Subnanomolar indazole-5-carboxamide inhibitors of monoamine oxidase B (MAO-B) continued: indications of iron binding, experimental evidence for optimised solubility and brain penetration

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Introduction

Alzheimer’s disease (AD) and Parkinson’s disease (PD) are the most prevalent, aging-related neurodegenerative disorders of the central nervous system (CNS), currently affecting over 8% of individuals aged ≥65 years worldwide. Despite their differences in pathogenesis and symptoms, AD and PD share common underlying features such as chronic, irreversible, and progressive neuronal degradation in the human brain caused by complex pathophysiological processes, including oxidative stress, neuro-inflammation, excitotoxicity, mitochondrial dysfunction, and proteolytic stress. The currently approved anti-AD and anti-PD drugs have an impact on several symptoms in different disease stages and improve quality of life for patients, but do not stop the disease progression nor exhibit a neurorestorative effect.

Monoamine oxidases (MAOs, EC 1.4.3.4) are mitochondrial flavoenzymes that play a key role in the metabolism of monoaminergic neurotransmitters. Two isoforms of MAOs are present in mammals, MAO-A and MAO-B, differing by their distribution in the human brain, substrate preference, and selectivity to different inhibitors. The expression levels and activity of MAO-B, but not of MAO-A, increase with aging, leading to an enhanced production of reactive oxygen species (ROS). Some studies suggest that high-level accumulation of iron may also contribute to higher ROS production and neurotoxicity. Overproduction of ROS is associated with oxidative stress and neuronal death in patients with AD and PD. Therefore, selective inhibition of MAO-B is a well-established approach for treatment of PD. For example, the irreversible MAO-B inhibitor selegiline and the reversible inhibitor rasafinamide are approved for the treatment of PD. (Figure 1) Recently we have discovered a series of selective MAO-B or dual MAO-A/B inhibitors with nanomolar potency several compounds of structurally related N-unsubstituted indazole-5-carboxamides (subclass I) and N1-methylated indazole-5-carboxamides (subclass II) were identified as best-in-class MAO-B inhibitors (Figure 1).

MOA inhibition assay

The evaluation of human MAO inhibitory activity of the indazole-5-carboxamides and reference inhibitors was assessed by a fluorescence-based assay measuring their effects on the production of hydrogen peroxide (H₂O₂) from p-tyramine, a common substrate for both MAO isoforms, using microsomes of baculovirus-infected insect cells (BTI-TN-5B1-4) as sources for the human recombinant MAO isoforms and the Amplex Red MAO assay kit (Molecular Probes Inc., Eugene, OR). The standard drugs clorglyline (MAO-A) and selegiline (MAO-B) were used as positive controls. The kinetics of the hMAO-B enzyme reaction were determined in the presence of different p-tyramine concentrations (0.12–1.0 mM). In our...
experiments, hMAO-B displayed a Michaelis constant \( K_m \) of 118.8 ± 1.23 μM and a maximal velocity \( V_{\text{max}} \) of 40.4 ± 1.13 nmol p-tyramine/min/mg protein \((n = 3)\). Determined in vitro inhibitory potencies \( I_{50} \) values, selectivity towards hMAO-A and hMAO-B (expressed as the selectivity index, SI), together with the respective \( K_i \) values at hMAO-B (calculated from the mean \( IC_{50} \) for all compounds and reference inhibitors are reported in Table 1. In addition, we estimated the affinities \( K_{\text{HYDE}} \) ranges obtained from the best scored compounds’ docking solutions (see molecular modelling studies) and the thermodynamic parameters of binding to hMAO-B using a novel free energy approximation “HYDE” embedded in SeeSAR v.5.5 software.

**Molecular modelling studies**

Docking studies, estimation, and visualisations of hMAO-B binding affinities were carried out according to a recently developed modelling workflow using LeadIT v.2.1.8 and SeeSAR v.5.5 software packages (both from BioSolveIT GmbH, Sankt Augustin, Germany) with HYDE visual affinity assessment.

**Ligand and protein preparation**

Ligand input structures were taken from the respective single molecule crystal X-ray structures of compounds NTZ-1006, 1034, 1091, and 1041 and used without further preparation. The crystal structure of the hMAO-B enzyme in complex with safinamide (PDB: 2V5Z) was obtained from the Protein Data Bank (PDB).

**Pose generation and docking**

Docking experiments were performed using the FlexX docking module in LeadIT v.2.1.8 with the same procedure as reported previously. LeadIT has accurately reproduced the experimental binding mode of safinamide in 2V5Z and yielded very plausible and well-scored poses on high ranks for all docked compounds discussed in this and earlier works. The ranking of the generated poses corresponds always with the measured binding affinity \( I_{50} \) values of the tested compounds and their best docking conformations. For some compounds, the SeeSAR-integrated docking engine was applied to generate a maximum of 10 poses as output.

**Hyde scoring and visualisation**

The top 23 LeadIT poses were loaded into SeeSAR for post-scoring with HYDE visual affinity assessment. Compounds NTZ-1032 and NTZ-1471 were built using the SeeSAR-integrated editor. SeeSAR visualises the estimated free energy of binding \( \Delta G \) using “coronas” that range from dark red (very unfavourable) to large dark green spheres (very favourable for affinity). The selection of the best poses was based on their visual HYDE scores while also considering a statistics-based torsional analysis. The software enables an interactive assessment of torsions and energies (in kJ/mol) including the desolvation (dehydration, \( -T\Delta S \)) and enthalpic (interaction, \( \Delta H \)) contributions to binding for both protein and ligand. Furthermore, SeeSAR can visualise and quantitatively report the energy contributions for all heavy atoms (with a united atom approach for bound H-atoms) and allows a semi-quantitative estimation of the thermodynamic profile for all tested compounds and safinamide in the co-crystal structure 2V5Z (Figure 2).

**Ligand ADME**

Kinetic solubility of compound NTZ-1034 and octanol-water distribution coefficients (logD) of NTZ-1034, 1091, 1441, and 1471 were determined using a protocol described earlier. Ligand physico-chemical estimations were carried out using the StarDrop model runner algorithms in SeeSAR v.5.5. The in silico physicochemical, drug-like, and in vitro ADME properties of investigated and reference MAO-B inhibitors are summarised in Table 2.

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**Table 1. MAO inhibitory activity and HYDE estimated affinities against hMAO-B.**

| Compound  | IC\textsubscript{50} (nM) | Binding affinity (kJ/mol) |
|-----------|--------------------------|---------------------------|
|           | hMAO-A                  | hMAO-B                    | SI\textsuperscript{a} | \( K_i \) (nM)\textsuperscript{c} | \( K_{\text{HYDE}} \) (nM)\textsuperscript{b} | \( \Delta H \) | \( -T\Delta S \) | \( \Delta G \) |
| NTZ-1006  | >10,000                  | 0.59 ± 0.09               | >16959                | 0.26 ± 0.04               | 2–185            | -38.0          | -6.2           | -44.2          |
| NTZ-1032  | >10,000                  | 0.68 ± 0.04               | 14706                 | 0.30 ± 0.02               | 4–400            | -38.0          | -3.8           | -42.2          |
| NTZ-1034  | >10,000                  | 1.59 ± 0.16               | >6289                 | 0.70 ± 0.07               | 5–464            | -38.0          | -9.9           | -41.8          |
| NTZ-1091  | >10,000                  | 0.39 ± 0.05               | 25,641                | 0.17 ± 0.02               | 0–37             | -38.0          | -7.5           | -45.5          |
| NTZ-1441  | >10,000                  | 0.66 ± 0.06               | >15,151               | 0.29 ± 0.03               | 1–103            | -38.0          | -7.3           | -45.3          |
| NTZ-1471  | >10,000                  | 1.52 ± 0.18               | 6579                  | 0.67 ± 0.08               | 1–112            | -38.0          | -7.3           | -45.3          |
| Selegiline| 1700\textsuperscript{d} | 6.59 ± 1.09\textsuperscript{d} | 258                  | 2.91 ± 0.48               | 8–757            | 0.0            | -41.4          | -41.4          |
| Safinamide| 45,000\textsuperscript{d} | 5.18 ± 0.04\textsuperscript{d} | 5000\textsuperscript{d} | 2.29 ± 0.02               | 2–187            | -21.8          | -22.5          | -44.3          |

\textsuperscript{a}The values are the mean ± SEM \((n = 3)\).

\textsuperscript{b}SI: Selectivity Index = \( IC_{50} \) (hMAO-A)/\( IC_{50} \) (hMAO-B).

\textsuperscript{c}\( K_i \) values were calculated from the experimentally measured \( IC_{50} \) hMAO-B values according to the equation by Cheng and Prusoff: \( K_i = IC_{50} \ (1 + [S]/K_m) \) with substrate concentration of p-tyramine \([S] = 150 \mu M\) and Michaelis constant \( K_m = 118.8 \mu M\).

\textsuperscript{d}\( K_{\text{HYDE}} \) values: estimated HYDE \( K_i \) range values from the compounds’ best docking conformations within the human MAO-B (PDB: 2V5Z).

\textsuperscript{e}\( \Delta G; \) Gibbs free energy.
Solubility studies

The solubility of selected compounds in pure water (Purelab flex) and 50% methanol was measured by a combined HPLC-UV/ESI-MS analysis using different measurement techniques as described in the Supporting Information.

Parallel artificial membrane permeability assay (PAMPA)

The PAMPA permeability of selected compounds and standard drugs across the artificial blood-brain barrier (BBB) lipid membrane was performed by measuring the UV–visible absorbance of compounds in both donor and acceptor compartments, using the PAMPA Explorer kit (Pion Inc., Billerica, MA). Measurements were performed at room temperature with incubation for 4 h under stirring. The $P_e$ and $–\log P_e$ values were processed using the PAMPA Explorer software v.3.8 (Pion) and the data are presented as the mean ± SD (Table 3, Figure 4). The permeability data obtained for the standard drugs were used for method validation (see Table S2, Supporting Information). Among these, verapamil and theophylline were used as references for compounds with high and low permeability, respectively. The plot of the experimental permeability versus the reported values of these commercial drugs gave a good linear correlation with $–\log P_e (\text{rep}) = 1.007 - 0.494 \log P_e (\text{rep}) + 0.1334$ and $R^2 = 0.9046$ (see Figure S3). Based on this equation and considering the well-established limit for BBB permeation ($P_e > 4 \times 10^{-6} \text{ cm/s}$), compounds NTZ-1091, 1441, and 1471 can be classified as highly BBB permeable, indicating that these should cross the BBB and reach the CNS therapeutic targets (CNS+).

Metal binding studies

Binding ability of compounds with the Fe$^{2+}$ and Fe$^{3+}$ ions was determined by measuring the UV–vis absorbance spectra of the respective compound in the absence and in the presence either of FeCl$_3$ or FeSO$_4$. Deferiprone (DFP) was used as a standard chelating agent.

Results and discussion

All compounds under investigation were prepared by amide coupling reactions of differently substituted amines with $N$-unsubstituted carboxylic acids or their $N1$-methylated analogues for the formation of $N$-unsubstituted indazole-5-carboxamides (designated subclass I, compounds NTZ-1006, 1032, and 1034) or $N1$-methylated indazole-5-carboxamides (subclass II, NTZ-1091, 1441, and 1471), respectively. Therefore, the main goal of this study was to investigate the role of the indazole N1 position for MAO-A/B selectivity and inhibitory activity as well the effects of this structural modification on different physicochemical and
biophysical properties within indazole-5-carboxamides of subclass I and II. In general, all tested compounds are highly potent and selective inhibitors of hMAO-B isoenzyme with IC_{50} values in the subnanomolar or even picomolar range (Table 1). The 3,4-dichlorophenyl-substituted compounds NTZ-1006 (hMAO-B, IC_{50} = 0.56 nM; SI >16,000) and NTZ-1091 (hMAO-B, IC_{50} = 0.39 nM; SI >25,000) were found to be the most potent and selective MAO-B inhibitors in both series, being ~9-fold and >13-fold more potent against hMAO-B than the approved drug safinamide. Compared to the irreversible MAO-B inhibitor selegiline, compounds NTZ-1006 and NTZ-1091 display a >11- and ~17-fold increase in inhibitory activity against hMAO-B, respectively. The IC_{50} values and SI slightly decrease within both subclasses with decreasing the lipophilic character (Cl vs. F) of the compounds as follows: 3,4-di-Cl (NTZ-1006, 1091) > 3-Cl, 4-F (NTZ-1032, 1441) > 3,4-di-F (NTZ-1034, 1471). The introduction of a methyl substituent at the indazole N1 position of subclass I compounds led to a slight increase in inhibitory potency and selectivity of subclass II compounds toward hMAO-B, indicating that small lipophilic substituents at the indazole N1 atom are well-tolerated by hMAO-B. None of the tested compounds exhibited inhibitory activity against the hMAO-A isozyme at the initial tested concentration of 10 μM.

In order to validate the biological testing results and to gain insight into the binding thermodynamics of the investigated compounds against hMAO-B, we computed their binding modes with LeadIT using the X-ray co-crystal structure of hMAO-B with the inhibitors NTZ-1006 and NTZ-1091, an elevated fluorescence of NTZ-1006 and 1091. The obtained results clearly indicate that the compounds NTZ-1006 and NTZ-1091 are reversible inhibitors of hMAO-B. The binding modes and estimated affinities strongly suggest that compounds NTZ-1006 and 1091 occupy the same substrate cavity region within the binding pocket of hMAO-B, provided that both ligands are very compatible with the active site and do not covalently bind to flavin adenine dinucleotide (FAD) cofactor (Figure 2(A)).

### Table 3: Solubility and PAMPA-BBB data for selected compounds.

| Compound | Solubility in water | Solubility in 50% methanol | PAMPA-BBB | P_{bb} (×10^{-6} cm/s) |
|----------|---------------------|---------------------------|-----------|------------------------|
| NTZ-1091 | 5.62 ± 0.17         | 17.6 ± 0.5                | 156 ± 19  | 0.49 ± 0.06             |
| NTZ-1441 | 42.1 ± 2.3          | 136 ± 5                   | 609 ± 49  | 2.04 ± 0.18             |
| NTZ-1471 | 61.6 ± 9.6          | 215 ± 33                  | 610 ± 26  | 2.12 ± 0.09             |

The values are the mean ± SD (n > 3).

PAMPA blood-brain barrier (BBB) values were determined under stirring at the pH value of the Prisma HT buffer (Pion).
drug and radioligand candidates. Overall, the physicochemical properties for the investigated compounds are in the suggested strict limits for drug-likeness of CNS active (CNS+) drugs (MW ≤400, HBA <7, HBD <3, and tPSA <70 Å²)\(^{(16,25–27)}\). Oral bioavailability is a multifactorial property, primarily driven by the gastrointestinal (GI) absorption (expressed as %ABS). Furthermore, the topological polar surface area (tPSA) is a key descriptor that correlates with passive transport through membranes (GI and BBB) and, therefore, used for calculation of %ABS\(^{(28)}\). The tPSA values for all compounds are in the range of 46.9–57.8 Å² and, consequently, the %ABS ranges from 89.1 to 92.8%, indicating that all investigated compounds are expected to be orally bioavailable (%ABS ≥60%) and classified as good brain penetrable (tPSA ≤60 Å²) CNS drug candidates\(^{(25,29,30)}\). To further predict the BBB permeability of compounds, we also calculated their blood (plasma)-brain coefficients (logBB)\(^{(31)}\). The logBB values for all tested compounds are in the suggested limit for BBB permeable drugs (logBB >–1)\(^{(25)}\), being even higher than the one of the approved drug safinamide (logBB = –0.083).

Solubility and lipophilicity are key ADME parameters that affect pharmacokinetics and pharmacodynamics of drugs. The measured and calculated water-solubility (expressed as logS\(_{7.4}\)) and distribution coefficients (logD\(_{7.4}\)) for the tested compounds are in the ideal range (logS\(_{7.4}\) ≥–5.0, logD\(_{7.4}\) = 1–4)\(^{(31)}\), suggesting a good
The solubility-lipophilicity balance that is suitable for GI absorption by passive membrane permeation after oral administration is a key consideration. The ligand-lipophilicity efficiency (LLE), a drug-like multiparameter that combines lipophilicity and in vitro potency (pIC50 or pKᵢ), is critical for further in vivo evaluation. The investigated compounds show optimal physicochemical, drug-like, and ADME properties. Compounds NTZ-1441 and 1471 can be highlighted because of their improved drug-likeness (MW = 287–303, %ABS = 92.8%; logS > 4.0, logD > 3, LLE > 6–7) and predicted brain permeability combined with optimal hMAO-B inhibitory activity (pIC50 > 8.0). Subsequently, we evaluated subclass II compounds for their BBB permeability using PAMPA-BBB assay.

Figure 5. UV–visible studies of Fe²⁺ or Fe³⁺ chelating ability of deferiprone (DFP) in the absence or presence of 10 mM FeSO₄ (A) and FeCl₃ (B) and compounds NTZ-1006 (black line), NTZ-1032 (red line), and NTZ-1441 (blue line) before and after addition of 40 μL of 10 mM FeSO₄ (C), or FeCl₃ (D). Superposition UV–visible spectra were obtained by measurement of absorbance of compound alone (50 μM) and FeSO₄ or FeCl₃ without compound (both 130 μM) in DMSO 50%.

Considering the growing interest in the development of multi-efficient drugs including iron chelating agents for the treatment of AD, we have preliminary investigated compounds NTZ-1006, 1032, and 1441 for their ability to bind di- and trivalent iron ions (Figure 5). Therefore, we measured the UV–visible absorbance of these compounds in the absence and in the presence either of Fe²⁺ or Fe³⁺ and compared this behaviour with that obtained for the reference drug DFP. As expected, DFP exhibited higher chelating affinity against Fe³⁺ ions. However, its UV–visible spectra were significantly changed by the addition either of FeSO₄ or FeCl₃ resulting in an increase in the specific profile at 521 or 565 nm, respectively (Figure 5(A) and (B)). The N₂-methylated compound NTZ-1441 did not have the ability to bind iron cations. Compounds NTZ-1006 and 1032 show similar chelating ability. The maximum absorbance of both compounds was not modified by the addition of Fe³⁺, whereas a slight increase of the maximum absorbance was observed when FeSO₄ was added to the respective solutions of NTZ-1006 or 1032, indicating that both compounds selectively interact with Fe²⁺ ions.

Conclusion
In conclusion, newly discovered N'-unsubstituted indazole-5-carboxamide derivatives (subclass I compounds) and their N1-methyl
analogues (subclass II compounds) are subnanomolar potent, reversible and competitive MAO-B inhibitors with the ability to selectively bind Fe^{3+} ions, as measured for compounds NTZ-1006 and 1032. The reversible mode of binding within the binding pocket of the human MAO-B enzyme was investigated using time-dependent studies; it was confirmed by applying a novel modelling and visualisation technique. In general, all investigated compounds exhibit suitable drug-like properties required for CNS active drugs (LLE >5.5, logD = 2–4, MW 287–320 Da). Optimisation of the indazole moiety in subclass I compounds has resulted in the discovery of compounds NTZ-1441 and NTZ-1471, highly BBB permeable, water-soluble MAO-B inhibitors that combine both well-balanced physicochemical and in vitro ADME properties with remarkable hMAO-B activity and selectivity against the hMAO-A isoform. The compounds all together appear as promising drug and radioligand candidates for the therapy and diagnosis of PD, AD, and other CNS diseases.

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Disclosure statement
The authors declare the following interests: N.T.T. is founder of NTZ Lab, a startup research and development company that synthesises and explores markets for the presented compounds.

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