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Identification of a series of highly potent activators of the Nurr1 signaling pathway

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Abstract—The nuclear receptor Nurr1 (NR4A2) is critically involved in the development and maintenance of midbrain dopaminergic neurons and is believed to function independently of endogenous activation. The hit identification and SAR studies leading to isoxazolo-pyridinone **7e**, a highly potent, brain penetrable activator of the Nurr1 signaling pathway, are described.

Nurr1 is a member of the family of orphan nuclear hormone receptors and plays a critical role in the functional differentiation and survival of midbrain dopaminergic neurons. Homozygous Nurr1 knockout mice die shortly after birth because of profound hypoactivity and impairment of suckling behavior.[1] In these animals, differentiated dopaminergic neurons are absent in the substantia nigra and the ventral tegmental area. Consequently, the striatal dopamine levels are reduced by 98%. In heterozygous Nurr1-deficient mice, striatal dopamine levels and locomotor activity progressively decrease with increasing age.[1] In addition, the reduced Nurr1 expression increases the vulnerability of midbrain dopaminergic neurons in the acute MPTP mouse model of Parkinson's disease.[2] Nurr1 was shown to be directly involved in the regulation of genes such as those coding for aromatic amino acid decarboxylase, tyrosine hydroxylase, and the dopamine transporter.[3] Recently, it was shown that Nurr1 immunoreactivity in PD patients is significantly decreased in nigral neurons containing α-synuclein immunoreactive inclusions.[4] All these observations led to the hypothesis that activating the remaining Nurr1 protein in degenerating dopaminergic neurons by a drug might delay or even prevent the onset of Parkinsonian symptoms.

In spite of sharing the general features of the classical ligand activated nuclear receptors, Nurr1 is considered to be constitutively active. In contrast to other ligand activated nuclear receptors, the ligand binding domain (LBD) of Nurr1 does not present a cavity for ligand binding and possesses an intrinsic conformation that resembles that of ligand-bound, transcriptionally active nuclear receptors.[5] Recently, however, a novel hydrophobic interaction surface has been identified that could serve not only for co-activator binding but also as molecular target for Nurr1 activating compounds.[6]

To date, only one activator of Nurr1, 6-mercapto-purine (an anti-leukemic agent), has been reported.[7] This compound, however, has many additional cellular activities including anti-proliferative and cytotoxic effects that result mainly from the inhibition of purine biosynthesis and, in addition, it displays only a modest (μM) potency for Nurr1 activation.[7] Other compounds have recently been identified which either activate the Nurr1 NGFI-B heterodimer[8] or another member of this receptor superfamily NOR1.[9]

Herein, we report the discovery and initial lead optimization of a class of highly potent activators of the Nurr1 pathway which show cellular activities in the low nanomolar range.

Nurr1 overexpressing MN9D cells, a murine midbrain dopaminergic neuronal cell line, were used to set up a reporter gene assay to screen the Novartis collection.[10] Briefly, three copies of the Nurr1 specific DNA binding

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element[11] were cloned in front of a TK minimal promoter of a luciferase reporter gene construct. The screening led to the identification of a number of compounds capable of activating the basal Nurr1 dependent reporter gene activity. A subset of about 120 compounds of different chemotypes displayed EC$_{50}$s below 30 μM. Specificity of the effect was confirmed by measuring the activity of a second reporter gene lacking the Nurr1 responsive elements in front of the TK minimal promoter.

Since Nurr1 is believed to be constitutively active and lacks the classical LBD,[5] it was no surprise that the activation levels induced by the compounds were rather modest and mostly limited to about a 2-fold increase over basal activity. Isoxazolopyridinone **1** (Fig. 1), with an EC$_{50}$ of 78 nM, seemed to be particularly attractive as a starting point for further derivatization. Besides increasing the potency, the need to improve the rather low solubility of the hit compound (see Table 1) was identified as a main goal for the optimization efforts.

Examination of the screening data and evaluation of further compounds from the substance collection revealed that three pharmacophoric elements present in the hit were important for sub-micromolar activity: two aryl-substituents linked by a heterocyclic, hydrogen-bond acceptor containing system resulting in a rather flat system (Fig. 1). Furthermore, it became obvious that the amide group could be alkylated without loss of potency and that a variety of substitutions of the 6-phenyl-ring (mainly in the 4-position) were tolerated, offering a chance to incorporate solubilizing groups.

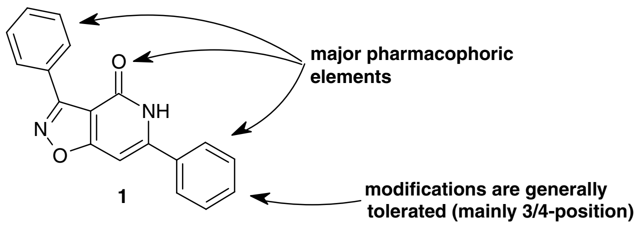
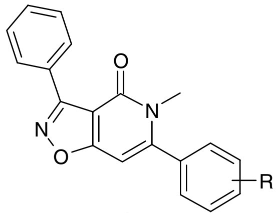


Figure 1. The potent screening hit **1**.

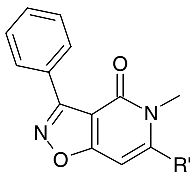
The original reaction sequence[12] is exemplified in Scheme 1 for the synthesis of the screening hit **1**. Briefly, the commercially available 5-methyl-3-phenyl-isoxazole-4-carboxylic acid (**2**) was converted into the acid chloride followed by quenching with ethanolic ammonia solution leading to the corresponding amide **3** in two steps with excellent yields. The amide was then transformed into the di-anion which reacted readily with benzonitrile to the corresponding imine, which was cyclized under strongly acidic conditions (HCl/dioxane) to give the desired product **1** albeit in rather poor yield. In all cyclization reactions performed under these conditions, the corresponding amino-pyridine **4** was formed as a by-product by water elimination from the tetrahedral intermediate.

To have an improved access to a large number of test compounds also in larger quantities, an alternative synthetic route toward this class of isoxazolo-pyridinones was developed by replacing the nitrile with a number of other electrophiles. Whereas using acid chlorides or esters as acylating agents did not improve the overall yield of the sequence, the use of *Weinreb*-amides improved the overall yield considerably. The optimized synthetic access to **7e** as a representative example is outlined in Scheme 1. Briefly, methyl-amide **5** was converted into the di-anion with an excess of butyl-lithium

Table 1. Nurr1 activation of selected compounds



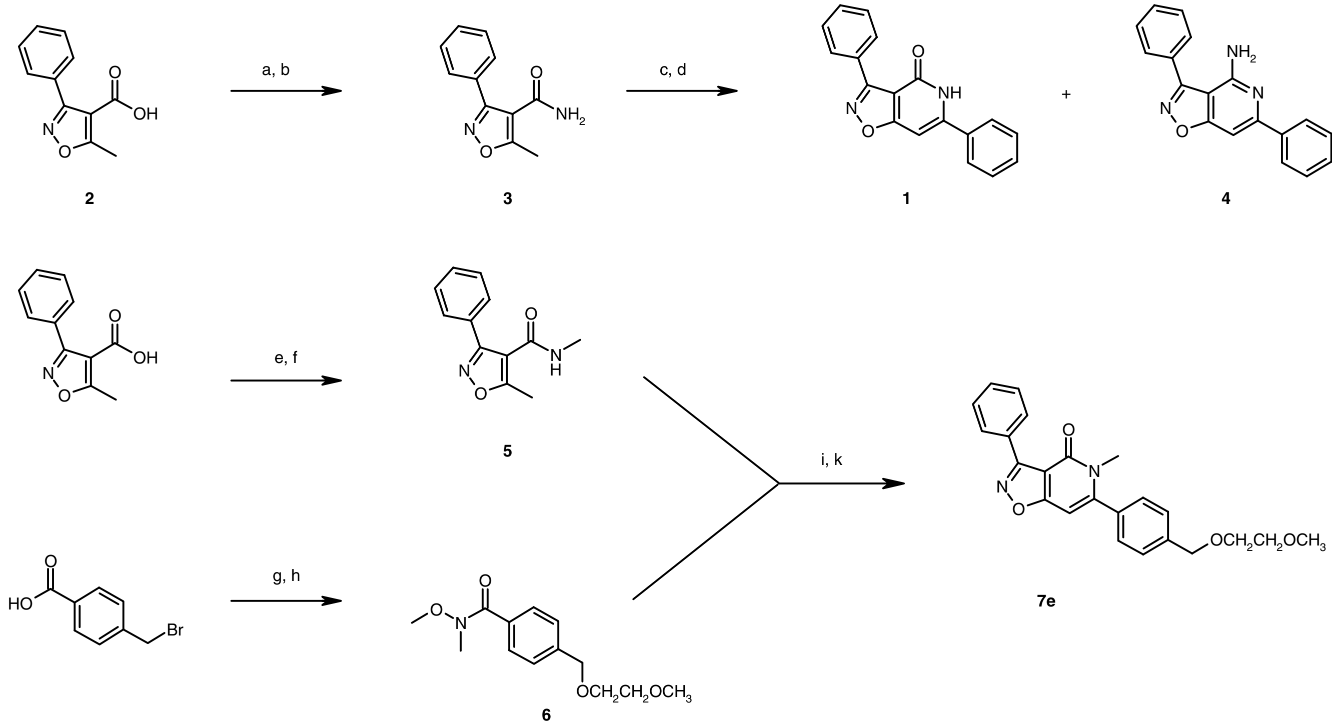
7a-i



8a-b

Compound	R (**7**) or R′ (**8**)	EC$_{50}$[a] (nM)	Max effect[a,b] (%)	Solubility at pH 7.6 (mg/L)[13]	Solubility at pH 1.0 (mg/L)[13]
1	—	77.5 (±26.8)	207 (±21)	0.3	14
7a	4-OCH_3	4.1 (±2.0)	185 (±20)	3	6
7b	2,4-$(OCH_3)_2$	59.2 (±26.1)	202 (±38)	7	18
7c	3,4-(-OCH_2O-)	0.8 (±0.1)	202 (±27)	2	4
7d	4-$CH_2OCH_2CH_2OH$	3.5 (±1.9)	226 (±14)	8	19
7e	4-$CH_2OCH_2CH_2OCH_3$	3.9 (±1.6)	208 (±21)	20	20
7f	4-$CH_2OCH_2CH_2OCH(CH_3)_2$	113.1 (±38.1)	173 (±26)	10	10
7g	4-OPh	>2000	—	0.4	8
7h	4-$CH_2N(CH_3)_2$	26.0 (±11.4)	171 (±39)	50	100
7i	4-CH_2-(4-CH_3-piperazin-1-yl)	31.9 (±12.7)	163 (±23)	100	>200
8a	pyrid-4-yl	129.9 (±47.5)	198 (±13)	6	15
8b	CH_3	>2000	—	20	20

[a] Values are means of four experiments, standard deviation is given in parentheses.
[b] % of control (DMSO = 100%).



Scheme 1. Original synthesis of hit compound **1** (top) and improved synthesis exemplified for **7e** (bottom). Reagents and conditions: (a) $SOCl_2$, $ClCH_2CH_2Cl$, 80 °C, 2h; (b) 2 M NH_3 in EtOH, 0 °C to rt, 14 h (92%, two steps); (c) 1.6 M BuLi in hexane, THF, 0 °C, 1 h; then benzonitrile, 0–5 °C, 1 h; (d) 4 M HCl in dioxane, rt, 16 h (**1**: 7%, two steps); (e) $SOCl_2$, $ClCH_2CH_2Cl$, 80 °C, 2 h; (f) $MeNH_2$ in EtOH, 0 °C to rt, 12 h (98%, two steps); (g) KOH, 2-methoxyethanol, 0 °C to rt, 16h; (h) $SOCl_2$, DMF cat., $ClCH_2CH_2Cl$, 80 °C, 1 h; then Hünig's base, MeONHMe, 0 °C to rt, 12 h (74%, two steps); (i) 1.6 M BuLi in hexanes, THF, −70 °C, 1 h; then **6**, −70 °C to rt, 3 h; (k) HCl (4 M)/THF, rt, 16 h (42%, two steps).

solution at low temperature. Subsequent addition of the *Weinreb*-amide **6** yielded the intermediate ketone which was cyclized to the final product in a one-pot procedure by adding aqueous hydrochloric acid. With this novel synthetic route, a very general access to substituted isoxazolopyridinones became available. The required *Weinreb*-amides were easily prepared from the corresponding carboxylic acids in a one-pot sequence and in good yields via the corresponding acid chlorides.

The cellular biological activity of compound **1** and its newly synthesized derivatives was assessed in the same reporter gene assay system which was used for HTS.[10] Almost identical activities were obtained when the compounds were tested in an alternative cellular assay also based on the parental cell line MN9D in which the reporter gene luciferin luciferase was under the control of the rat tyrosine hydroxylase promoter, a gene known to be sensitive to Nurr1 activation (data not shown).

Early on, it was decided to focus optimization efforts on alkyl-amides because the NH-group was suspected to be one reason for the low solubility. And, indeed, N-methylation of the hit **1** increased the solubility from 0.3 to 10 mg/L (pH 7.6).

The SAR of modifications of the 6-phenyl-substituent of this chemotype was investigated in more detail and a representative selection of the results is given in Table 1. In general, small substituents were well tolerated especially in the 3- and 4-position, whereas larger substituents were tolerated only in the 4-position.

The 4-methoxy-derivative **7a** showed an excellent, single digit nanomolar potency. Incorporation of larger ether substituents with the potential to increase the aqueous solubility was also well tolerated. For instance, the benzylic ether **7d**, as well as the corresponding methylated derivative **7e**, showed a doubling of the basal luciferase activity in our cellular assay with a low-nanomolar EC_{50}. Bulkier ether substituents were not tolerated in this position and led to a drop in potency by two or more orders of magnitude (**7f**, **g**).

Fortunately enough, the increase in potency by incorporation of ether side chains was paralleled by a higher solubility at both pH 7.6 and 1. For instance, compound **7e** showed a reasonable solubility of 20 mg/L, an increase of more than two orders of magnitude compared to the original screening hit **1**.

Next, we investigated the influence of multiple alkoxy-substituents on the 6-phenyl ring. The 2,4-di-methoxy derivative **7b** confirmed our hypothesis that in the alkyl-amide series ortho-substituents were detrimental for potency. Incorporation of a [1,3]-dioxolane moiety into the 3,4-positions, however, yielded the most potent compound we ever identified (**7c**) with a subnanomolar EC_{50}. Unfortunately, its solubility was rather low thereby limiting its further use.

We then focused our attention on amines as potential solubilizing groups. As expected, tertiary benzylic amines **7h** and **7i** showed much higher solubilities than the ether derivatives but this improvement was accompanied by a slight drop in potency. Replacing the phenyl- by a 4-pyridyl group (**8a**) did not improve the solubility and caused a significant drop in potency. Finally, complete removal of the hydrophobic aryl-substituent to lower the lipophilicity turned the resulting isoxazolo-pyridinone **8b** completely inactive, as was originally postulated in our pharmacophore hypothesis.

Due to its in vitro profile, compound **7e** seemed to be a promising candidate for further investigations. Indeed, in a preliminary pharmacokinetic experiment[14] it was found to have an excellent oral bioavailability of 95% in mice and a rapid and extensive brain uptake, reaching levels more than two orders of magnitude higher than the cellular EC_{50} within 30 min.

In conclusion, this report describes the identification of a novel class of activators of the Nurr1 signaling pathway showing low-nanomolar activities in cellular assays. The molecular mode of action of these compounds is at the moment not clarified and work is in progress, to analyze a possible direct interaction with the newly described hydrophobic pocket of the receptor. In addition, efforts are ongoing to further optimize and characterize this promising compound class in vitro for later in vivo profiling in animal models.

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10. MN9D cells were stably transfected with a Nurr1 expressing plasmid under the control of a CMV promoter. A clone showing a 4-fold increase in tyrosine hydroxylase activity was used to generate the screening cell line by transfecting the cells with a reporter plasmid in which firefly luciferase expression was controlled by multiple copies of a Nurr1 specific DNA binding element.[11] To demonstrate that luciferase expression was under the control of Nurr1, a cell line was established in which the retinoic acid receptor RXRα was coexpressed. RXRα can form heterodimers with Nurr1 so that Nurr1 responsive elements can become retinoic acid inducible. In fact, the luciferase reporter activity became strongly inducible by 9-*cis* retinoic acid in this cell line while it was not in the screening cell line expressing only Nurr1. As a negative control to eliminate Nurr1 independent inducers, a plasmid, without any Nurr1 specific DNA binding sites, was co-transfected. Compounds were tested by incubating the MN9D cells with various concentrations in triplicates (controls received the same amount of vehicle). After 24 h, the cells were processed for luciferase activity. The EC_{50} values for the stimulation over the basal activity were calculated after fitting of the curves.
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14. Mice were dosed with **7e** (10 μmol/kg) dissolved in 10% Tween 80/water iv and po. Plasma and brain samples were collected at 7 time points between 5 min and 24 h ($N = 3$). Samples were extracted and analyzed by HPLC, and bioavailability calculated, following standard procedures.