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Targeting Bacterial and Human Levodopa Decarboxylases for Improved Drug Treatment of Parkinson's Disease: Discovery and Characterization of New Inhibitors

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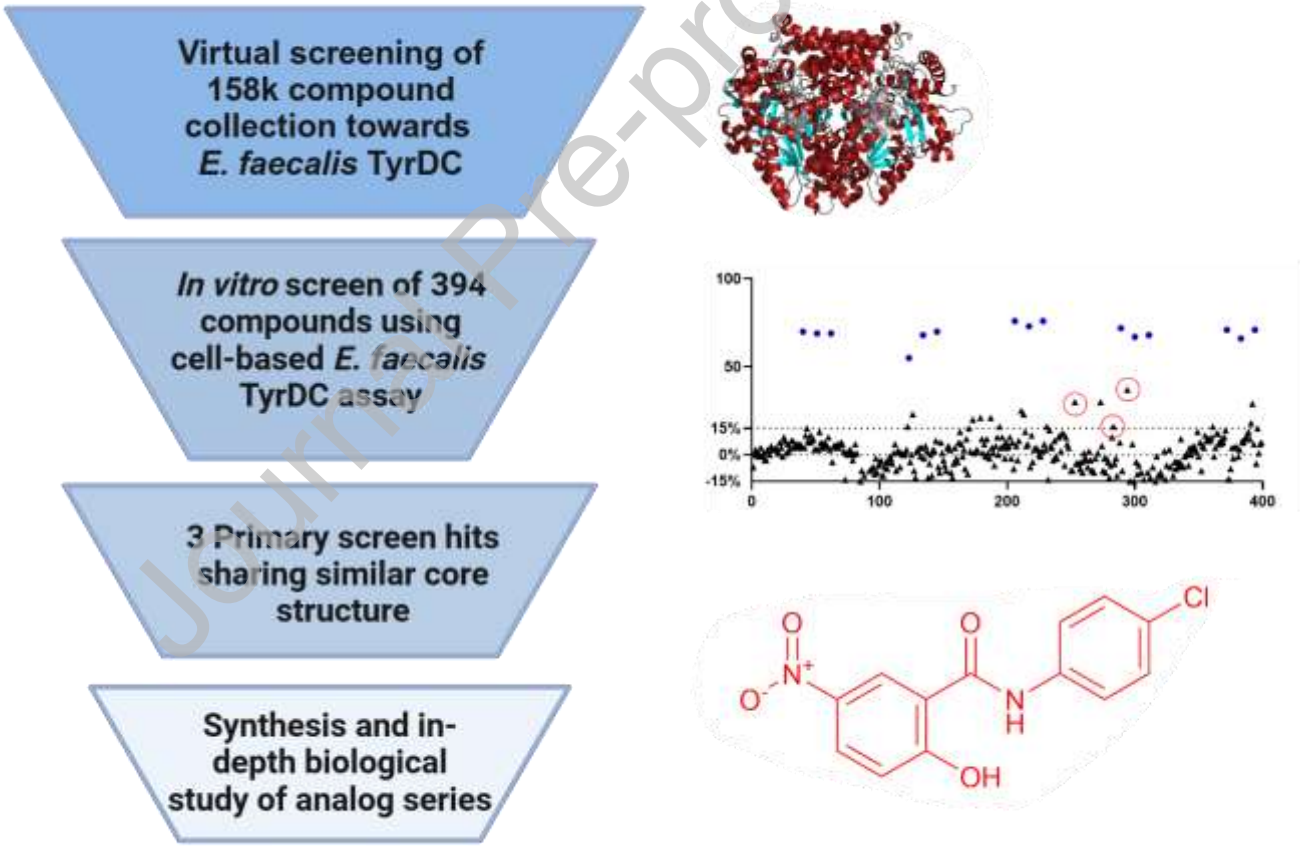
Graphical Abstract



ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disease, typically treated with levodopa, which alleviates the motor symptoms of the disease. However, levodopa metabolism in peripheral tissues hampers its bioavailability and leads to undesired side-effects. Therefore, co-administration of amino acid decarboxylase (AADC) inhibitors is necessary, but still, up to 50% of levodopa may not reach the brain. Recent evidence suggests that gut microbes, especially *Enterococcus faecalis*, are also able to metabolize levodopa and affect the bioavailability by utilizing microbial tyrosine decarboxylase (TyrDC) enzyme. The main aim of this study was to develop inhibitors targeting gut microbial and host decarboxylation of levodopa. First, a virtual screen of a library of 158,000 compounds against *E. faecalis* TyrDC was conducted, combining three methods: molecular docking against the *E. faecalis* TyrDC homology model, structure-based pharmacophore model, and shape similarity searches based on levodopa, carbidopa (AADC inhibitor) and (*S*)-*α*-fluoromethyltyrosine (TyrDC inhibitor). A total of 394 compounds were selected and tested *in vitro* by using a cell-based *E. faecalis* assay measuring inhibition of levodopa metabolism. Three most active compounds (49-92% inhibition at 100 μM) sharing a similar scaffold and a set of commercially available and in-house synthesized analogs were then assessed against purified TyrDC and AADC enzymes. The IC_{50} values for the most potent compounds for TyrDC and AADC inhibition were 23 μM / 144 μM (compound **1**), 42 μM / 199 μM (compound **2**) and 51 μM / 182 μM (compound **3**), respectively. These compounds also displayed cytotoxic effects on HeLa cells and modest antibacterial activity against *E. faecalis* at the same concentration range. The core structure of the compounds presented here can serve as a starting point for the development of a new inhibitor class against TyrDC and AADC, and offers a promising avenue for personalized PD treatment, particularly for patients with high levels of gut microbes expressing the levodopa metabolizing TyrDC enzyme.

Keywords: Levodopa, Parkinson's disease, Gut microbiome, Tyrosine decarboxylase

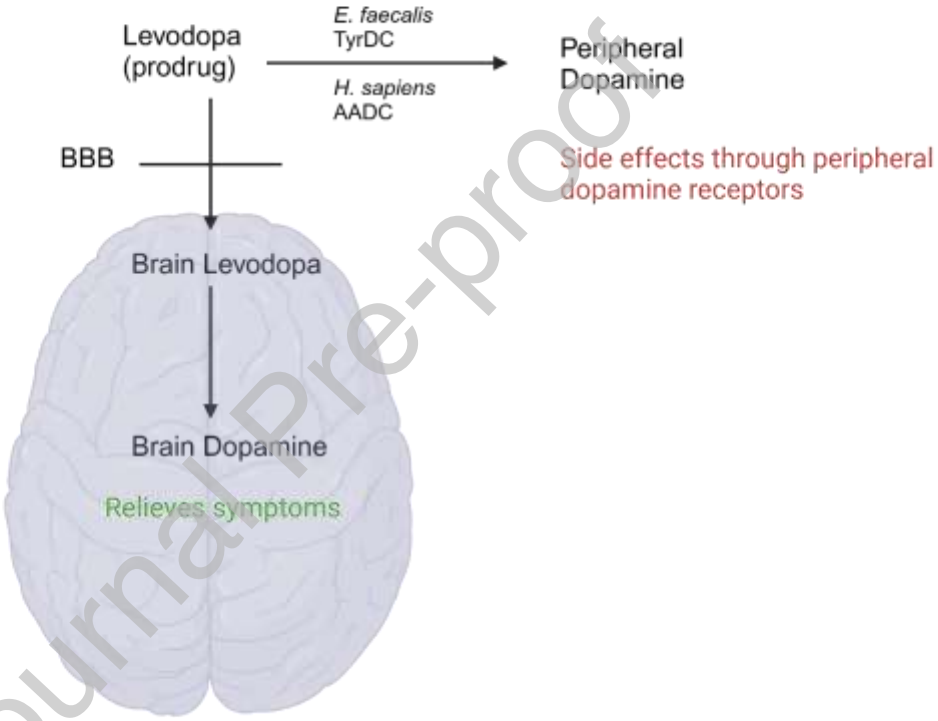
1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease that affects approximately 1% of people over 60 years of age (Hornykiewicz, 2017; Post et al., 2007). PD pathology is characterized by dopaminergic neurodegeneration in the substantia nigra area of the brain and accumulation of protein aggregates known as Lewy bodies in neurons that result in hallmark symptoms of the disease: tremor, rigidity, and bradykinesia (Kalia and Lang, 2015). Levodopa, a prodrug of dopamine, is the most prescribed drug used to manage the motor symptoms of PD that result from dopaminergic neuron death (Hornykiewicz, 2017). Levodopa is converted into dopamine by the enzyme aromatic amino acid decarboxylase (AADC) both in the brain and in the peripheral tissues (Morgan et al., 1971) (Fig. 1). However, dopamine formed in the periphery cannot enter the brain because of the presence of the blood-brain barrier (BBB), which is why peripheral AADC inhibitors such as carbidopa or benserazide are typically co-administered with levodopa (Deleu et al., 2002). Without the use of AADC inhibitors, only about 1-5% of levodopa can reach the central nervous system (Morgan et al., 1971; Nutl and Fellman, 1984). Moreover, as peripherally formed dopamine is not able to pass through BBB, it can cause multiple side effects such as hypotension, nausea, vomiting, and cardiac arrhythmia through the activation of vascular dopamine receptors and *α*-adrenoreceptors or *β*-adrenoreceptors (Goldenberg, 2008). AADC inhibitors increase the amount of levodopa that reaches the brain and also reduce unwanted side effects caused by excess peripheral metabolism of levodopa (Hauser, 2009; Whitfield et al., 2014). However, despite the use of AADC inhibitors, up to half of levodopa is metabolized into dopamine in the gut (Nutl and Fellman, 1984).

Gut microbiota can alter the pharmacokinetics of various drugs by metabolizing them in the gastrointestinal lumen (Spanogiannopoulos et al., 2016). Gut microbes harboring tyrosine decarboxylase (TyrDC) enzyme can convert levodopa into dopamine, potentially further reducing its bioavailability (Maini Rekdal et al.,

2019; van Kessel et al., 2019). Van Kessel et al. discovered that TyrDC gene abundance in fecal samples of PD patients positively correlates with higher daily levodopa dosage (van Kessel et al., 2019). Further, a recent clinical study conducted by Zhang et al. involving 101 PD patients showed that higher abundance of microbial TyrDC gene in fecal samples of PD patients was associated with lower levodopa responsiveness levodopa challenge test, which is used to assess the drug response of PD patients (Zhang et al., 2022). Hence, compounds inhibiting TyrDC could offer a novel strategy for personalized medical treatment for PD patients with a high abundance of TyrDC-expressing bacteria.

Fig. 1. Metabolism of levodopa to dopamine in the periphery and brain. When levodopa is administered, it



crosses the blood-brain barrier (BBB) and is metabolized to dopamine which relieves the PD symptoms. This is crucial because dopamine itself cannot cross the BBB, which is why levodopa is used as the treatment instead. However, levodopa is also decarboxylated peripherally by both gut bacterial and human decarboxylases. This results in reduced bioavailability and increased side effects that are caused by formation of peripheral dopamine in the gut.

TyrDC and AADC enzymes both belong to the α-family of pyridoxal-5'-phosphate (PLP)-dependent enzymes (Christen and Mehta, 2001). They catalyze decarboxylation reactions and utilize the phosphorylated form of pyridoxal (vitamin B_6) as cofactor (Sandmeier et al., 1994). TyrDC gene has been identified in different microbial species, most of which belong to the genera *Lactobacillu*s and *Enterococcus* (Maini Rekdal et al., 2019). In the human gut microbiome, most levodopa metabolizing TyrDC enzyme activity has been identified in the genus *Enterococcus*, particularly *Enterococcus faecalis* (Maini Rekdal et al., 2019). The TyrDC enzyme is involved in the amino acid metabolism of bacteria, converting L-tyrosine into tyramine. From an evolutionary point of view, the decarboxylation of L-tyrosine into tyramine gives

microbes utilizing the TyrDC system a competitive edge in acidic and nutrient-depleted environments (Pereira et al., 2009).

Current human AADC inhibitors are mainly substrate analogs that bind covalently to the PLP cofactor of AADC (Wu et al., 2011). However, bacterial TyrDC is not inhibited by these compounds (Maini Rekdal et al., 2019; van Kessel et al., 2019). Maini Rekdal et al. were able to identify a small molecule capable of inhibiting TyrDC, known as (*S*)-*α*-fluoromethyltyrosine (AFMT) (Maini Rekdal et al., 2019). Nevertheless, AFMT does not inhibit the human AADC enzyme (Maini Rekdal et al., 2019). Therefore, our main aim for this study was to discover enzyme inhibitors against both gut microbial and host decarboxylation of levodopa that could potentially further increase the bioavailability of levodopa treatment and reduce unwanted side effects caused by the peripheral metabolism over the standard levodopa /human AADC inhibitor treatment.

Previously, the metabolism of levodopa by *E. faecalis* was studied under anaerobic conditions (Maini Rekdal et al., 2019; van Kessel et al., 2019). To develop a method that is suitable for screening with an increased throughput, we developed an *E. faecalis* cell-based screening assay that works under aerobic conditions. The compounds screened experimentally were selected by virtual screening from the Institute for Molecular Medicine Finland (FIMM; library version 2021) compound collection. Based on the virtual screening results, 394 compounds were tested *in vitro* using the *E. faecalis* assay. The three most promising compounds along with 13 similar compounds were further studied by target-based assays using recombinant *E. faecalis* TyrDC and *H. sapiens* AADC enzymes as well as for mammalian cell toxicity and antibacterial effects.

In the present study, by using a combination of *in silico* and *in vitro* evaluation methods, we identified and characterized a novel class of compounds that inhibit host and gut microbial metabolism of levodopa. The core structure of the compounds presented here can serve as a starting point for the development of a new inhibitor class against TyrDC and AADC enzymes.

2. Materials and methods

2.1 *In silico* selection and primary screen *in vitro*

A virtual screening protocol was first developed to identify potential TyrDC enzyme inhibitors (see Supporting Information S1. for details). This protocol involved three complementary computational approaches: molecular docking, structure-based pharmacophore filtering, and shape similarity searching, all implemented using Schrödinger computational modeling tools (Schrödinger LLC, NYC, NY, USA). The virtual screening was conducted on a diverse compound library from the Institute for Molecular Medicine Finland (FIMM, library version 2021), comprising 158,000 compounds that are readily available for subsequent experimental testing.

We first constructed a homology model of *E. faecalis* TyrDC based on the crystal structure of the holo form of *Lactobacillus brevis* (75% sequence identity, PDB code: 5HSJ), the best template available at the time (Supporting Information S1.1, Fig. S1-S4). The binding pocket was defined around critical residues involved in TyrDC substrate binding. Molecular docking of the FIMM library against the *E. faecalis* TyrDC homology model was performed using GLIDE (Schrödinger LLC, NYC, NY, USA) and standard parameters. A structure-based pharmacophore model was developed using PHASE (Schrödinger LLC, NYC, NY, USA) (Supporting Information S1.2, Fig. S5). This model was derived from essential protein residues required for TyrDC substrate binding. The pharmacophore model was then employed to filter the FIMM library, selecting compounds that matched the defined pharmacophoric features. A shape similarity search was conducted using SHAPE (Schrödinger LLC, USA). Three shape queries were generated from the known inhibitors carbidopa, levodopa, and (*S*)-*α*-fluoromethyltyrosine. The compounds in the FIMM library were aligned to these queries based on overlapping hard-sphere volumes, and similarity scores were computed.

The highest-scoring molecules (80 per model) were selected from each list of chosen molecules. Overlapping molecules that were present across models were not selected twice; instead, in case of an overlap the next highest-scoring unique molecule was chosen, resulting in a total of 400 compounds for *in vitro* evaluation. The compounds were plated by using an Echo liquid handler (Beckman Coulter, CA, USA) at the Institute for Molecular Medicine Finland (FIMM), High Throughput Biomedicine Unit. A total of 394 compounds (Table S1) could be physically obtained and were tested experimentally at 10 μM in singles using *E. faecalis* cell-based assay (see below). Primary screen hits were then repurchased from various suppliers (Table S2) and retested to confirm activity. As a result, three compounds, compounds **1-3**, were identified as active and selected for follow-up studies (see Fig. 3 for compound structures; Supporting Information S5.3 for interactions suggested by molecular docking, Fig. S6-S8).

2.2 Compounds 1-16

Compounds **1-3** that were identified as actives in the first stage were re-purchased from Specs (Zoetermeer, The Netherlands). Compounds **4-8** were synthesized in-house based on the structures of the active compound class (Supporting Information S2, Fig. S9). Compounds **9-12** were commercially available analogs identified by performing a substructure search on the FIMM compound collection as well as ZINC and ChemDiv databases using the shared core substructure as query. These were obtained from ChemBridge (San Diego, CA, USA; compound **9**) and Specs (Zoetermeer, The Netherlands; compounds **10-12**). Compounds **13-16** are known antiparasitic drugs in clinical use sharing structural similarity to the active compound class and were purchased from Ambeed, Inc., IL, USA. The commercially purchased compounds (compounds **1-3**, and **9-16**) were reported to be > 95% pure by the suppliers. Compounds were dissolved in DMSO at 10 mM concentration and diluted into assay buffer during the experiments.

2.3 *Enterococcus faecalis* cell-based assays

Compounds were initially screened at 10 μM in singles using cell-based assay in half-area 96-well microplate and 100 μL final volume per well. *Enterococcus faecalis* ATCC 29212 was maintained on LB-agar at 4 °C. On the day before the experiment, a single colony was inoculated into 20 mL of Brain Heart Infusion (BHI) broth and incubated overnight at 37 °C, 220 rpm shaking under aerobic conditions. On the following morning, cell-density was measured using a densitometer (DEN-1, BioSan, Riga, Latvia) and the bacteria were diluted to a final concentration of 1.5×10^6 bacteria/well in BHI supplemented with 100 μM levodopa and 10 μM of the test compound. The bacteria were then incubated for 12 h at 37 °C and 220 rpm shaking. After incubation, the well plates were centrifuged at 2000 × *g* for 10 min to pellet down the bacteria and samples of supernatant were collected and analyzed using high performance liquid chromatography system with electrochemical detection (HPLC-ECD). Percent inhibition was calculated from dopamine produced relative to no inhibitor control using 15% as the hit cut-off limit. To confirm primary screen hits, follow-up assays followed the same protocol, with the exception that compounds were retested at 100 μM, 30 μM and 10 μM (Table S2). AFMT (Hong Kong Chemhere Co., Ltd., Hong Kong, China) was used as a positive control in all experiments. The amount of solvent (DMSO) was always adjusted to match controls. Percent inhibition was calculated from dopamine formed relative to the non-inhibited controls. Potential antibacterial effects of compounds **1-16** against *E. faecalis* were assessed by measuring the absorbance at 620 nm during the assays.

2.4 HPLC-ECD analysis of samples

The system consisted of a mobile phase (0.1 M NaH_2PO_4, pH 4.5, 8% v/v MeOH and 300 mg/mL sodium 1-octanesulfonate), electrochemical detector (ESA Coulochem model 5100A detector and a model 5014B cell; Esa InC., Chelmsford, MA, USA) and a reverse-phase C18 column (Kinetex 150 mm × 4.6 mm, 2.6 μm particle size; Phenomenex, CA, USA) which was kept at 45 °C. Supernatant samples from cell-based assays were filtered and 20 μL of the filtrate was injected directly into HPLC system using a flow rate of 0.8 mL/min.

2.5 Expression and purification of *E. faecalis* TyrDC and *H. sapiens* AADC

E. coli BL21(DE3) strains harboring pET-28 plasmid encoding either *E. faecalis* MMH594 TyrDC or *H. sapiens* AADC gene as cDNA were received as a gift from Prof. Emily Balskus, Harvard University, and were verified by Sanger sequencing. Starter cultures were grown from individual colonies overnight in 20 mL of Terrific Broth (TB) containing 50 µg/mL kanamycin and were then inoculated 2% v/v into 1 L of TB + 50 µg/mL kanamycin. Bacteria were grown in shaking flasks at 37 °C and 220 rpm until OD600 reached 0.4 – 0.5, at which point isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added at 0.2 mM to induce the protein expression. After induction, bacteria were grown for an additional 18 h at 16 °C with 220 rpm shaking.

The protein purification was carried out using Bio-Rad NGC system (CA, USA). For purification of recombinant TyrDC or AADC, the culture was harvested by centrifugation (5000 × *g* for 10 min), and the resulting pellet was resuspended in 40 mL of 50 mM Tris pH 7.4 containing 0.25 M NaCl, 150 µg/mL lysozyme and protease inhibitors (Roche, Basel, Switzerland), followed by lysis using sonicator (10 bursts of 6 s with 20 s cooling between pulses). Cell debris was removed by centrifugation (13 000 × *g*, 45 min) and the clarified lysate was loaded onto 2 mL of NiExcel resin (Cytiva), washed using 40 mM imidazole and eluted using 500 mM imidazole (in 50 mM Tris pH 7.4 containing 0.25 M NaCl). Eluted fractions containing protein were combined, filtered and concentrated through Amicon 50 000 MWCO filters, followed by gel filtration through Superdex 200 Increase 10/300 GL (Cytiva) at 0.5 mL/min in 25 mM Tris pH 7.4 + 150 mM NaCl. Eluted fractions containing protein were analyzed using SDS-PAGE (Fig. S10), and fractions containing pure TyrDC or AADC were combined and stored at –80 °C in aliquots at a final concentration of 24 µM (TyrDC) or 8 µM (AADC).

2.6 Enzyme assays and IC_{50} determination

The assay for TyrDC inhibitory activity was carried out in 0.1 M sodium acetate buffer, pH 5.5. The final concentrations in the reaction were 500 µM of levodopa, 150 nM PLP, 150 nM TyrDC, and varying concentration of test compounds. The reaction mixture was first preincubated for 15 min at room temperature without levodopa, followed by 15 min incubation with the substrate after which the reaction was quenched by diluting the mixture 1:2 in MeOH. The amount of solvent (DMSO) was always adjusted to match controls. To measure the inhibitory activity of compounds towards AADC, the same reaction conditions and concentrations were used, except that the assay was conducted in 0.1 M Na_2HPO_4 buffer at pH 7.4.

The final inhibitory activity in both enzyme assays was determined by measuring the amount of dopamine formed using a fluorometric assay modified from Medici et al. (Médici et al., 2011). Briefly, 80 µL of the sample from the assay was added into 155 µL of detection reagent solution containing 1 mM 1,2-diacetylbenzene and 1 mM *β*-mercaptoethanol. After 3 h incubation at room temperature, fluorescence intensity of the formed product was measured using wavelengths λ_{ex} = 355 nm and λ_{em} = 445 nm (Varioskan Lux, Vantaa, Finland). Percent inhibition was calculated as dopamine formed relative to the non-inhibited control reaction. AFMT was used as a positive control in TyrDC inhibitory assays while carbidopa (Orion Pharma, Espoo, Finland) was used as control in AADC assays. Dose-response curves (Fig. S11) and IC_{50} values were calculated in GraphPad Prism (version 9.5, San Diego, CA, USA) using nonlinear curve fit. Enzyme assays were performed in triplicates and repeated once independently.

2.7 Cytotoxicity assay and CC_{50} determination

HeLa cells (ECACC 93021013) were seeded in 96-well plates in 200 µL growth medium (MEM medium supplemented with 10% FBS) at 5000 cells/well and incubated at 37 °C in 5% CO_2 incubator for 24 h. Cells were treated with various concentrations of compounds and cytotoxicity was evaluated at 48 h post treatment using ATP-based CellTiter-Glo® assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions. To calculate relative cytotoxicity, the readings for each compound concentration were

normalized to vehicle (DMSO) controls. Camptothecin (Sigma-Aldrich, MA, USA) was used as a positive control at 10 µM. Dose-response curves and CC_{50} values were calculated in GraphPad Prism (version 9.5, San Diego, CA, USA) using nonlinear curve fit. Cytotoxicity assays were performed in triplicates over a range of eight concentrations and repeated once independently.

3. Results and discussion

Parkinson's disease is amongst the most common neurodegenerative disorders worldwide. One of the primary therapeutic strategies for PD involves increasing dopamine levels in the substantia nigra of the brain, commonly accomplished through oral administration of levodopa. It has been shown previously that gut microbes, particularly *Enterococcus faecalis*, harboring TyrDC enzyme are able to metabolize levodopa rapidly into dopamine. Here, we aimed to discover TyrDC inhibitors by starting with *in silico* selection and cell-based primary screen, and followed by synthesis and search for commercially available analogs, evaluated by a comprehensive set of follow-up studies.

3.1 *In silico* selection and primary screening *in vitro*

A virtual screen was conducted, combining different search methodologies. Very limited overlap was found between the methods, which have a very low probability to pick the same compounds in a pool of 158.000 compounds. In the end five hit lists of 80 compounds were selected, totaling 400 compounds.

Based on the virtual screening of FIMM compound collection, 394 compounds (Table S1) that were available through FIMM were screened experimentally for inhibition of levodopa conversion using the cell-based *E. faecalis* assay (Fig. 2). Prior to the screen, the assay was validated by measuring the efficiency of levodopa metabolism, bacterial growth and the inhibitory effect of the known inhibitor AFMT over time (Fig. 3A). As expected, AFMT at 100 µM effectively inhibited the levodopa conversion to dopamine, displaying 88% inhibition of levodopa conversion relative to the non-inhibited bacteria after 8 h incubation (Fig. 3A).

The primary screen at 10 µM concentration indicated 17 compounds showing > 15% inhibition (Fig. 3C, Table S1), reaching at max. 37% inhibition. Positive control, AFMT, at 10 µM inhibited the levodopa conversion by 69% ± 5% during the screen (Fig. 3C). The 17 primary hits were repurchased from various suppliers (Table S2) and retested at 100 µM, 30 µM and 10 µM to confirm the activity. Twelve compounds did not show any notable inhibition (Table S2) in the follow-up assays, but three compounds (**1**-**3**, Fig 3A) sharing similar nitrobenzamide core structure were active upon retesting. Next, we established target-based assays for both bacterial TyrDC and human AADC enzyme assays for the follow-up phase. Metabolism of levodopa by these enzymes was assessed by using a fluorometric assay modified from Medici et al. (Médici et al., 2011). Both enzymes metabolize levodopa, but the substrate shows higher affinity towards AADC compared to TyrDC (Fig. 3B).

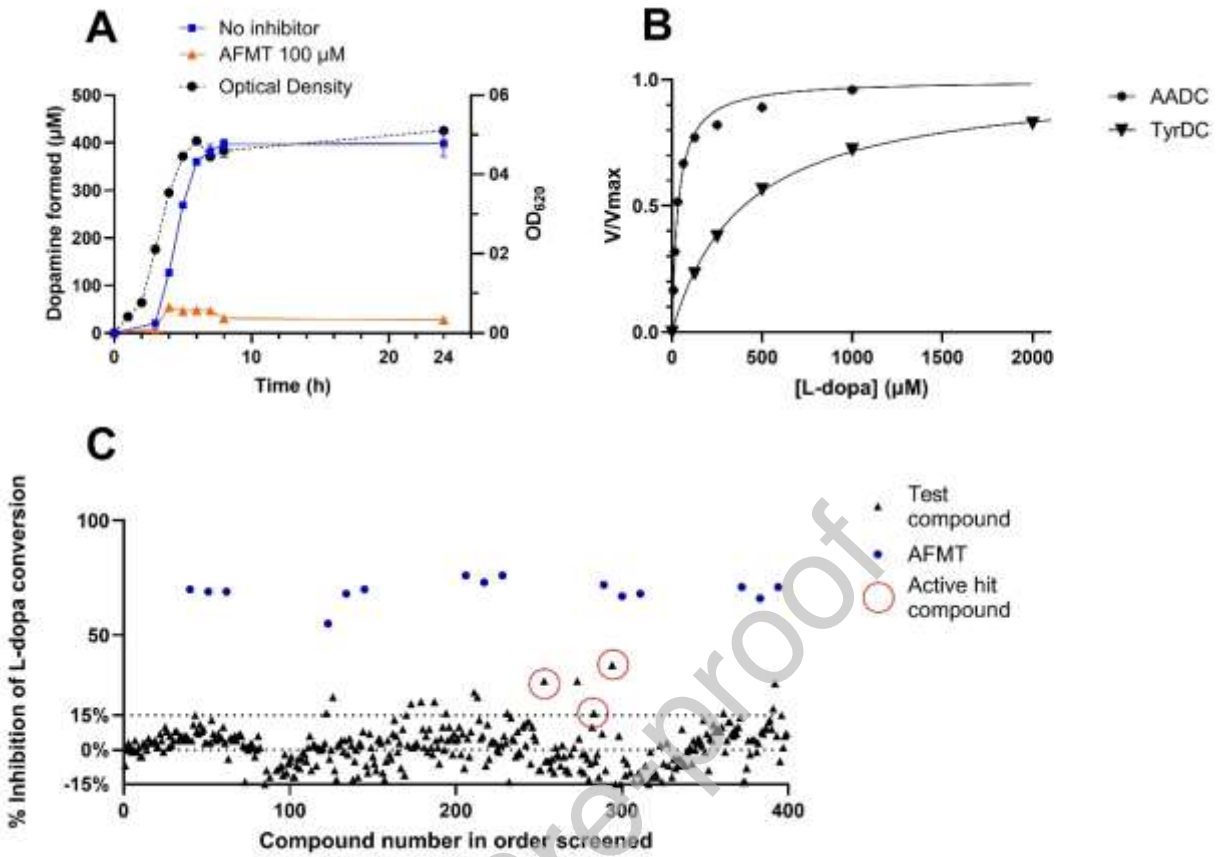


Fig. 2. **Metabolism of levodopa by *E. faecalis* ATCC 29212 and AADC/TyrDC enzymes and results of the initial FIMM compound *in vitro* screen**. (A) Formation of dopamine over time in *E. faecalis* ATCC 29212 culture without inhibitor and within the presence of 100 µM TyrDC inhibitor AFMT. The bacteria were grown in BHI broth supplemented with 500 µM levodopa under aerobic conditions, and optical density (OD) readings were measured at 620 nm to assess bacterial growth. Data represents mean of three technical replicates. (B) Michaelis-Menten enzyme kinetics of levodopa towards purified TyrDC and AADC enzymes. (C) Results of primary *in vitro* screen of 394 FIMM library compounds. AFMT (10 µM) was used as a positive control.

3.2 Follow-up studies on compounds 1-3

We co-incubated *E. faecalis* with levodopa and compounds **1-3** at various concentrations. We found that compounds **1-3** dose-dependently inhibit the conversion of levodopa into dopamine in *E. faecalis* ATCC 29212 culture supplemented with levodopa (Fig. 3B). Compounds **1**, **2** and **3** displayed up to 78%, 77% and 67% inhibition of levodopa conversion at 300 µM, respectively. These compounds exhibited good activity also in TyrDC enzyme assay (Fig. 3C, Table 1) with IC_{50} values at low micromolar concentrations similar to AFMT. Moderate activity was observed towards the AADC enzyme with IC_{50} values at higher micromolar concentrations (Fig. 3D, Table 1). Interestingly, compounds **1-3** share similar chemical features, i.e., *para*-nitrophenyl and benzamido moieties.

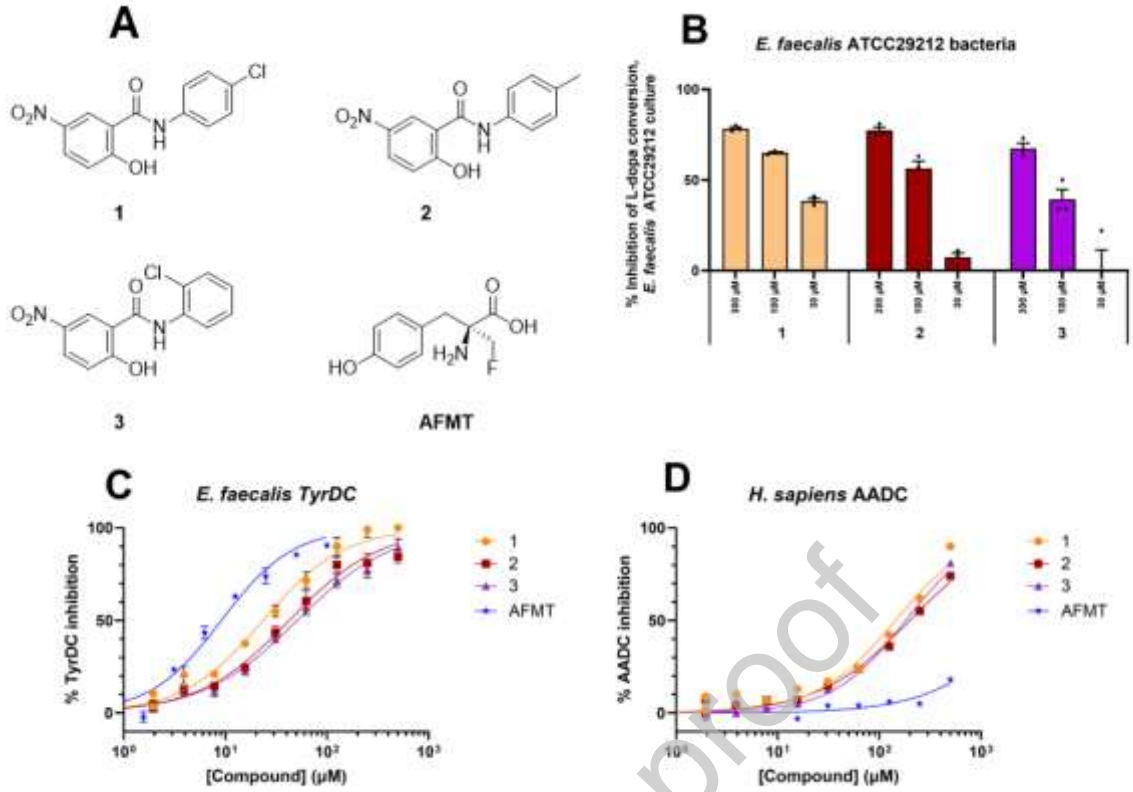


Fig. 3. Enzyme-catalyzed conversion of levodopa into dopamine is inhibited by compound class containing *para*-nitrophenyl and benzamido moieties. (A) Chemical structures of active compounds **1-3** identified in primary cell-based *E. faecalis* screen along with AFMT. (B) Inhibitory effects of AFMT and compounds **1-3** (at 30-300 μM) on conversion of levodopa into dopamine in *E. faecalis* ATCC 29212 culture supplemented with levodopa. (C) Dose-response curves of compounds **1-3** and AFMT towards recombinant *E. faecalis* MMH594 tyrosine decarboxylase. (D) Dose-response curves of compounds **1-3** and AFMT towards recombinant *H. sapiens* AADC. The percent inhibition was measured as dopamine produced relative to no inhibitor control and error bars represent the mean ± SEM of three replicates.

3.3 TyrDC and AADC inhibition by synthesized and commercially available analogs

To gain preliminary insights into the structure-activity relationships (SARs), we synthesized a small set of analogs (compounds **4-8**, Fig. 4)**,** changing the position of hydroxyl substituent and/or introducing an additional hydroxyl group in our hit compounds. Among these, compounds **4** and **6** have an additional hydroxyl group, while compounds **5** and **7** have hydroxyl groups at different positions compared to the original hit compound **1**. Additionally, we synthesized compound **8** to examine the effect of removing the nitro group present in hit compound **1**.

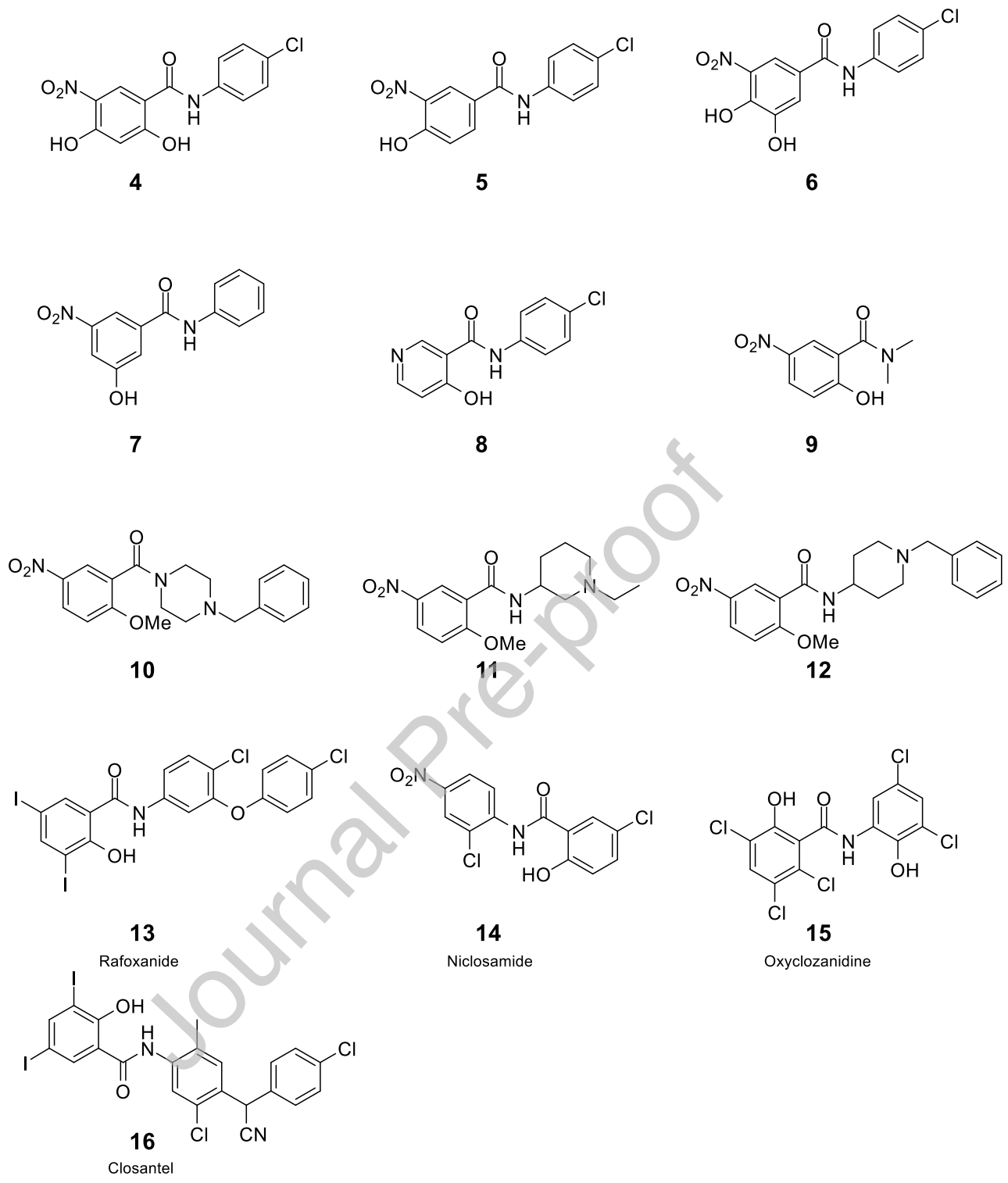


Fig. 4. Chemical structures of analog of compounds **1-3**. Compounds **4-8**, synthesized analogs. Compounds **9-12** , commercially available analogs. Compounds **13-16,** are approved drugs sharing structural similarity.

Compounds **4-8** were evaluated for inhibitory activity towards TyrDC and AADC enzymes (Table 1). Compound **4** having an additional hydroxyl group showed almost similar potency towards TyrDC (IC_{50} 27 µM) and AADC (IC_{50} 80 µM) compared to the original hit compound **1** (TyrDC IC_{50} 23 µM, AADC IC_{50} 102 µM). Compound **6**, which also has two hydroxyl groups but none at the para position relative to the nitro group, exhibited improved potency against TyrDC (IC_{50} 255 µM) although improvement in potency against

AADC (IC_{50} 25 μM) was observed compared to compound **1**. In contrast, shifting of the hydroxyl group to the ortho position (compound **5**) or meta position (compound **7**) resulted in a complete loss of potency towards TyrDC (IC_{50} > 500 μM) and weak potency was observed against AADC (IC_{50} 393-453 μM) compared to compounds **1-3**. Compound **8**, lacking the nitro group present in hit compound **1**, showed a complete loss of inhibition towards both enzymes.

Table 1. Bioactivity results for compounds **1-16** towards TyrDC and AADC enzymes along with their efficacy in whole-cell-based *E. faecalis* assay, cytotoxicity towards HeLa cells, and antibacterial effects against *E. faecalis*.

Compound	IC_{50} *E. faecalis* TyrDC (μM)[a]	IC_{50} *H. sapiens* AADC (μM)[b]	% Inhibition of levodopa conversion (100 μM)	CC_{50} HeLa (μM)[c]	% Inhibition of *E. faecalis* proliferation (100 μM)
1	23 ± 1.8	102 ± 21	92 %	17 ± 0.7	67 %
2	42 ± 3.2	154 ± 19	49 %	20 ± 1.4	55 %
3	51 ± 3.1	182 ± 16	81 %	18 ± 1.5	63 %
4	27 ± 2.0	80 ± 14	95%	14 ± 1.2	94 %
5	> 500	453 ± 82	39 %	> 100	-
6	255 ± 50	25 ± 2	60 %	> 100	17 %
7	> 500	393 ± 67	98 %	> 100	70 %
8	509 ± 42	> 500	25 %	> 100	-
9	386 ± 69	192 ± 30	16 %	> 100	-
10	> 500	> 500	-	> 100	-
11	> 500	> 500	-	> 100	-
12	> 500	> 500	-	> 100	-
13	332 ± 32	37 ± 8	99 %	19 ± 1	88 %
14	> 500	104 ± 9	84 %	25 ± 2.2	81 %
15	> 500	98 ± 14	94 %	18 ± 1.5	89 %
16	504 ± 54	14 ± 1.4	98 %	31 ± 0.9	89 %
AFMT	9 ± 0.9	> 500	86 %	> 100	-
Carbidopa	30 ± 10	0.04±0.002	-	> 100	-

IC_{50}, Half maximal inhibitory concentration; CC_{50}, Half maximal cytotoxic concentration; AFMT, a well-known TyrDC inhibitor, was used as a positive control; Carbidopa, a well-known AADC inhibitor, was used as a positive control. [a,b,c]Assays were performed in triplicates and repeated once independently.

Based on the above results, we purchased compounds **9-16** (Fig. 4) to expand the scope of the SARs exploration (Table 1). Compounds **9-12** were retrieved using the identified shared substructure of compounds **1-3** to perform *in silico* search on the FIMM database. In addition, four compounds (**13-16)** that are drugs in clinical use sharing structural similarity to the active compound class were tested. As a result, compound **9** demonstrated weak potency towards both enzymes, highlighting the importance of the bulky phenyl ring (as benzamide) of compounds **1-3** over the small dimethyl substituents of compound **9** for achieving good potency. Both the presence of a methoxy substituent instead of a free hydroxyl group and a substituted piperidine ring instead of the phenyl ring (as benzamide) in compounds **10-12** resulted in the complete loss of inhibition towards both enzymes. Compounds **13-16** having *N*-phenylbenzamido scaffold as the main core structure and presence of specific functional groups, such as Cl, -OH, -NO_2, etc., similar as compounds **1-3**, showed moderate to loss of inhibition towards TyrDC although all these four compounds showed selective inhibition towards AADC. Especially compounds **13** and **16** have moderate potency towards AADC (IC_{50} 37

μM and 14 μM, respectively) and weak potency towards TyrDC (IC_{50} 332 μM and 504 μM, respectively). Interestingly, both **13** and **16** have additional phenyl ring that might be the one of the reasons contributing to enhanced potency towards AADC over TyrDC. In contrast, to achieve improved potency towards TyrDC over AADC as found in original hit compounds **1-3**, possibly *N*-phenylbenzamido scaffold as the main core structure is needed along with free hydroxyl substituent.

3.4. Efficacy in cell-based *E. faecalis* assay, mammalian cell toxicity and antibacterial effects

Furthermore, all compounds that showed moderate to good potency against either TyrDC or AADC or both enzymes were found to block the metabolism of levodopa conversion by *E. faecalis* ranging from moderate to excellent inhibition (Table 1). However, in the cytotoxicity assay carried out on HeLa cells, all compounds that showed good potency against either TyrDC (compounds **1-3**) or AADC (compounds **4**, **6** and **13-16**) were found to be toxic as their CC_{50} values (ranging from 14 to 31 μM) were similar to their respective IC_{50} in the enzymatic assays. However, it should be mentioned that compounds **13 – 16** are known antiparasitic drugs already in clinical use. Although the known AADC inhibitor carbidopa had an IC_{50} value also in the low micromolar range towards TyrDC (30 μM), it had no effect on levodopa metabolism by *E. faecalis* or bacterial growth (Table 1). Similar results where human AADC inhibitors lack efficacy towards *E. faecalis* have been reported previously (Maini Rekdal et al., 2019; van Kessel et al., 2019), and one reason for this could be a poor permeability of carbidopa through the bacterial cell wall combined with a notably lower enzyme affinity towards TyrDC (≈ 7500-fold selectivity for AADC over TyrDC).

The inhibitors discovered in this study share similar structural features and have their IC_{50} values in the low micromolar range towards *E. faecalis* TyrDC enzyme, and many of them also showed activity towards human AADC at micromolar concentration range. They were effective in cell-based *E. faecalis* assay, showing a dose-dependent inhibitory effect on the conversion of levodopa into dopamine, and many of the compounds were also able to inhibit the proliferation of *E. faecalis*. However, some of the compounds also displayed a reduction in human cell viability at similar bioactive concentration range. As the inhibition of AADC or TyrDC alone is not known to lead to toxic effects (Maini Rekdal et al., 2019), this might imply that the compounds are not entirely selective for these two decarboxylase enzymes.

Potential approaches to prevent the metabolism of levodopa by intestinal bacteria involve inhibiting their TyrDC or targeting the bacteria with antibacterial agents. Recently, it was reported that mito-*ortho*-HNK, which is a modification of a naturally occurring honokiol, was able to inhibit proliferation of *E. faecalis in vitro* (Cheng et al., 2024). The compound also increased brain dopamine levels *in vivo* after oral levodopa administration to mice, providing further evidence that the bioavailability of oral levodopa can be increased by targeting intestinal bacteria (Cheng et al., 2024). Similar findings were presented by Hu et al., who found that treating rats with piperine, which is a major component of the plant *Piper longum* L. was able to increase AUC of levodopa after oral administration to rats and decrease the amount of *E. faecalis* in the gut (Hu et al., 2024). Interestingly, in our study we found that commercial drugs **13 – 16** which all belong to group of antiparasitic drugs in clinical use were able to inhibit the metabolism of levodopa and proliferation of *E. faecalis* (Table 1). Since these compounds displayed only weak affinity towards TyrDC and AADC enzymes (Table 1), it can be assumed that their efficacy in inhibiting levodopa metabolism is based on their antibacterial effect. Nonetheless, even if efficacious, long-term treatment with these antibacterial agents might not be desirable since this can lead to disruption of the gut microbiota balance and growth of opportunistic pathogens. Thus TyrDC / AADC inhibitors without antibacterial effects would be of primary interest. To our knowledge, no compound class has been found so far that simultaneously inhibits both human and gut microbial enzymes involved in the metabolism of levodopa. Therefore, the core structure of the compounds presented here can serve as a starting point for the development of a new compound class of enzyme inhibitors. Noteworthy, *para*-nitrophenyl moiety present in our hit compounds is considered as a structural alert for potential liver toxicity, hence, necessitating cautiousness.

4. Conclusion

In conclusion, we utilized a combination of *in silico* and *in vitro* evaluation methods to identify a new class of inhibitors that effectively targets *E. faecalis* TyrDC and human AADC enzymes. The active compounds we discovered, along with their shared core structure, offer a foundation for developing pharmacologically active inhibitors to hinder both gut microbial and host metabolism of levodopa. Our findings demonstrate that core structure of the compounds presented here can serve as a starting point for the development of new tool compounds to study TyrDC and AADC enzymes, potentially enabling discovery of inhibitors to be used as a treatment for conditions related to levodopa metabolism.

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CRediT authorship contribution statement

SR: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Visualization, Conceptualization. LG: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. JZP: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. DRP: Writing – review & editing, Methodology, Formal analysis, Data curation. KA: Investigation, Formal analysis. JYK: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. MJ: Writing – review & editing, Supervision, Resources. HX: Writing – review & editing, Supervision, Resources, Funding acquisition. PP: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. PT: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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