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Identification of potent agonists of photoreceptor-specific nuclear receptor (NR2E3) and preparation of a radioligand

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Abstract—Agonists of NR2E3 (PNR, RNR) have been identified and optimized to $EC_{50} < 200$ nM. A tritiated analogue of one agonist was prepared to aid in the development of a binding assay.

Age-related macular degeneration (AMD) is a leading cause of blindness in the Western world which affects 10 million patients in the United States.[1] Loss of vision and eventual blindness has been related to the degeneration of photoreceptor cells in the macula, the center portion of the retina. The death of photoreceptor cells is associated with dysfunction of the retinal pigmented epithelium, which serves many critical roles in maintenance of rods and cones including cycling of visual pigments derived from retinol (vitamin A).[2]

Photoreceptor-specific nuclear receptor (PNR[3a], also known as RNR[3b] and NR2E3) is a recently discovered[3] orphan nuclear receptor which, as its name implies, is expressed exclusively in rod photoreceptor cells of the retina.[3a,4] In addition to its localization in the retina, several lines of evidence implicate NR2E3 in the progression of photoreceptor degeneration. For instance, NR2E3 mutations in humans have been correlated with various retinal diseases[5] and *rd7* mice, which contain a sporadic genomic deletion within the gene coding for NR2E3, exhibit abnormal development of rods and cones, and progressive reduction in their function.[4] Based on these associations and other evidence, NR2E3 is an attractive target for intervention in AMD.

High throughput screening of the Merck sample collection generated a series of leads which exhibited NR2E3 agonism.[6] Agonism was assessed using a GAL4DBD–NR2E3LBD chimera in a recently described transactivation-based β-lactamase assay.[6] Because no endogenous ligand for NR2E3 is known, this assay was developed in parallel with a functionally analogous thyroid hormone nuclear receptor GAL4DBD–TRLBD chimera assay. Since TR is well characterized and its native ligand is known, this pairing confirmed a functioning NR2E3 assay and enabled an initial determination of agonist selectivity. Structures of positive hits, which represent the first known agonists of NR2E3, are presented in Figure 1.

It is noteworthy that 13-*cis*-retinoic acid (**1**), a natural retinal pigment, exhibits limited properties of an NR2E3 agonist in cell-based assays and does so only at very high, non-physiological concentrations. Therefore, optimization efforts focused on the more potent compound **2**. An important complement to the NR2E3 β-lactamase transactivation assay, the NR2E3 NCOR release assay,[7] was based on NR2E3's role as a transcriptional repressor in its unligated state. This

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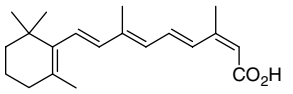
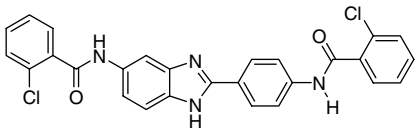


1, NR2E3 β-lactamase EC_{50} 25–50 μM



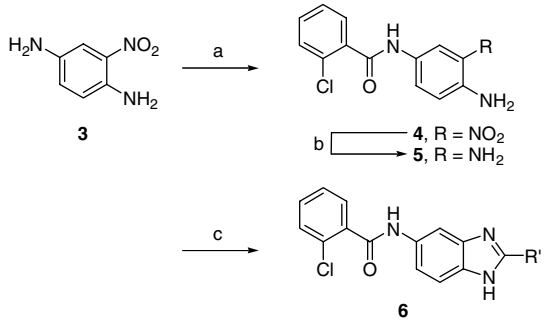
2, NR2E3 β-lactamase EC_{50} 1.3 μM

Figure 1. NR2E3 Screening leads.

assay quantitates the release of the co-repressor NCOR fragment from the NR2E3 ligand binding domain upon binding of an agonist.

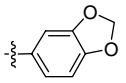
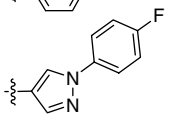
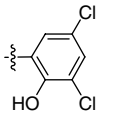
Initial investigation of lead **2** was carried out with libraries aimed at incorporating a diversity of benzimidazole 2-phenyl replacements, and preparation of these analogues is described in Scheme 1. Commercially available 2-nitro-1,4-diaminobenzene (**3**) was selectively acylated at the 4-amino position with 2-chlorobenzoyl chloride, and the resulting amide **4** was reduced under Zinin conditions to give **5**.[8] The diamine **5** was condensed with aromatic aldehydes (acetic acid, microwave irradiation, 170 °C, 20 min) to provide analogues **6**, a selection of which is shown in Table 1.

Potency in the β-lactamase assay suffered as a result of these changes; however, in the NCOR release assay several analogues retained some agonist activity. Reconciliation of these results is possible upon consideration that an agonist signal in the NCOR release assay is registered as decreased expression of the luciferase reporter; thus reduction in luciferase activity due to cell death caused by a toxic test compound could be misinterpreted as an agonist signal.[7] It was possible to confirm toxicity by testing the effect of compounds on cell survival in a control cell line; in the case of **6a**–**6c** this secondary screen confirmed toxicity and, importantly, established the criteria that test compounds were viable only if they exhibited activity in both the β-lactamase transactivation and the luciferase NCOR release assays, and were non-toxic in control cell lines.



Scheme 1. Reagents and conditions: (a) 2-chlorobenzoyl chloride, CH_2Cl_2, rt, 99%; (b) Na_2S, $NaHCO_3$, MeOH/H_2O, 60 °C, 96%; (c) R′CHO, 10% HOAc/trifluorotoluene, microwave, 170 °C, 20 min, 10–64%.

Table 1. NR2E3 agonist activity of selected analogues

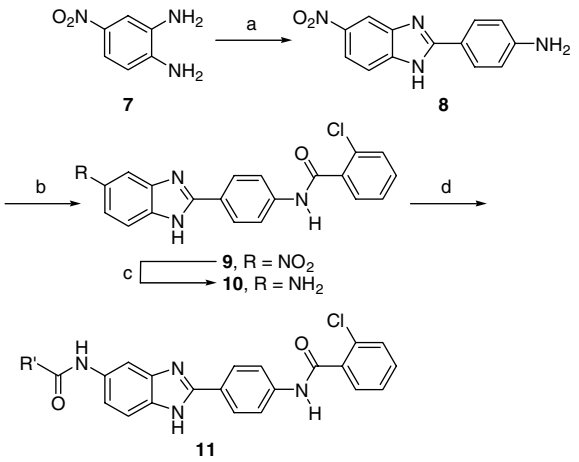
Compound	R′	EC_{50}[a] (nM)	
		NR2E3 β-lactamase	NR2E3 NCOR
6a		>25,000	7200
6b		>25,000	4100
6c		>25,000	4200

[a] Values are means of two experiments (na, not active).

Subsequent analogues retained the 2-phenylbenzimidazole core and focused on examining the two amide substituents. In this case (Scheme 2), 4-nitro-1,2-diaminobenzene was condensed with polymeric 4-aminobenzaldehyde in refluxing pyridine,[9] then converted to the 2-chlorobenzamide. Nitro reduction ($SnCl_2$) and amide formation generated the desired analogues; data are presented in Table 2.

Small ring amides provided the most potent compounds, with cyclopropyl amide **11a** conferring the greatest NR2E3 activity. Ring expansion (**11c** and **11d**) or opening (**11b**) significantly decreased activity, and alternative small alkyl groups (e.g., pivaloyl **11e**) exhibited diminished activity. The corresponding acetamide was devoid of any activity.

The cyclopropyl amide present in **11a** proved to be uniquely capable of providing NR2E3 agonism at $EC_{50} < 200$ nM. Table 3 indicates how sensitive NR2E3 activity is to substitution on the cyclopropane, with even small changes like mono- and difluorination resulting in a significant decrease in activity. Substitution with a phenyl group (**11j**) abolished NR2E3 activity and introduced cellular toxicity to the compounds.



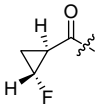
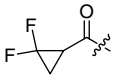
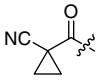
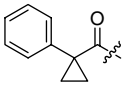
Scheme 2. Reagents and conditions: (a) 4-aminobenzaldehyde polymer, pyridine, 115 °C, 68%; (b) 2-chlorobenzoyl chloride, CH_2Cl_2, rt, 86%; (c) $SnCl_2$, EtOH/EtOAc, reflux; (d) R'COCl, CH_2Cl_2, rt, 10–50%.

Table 2. NR2E3 agonist activity of selected analogues

Compound		EC_{50}[a] (nM)	
		NR2E3 β-lactamase	NR2E3 NCOR
11a		141	35
11b		316	147
11c		1328	986
11d		3160	1320
11e		6270	2610

[a] Values are means of two experiments (na, not active).

Table 3. NR2E3 agonist activity of selected analogues

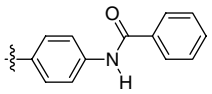
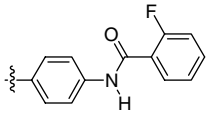
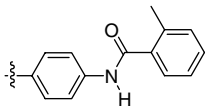
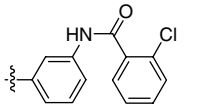
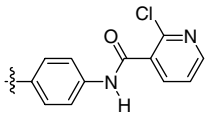
Compound		EC_{50}[a] (nM)	
		NR2E3 β-lactamase	NR2E3 NCOR
11f		720	260
11g		2100	3000
11h		2500	2900
11i		17,000	10,000
11j		>25,000	370

[a] Values are means of two experiments (na, not active).

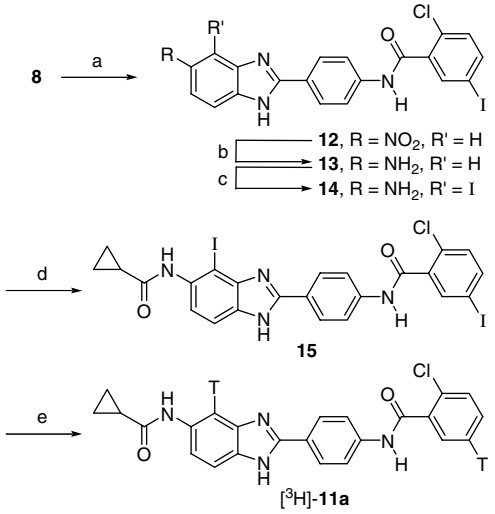
Having optimized the benzimidazole 5-amide, attention was directed toward improving potency by varying the right-hand anilide. These analogues were prepared by the route shown in Scheme 1 and NR2E3 data are given in Table 4. Benzamides were the most potent agonists, and while *o*-substitution with Cl increased activity 2× (**11a**), other *o*-substituents (e.g., F (**6e**) and Me (**6f**)) were equipotent with the parent benzamide **6d**. Relocation of the amide to the *m*-anilide position (**6g**) decreased potency by 100-fold, and incorporation of a 2-chloronicotinamide (**6h**) also decreased activity.

To this point, optimization of NR2E3 agonists was based on their activity in two cell-based assays, and we could not exclude the possibility that active compounds exert their effects on NR2E3 indirectly. The identification of **11a**, the most potent NR2E3 agonist known to date, presented the opportunity to develop a binding assay, which would confirm direct binding of test compounds to the ligand-binding domain of NR2E3 and simplify the evaluation of new compounds by eliminating the complications of membrane permeability and cellular toxicity. Toward this end, tritiated **11a** was prepared from a diiodinated precursor as outlined in Scheme 3. Intermediate **8** was acylated with 2-chloro-5-iodobenzoyl chloride (CH_2Cl_2, rt, 67%) before being reduced ($SnCl_2$) to the corresponding aniline. Incorporation of a second iodine substituent was achieved by treating **13** with benzyltrimethylammonium dichloroio-

Table 4. NR2E3 agonist activity of selected analogues

Compound	Structure	EC_{50}[a] (nM)	
		NR2E3 β-lactamase	NR2E3 NCOR
6d		270	610
6e		270	170
6f		260	330
6g		10,000	4100
6h		4800	2200

[a] Values are means of two experiments (na, not active).



Scheme 3. Reagents and conditions: (a) 2-chloro-5-iodobenzoyl chloride, CH_2Cl_2, rt, 67%; (b) $SnCl_2$, EtOH/EtOAc, reflux, 79%; (c) $BTMACl_2I$, $CaCO_3$; (d) cyclopropanecarbonyl chloride, CH_2Cl_2, rt; (e) tritium gas, 10% Pd/$CaCO_3$, DMF.

date[10] (4 equiv $BTMACl_2I$, MeOH, CH_2Cl_2, and $CaCO_3$), and iodination occurred exclusively in the benzimidazole 4-position. Tritiation was carried out using tritium gas in the presence of catalytic 10% Pd/$CaCO_3$, and after HPLC purification provided [^{3}H]-**11a** with a specific activity of 46 Ci/mmol.

In summary, a new class of potential NR2E3 agonists has been identified which is based on a 2-phenylbenzimidazole core. Compound **11a** exhibits agonism of NR2E3 in two cell-based assays and a tritiated analogue with high specific activity has been prepared. Development of a radioligand binding assay is in progress and will be reported in due course.

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