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Identification of a Potent Agonist of the Orphan Nuclear Receptor Nurr1

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The identification of potent small-molecule ligands to receptors and enzymes is one of the major goals of chemical and biological research. Two powerful tools that can be used in these efforts are combinatorial chemistry and structure-based design. Herein we address how to join these methods in a design protocol that produces a lead-finding library of compounds focused on the nuclear receptor target family, as well as a second-generation library directed against a specific member of this family for lead optimization.

The nuclear receptor family is a group of structurally related transcription factors that includes receptors for steroid hormones, vitamins, and thyroid hormone, as well as orphan receptors whose cognate ligands, if any, remain to be identified.[1,2] The ability to respond to such chemicals empowers these receptors with the capacity to coordinate tissue patterning, differentiation, and growth in response to remotely released molecules. This makes them important potential targets for therapeutic intervention.

NUR77-related protein 1 (Nurr1) is an orphan member of this superfamily that is expressed predominantly in the central nervous system.[3] The protein exhibits a close structural relationship to the orphan receptors NUR77[4] and neuron-derived orphan receptor.[1,5] These three proteins compose the NR4 A subfamily of nuclear receptors that bind to the same cis-acting consensus sequence—the nerve growth factor inducible β-response element (NBRE)—to regulate target gene expression.[6]

The essential role of Nurr1 in dopaminergic cell development was dramatically demonstrated in mouse gene knockout experiments in which homozygous mice lacking Nurr1 failed to generate midbrain dopaminergic neurons.[7] Nurr1 was shown to be directly involved in the regulation of genes coding for aromatic amino acid decarboxylase, tyrosine hydroxylase (TH), and the dopamine transporter.[8] These observations triggered the hypothesis that the Nurr1 protein might play a pathophysiological role in distinct conditions ranging from multiple inflammatory responses to dopaminergic nerve function and rescue.

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The identification of potent and selective agonists of Nurr1 may further clarify the role of Nurr1, and open new therapeutic interventions into central nervous system disorders and inflammatory conditions. Very recently 6-mercaptopurine (6-MP) was reported as a modest nonselective agonist of Nurr1.[9] In addition, isoxazolopyridinone-based agonists (EC_{50}: 40–70 nM) have also been disclosed.[10]

To find new ligands for the emerging members of the nuclear receptor family, we undertook the design and synthesis of a library of molecules oriented toward this family. Similar strategies, namely the creation of libraries directed toward families of receptors, have been described recently.[11,12] Such strategies were used to discover potent kinase inhibitors[13] or G-protein-coupled receptor (GPCR) ligands.[14] However, the vast majority of published examples of the combination of combinatorial chemistry and structure-based drug design are for libraries that were designed for a single target.[15–18]

The choice of the benzimidazole scaffold was based on several criteria, namely: physicochemical properties, modularity, and structural overlap with known nuclear receptor ligands. This scaffold, which represents a common motif found in many compounds of medicinal interest, is present in various GPCR ligands such as angiotensin II antagonists and NK1 antagonists.[19] It is also part of the core structure of retinoic acid receptor antagonists,[20] thrombin inhibitors,[21] and gp IIb/IIIa inhibitors.[22] Because it is composed of a basic functional group, it offers the potential to make salts, which would increase the water solubility of its derivatives. Undesirable physicochemical properties have often hampered the biological testing of first-generation combinatorial libraries or have hampered the successful use of screening hits discovered within them.[23] Further, the benzimidazole scaffold can be synthesized from three easily accessible building blocks, *o*-nitrophenylhalides, primary amines, and carboxylic acids or aldehydes.

To focus this library on nuclear receptors, we superposed the benzimidazole scaffold with a set of known nuclear receptor ligands (Figure 1). This experiment defined the substitution pattern and the potential scope of the side chains needed to mimic other known nuclear receptor ligands. A trisubstituted benzimidazole 5-carboxamide structure was finally chosen as scaffold for this library (Figure 2). Manual docking experiments of the designed compounds into 3D (e.g., estrogen receptors) and homology (e.g., PPAR) models of nuclear receptors confirmed the choice of the benzimidazole scaffold and further defined the size and shape of the potential substitution patterns. The final building blocks were chosen to allow a systematic assembly of pharmacogenic functions such as hydrophobicity, aromaticity, basicity, acidity, and hydrophilicity. By implementing structural elements that are able to engage in all fundamental nonbonded interactions at each of the variable sites of the core, we expected to partly cover the diversity of functions that are potentially present in nuclear receptor binding sites. This selection led to a virtual library of 4608 compounds. A further selection based on the rule of five[24] was applied to choose compounds with the best drug-like features, and a library of 3840 compounds was finally selected for synthesis.

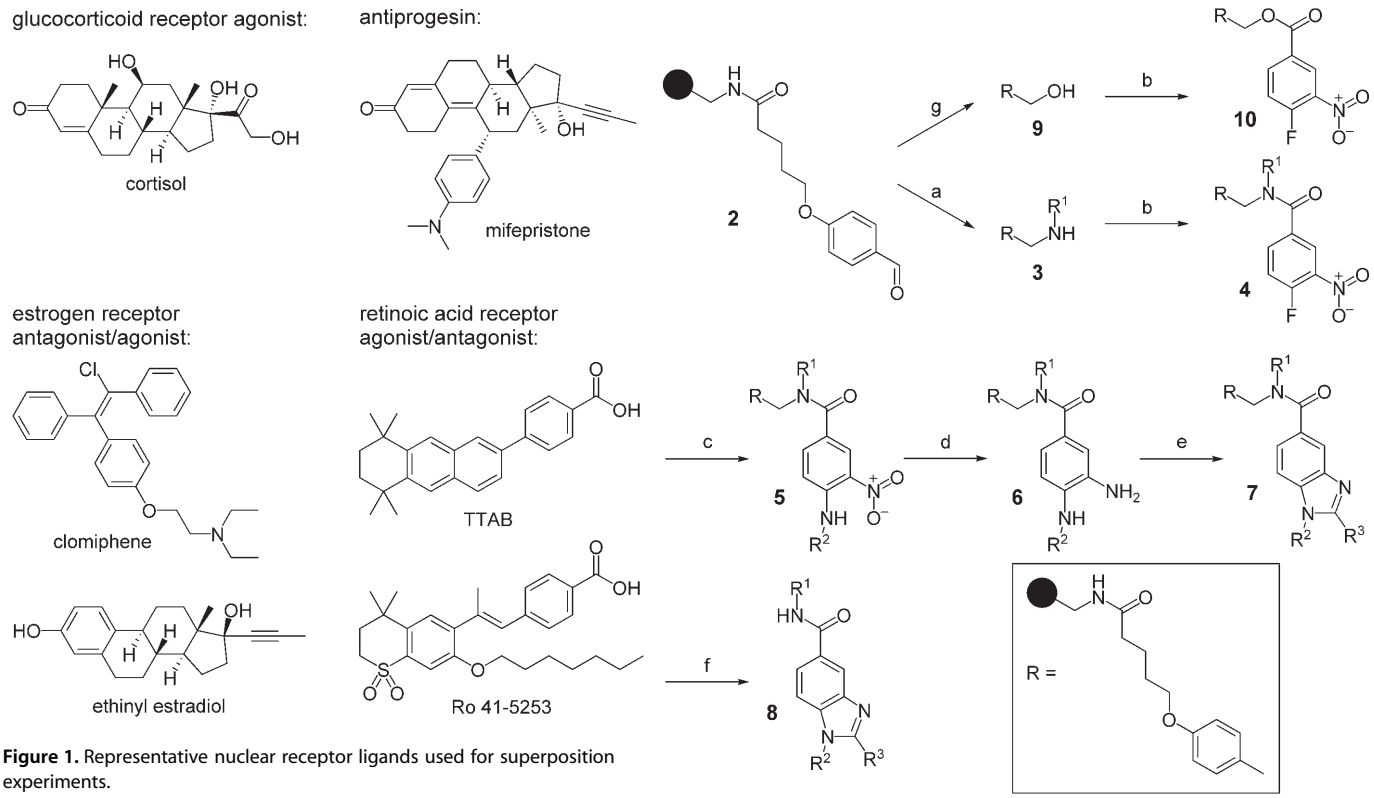


Figure 1. Representative nuclear receptor ligands used for superposition experiments.

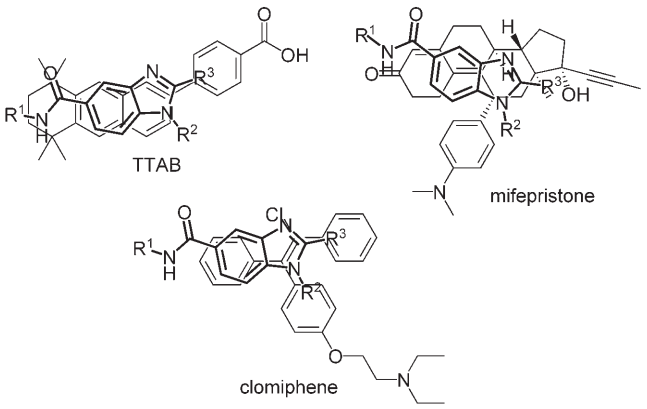


Figure 2. Representative superposition maps of the benzimidazole scaffold with nuclear receptor ligands.

Scheme 1. Reagents: a) R^1NH_2 (0.4 M), $NaBH_3CN$ (0.1 M), DCE/Ti(O*i*Pr)$_4$ (1:1), 60 °C, 6 h; b) HATU (0.2 M), NMM (0.3 M), 3-nitro-4-fluorobenzoic acid (0.2 M), DMF, 25 °C, 24 h; c) R^2NH_2 (0.2 M), DMSO, 50 °C, 48 h; d) $NaBH_2S_3$ (0.3 M), THF, 60 °C, 18 h; e) R^3CHO (0.2 M), DDQ (0.1 M), DMA/H_2O (9:1), 25 °C, 24 h; f) anisole (0.05 M), TFA, 25 °C, 18 h; g) $NaBH_4$ (0.1 M), DCE/Ti(O*i*Pr)$_4$ (1:1), 60 °C. DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, DMA = *N*,*N*-dimethylacetamide, DMF = *N*,*N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

Previously, a number of combinatorial syntheses of benzimidazoles with various substitution patterns have been reported.[25–27] They have led to the discovery of compounds that exhibit a number of biological activities including antimicrobial activity,[28] H1-antihistamine activity,[29] and inhibitors of the hepatitis C virus NS5B polymerase.[30]

Owing to their easy manipulation and washing, Mimotope's SynPhase double lanterns were used for the development of a six-step synthesis of the benzimidazoles. As outlined in Scheme 1, the starting lantern, TFA·NH_2-polystyrene lantern **1** (37 μmol per lantern), was treated with a mixture of dimethylformamide/triethylamine to deliver the free amine, which was treated with 4-(4′-carboxybutyloxy)-2-methoxybenzaldehyde, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), and *N*-methylmorpholine (NMM) to afford lantern-bound **2**. Lantern-bound **2** was then reductively aminated with the first building block and sodium cyanoborohydride to yield the lantern-bound amine **3**, which was acylated with 3-nitro-4-fluorobenzoic acid to yield lantern-bound *o*-nitrophenylfluoride **4**. On the other hand, when the aldehyde **2** was reduced with sodium borohydride, lantern-bound alcohol **9** was obtained. This alcohol was transformed into lantern-bound ester **10**, which enabled the synthesis of the 5-1*H*-benzamidizole carboxylic acid derivatives.

At this stage, the second building block was introduced by treating the *o*-nitrophenylfluoride **4** with the second amine. The resulting lantern-bound *o*-nitroaniline **5** was then reduced to the lantern-bound *o*-phenylenediamine **6**. Several published procedures based on transition-metal-catalyzed reductions ($FeCl_3$, $FeSO_4$, $SnCl_2$, Zn) were tried, but without success. The results were difficult to reproduce, and the products were always contaminated with a benzimidazole, which arose from the incorporation of a dimethylformamide or dimethylacetamide molecule during the reduction. After numerous attempts, we found two distinct procedures which allowed the clean and reproducible reduction of **5** to **6**. Tetrabutylammonium hy-

drogen sulfide in a toluene–ethanol mixture at 80 °C or $NaBH_2S_3$ in tetrahydrofuran at 60 °C proved useful. The final lantern-bound benzimidazole **7** was obtained from **6** by the reaction with an aldehyde and dichlorodicyanoquinone. Upon treatment with trifluoroacetic acid in dichloroethane (DCE), the final trisubstituted benzimidazoles were isolated from the lanterns. The 5-1*H*-benzamidizole carboxylic acid derivatives were prepared in a similar way, from the lantern-bound benzylic alcohol **9**.

After evaporation of the solvent, the products were purified by RP HPLC–MS. The target compounds were identified by electrospray ionization and collected by the automatic detect-before-collect routine. The system automatically reports the purity of a target compound in each well based on peak area ratio measurements, which were taken from the combined mass traces that were sampled over the complete fraction collection time.

Using the procedures described above, a library of 3840 benzimidazoles was prepared on Mimotope's double lanterns using the sort-and-combine strategy.[31] Each individual double lantern was coded by the insertion of a radio frequency (RF) chip, which allowed us to easily handle batches of compounds in large vessels. The manipulation of the lanterns during the simultaneous benzimidazole syntheses was guided by the online computer-aided scanning of the RF tag present in each lantern. Database registration of the RF tag codes before the synthesis allowed us to track each lantern in the library at each step of the synthesis, and to array the lanterns in 96-well racks for the acid-catalyzed cleavage of the benzimidazoles from the lanterns. All of the final products were purified by RP HPLC–MS, and the desired pure benzimidazoles, 3256 out of the 3840 expected, were obtained as trifluoroacetic acid salts in 30–70% yield, and in over 85% purity from the TFA·NH_2-functionalized lanterns.

Nurr1 is composed of three functional domains: a DNA-binding domain, a dimerization domain, and a ligand-binding domain. The DNA-binding and dimerization properties of Nurr1 are well characterized, however, no Nurr1 ligand is known. These properties were used to establish a specific reporter-gene assay for high-throughput screening. Briefly, MN9D cells,[32] a clonal hybrid cell line derived from the ventral mesencephalon, were stably transfected with Nurr1 expression plasmids. The expression of biologically functional Nurr1 was confirmed by measuring the TH activity levels of the individual clones. Single cell clones with increased TH activity were then selected and transfected with a reporter plasmid in which firefly luciferase expression is controlled by multiple copies of a Nurr1-specific DNA-binding element.[33] To demonstrate that luciferase expression is indeed under the control of Nurr1, a control cell line was established in which a retinoic acid receptor (RXRα) is also overexpressed. RXRα can form heterodimers with Nurr1 so that Nurr1-responsive elements can become retinoic acid inducible.[34] The luciferase reporter-gene activity in the resulting RXRα/Nurr1 overexpressing cell line was strongly induced by 9-*cis*-retinoic acid, in contrast to the Nurr1-expressing lines, which demonstrated that the reporter gene was under the control of Nurr1 response elements. As a negative control to distinguish between Nurr1-selective and general inducers, a renilla luciferase control plasmid (Promega) was co-transfected. This plasmid expresses renilla luciferase under the control of a thymidine kinase minimal promoter, and does not contain any Nurr1-specific DNA-binding sites.

A number of micromolar agonists (1–5 μM) of Nurr1 (Figure 3), which increased the luciferase reporter-gene activity

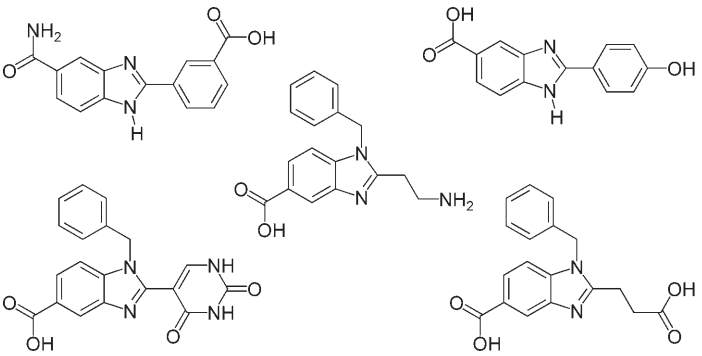


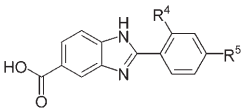
Figure 3. Selected Nurr1 agonists discovered in the first-generation library.

by a factor of 2, could be identified by screening the first generation library of 3256 compounds. To improve the potency of the first-generation hits, we designed and synthesized a second-generation library biased toward Nurr1. The limited structure–activity relationships gained from the first results indicated a clear preference for an acidic function in position 5 of the benzimidazole scaffold. The small number of building blocks chosen, four amines and 13 aldehydes, allowed a manual parallel synthesis of this library. Again, all final products were purified by RP HPLC–MS, and the desired benzimidazoles were obtained as trifluoroacetic acid salts in 55–68% yield from TFA·NH_2-functionalized lanterns.

The screening of this second-generation library in the reporter-gene assay revealed a number of very potent Nurr1 agonists (Table 1). The greatest increase in luciferase reporter-gene activity was observed for compound **11**, with an EC_{50} value of 8 nM.

Very potent Nurr1 agonists (EC_{50} 8–70 nM) were thus rapidly identified by designing, synthesizing, and screening a first-generation library oriented toward nuclear receptors, followed by a small, biased second-generation library. These results may

Table 1. Second-generation Nurr1 agonists.



Compd	R^4	R^5	EC_{50} [nM][a]
11	H	Cl	8
12	H	F	24
13	Me	OH	70

[a] Values represent the mean of three experiments.

sound surprising given the very recent finding that whereas Nurr1 shares the general features of the classical ligand-activated nuclear receptors, the ligand-binding domain of Nurr1 is filled with polar amino acid residues.[35] Recently, however, a novel hydrophobic interaction surface has been identified that could serve not only for coactivator binding, but also as molecular target for Nurr1-activating compounds[36] like those described herein. These new Nurr1 agonists might serve as useful tools to uncover the pathophysiological role of Nurr1.

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Keywords: benzimidazoles · combinatorial chemistry · compound libraries · nuclear receptors · Nurr1

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