable immunological parameters already established (*12–14, 65*) (e.g., specific cytokines, transcription factor activation, and so on), perhaps more insights into AhR cellular functions could be obtained with these compounds serving as molecular backbones in future QSAR studies.

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