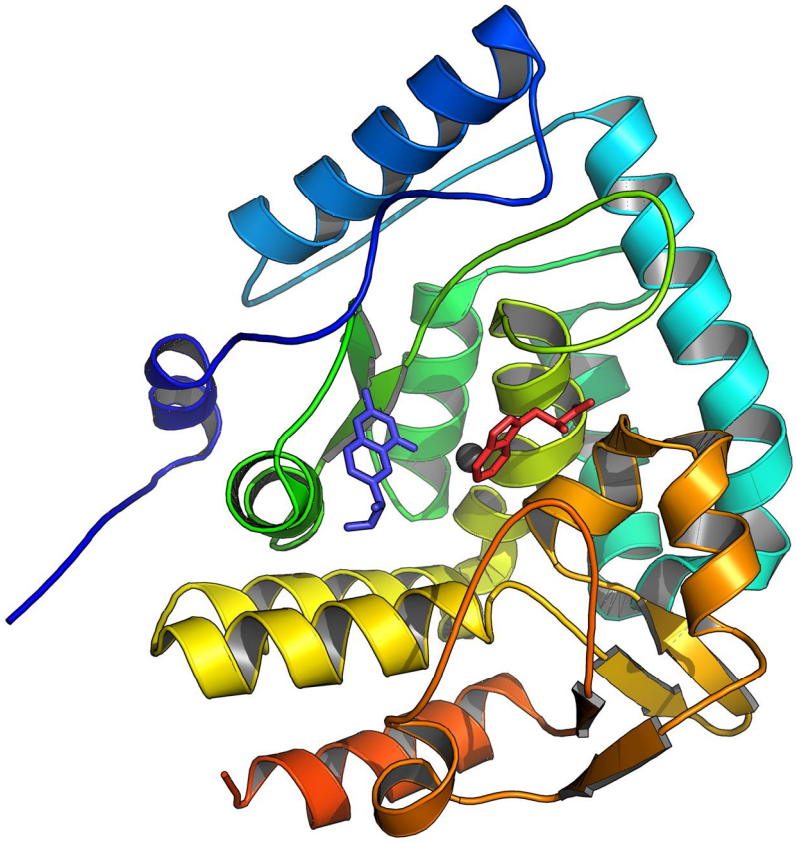
MASTER THESIS IN PHARMACY

IDENTIFICATION OF INHIBITORS OF

TRYPTOPHAN HYDROXYLASE 1



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MAY 2014

Acknowledgement

The work presented in this master thesis was performed in the Biorecognition group at the Department of Biomedicine at the University of Bergen. The project took place in the period August 2013-May 2014, and was the final part of my 5-year education in pharmacy.

First and foremost, I would like to express my sincere gratitude to my supervisor associate professor Knut Teigen, for amazing guidance throughout the learning process of this master thesis. His abilities in making difficult theory understandable are unique! Thank you for always having an open door and for your positive and good spirits. Furthermore, I would like to express my gratitude to my co-supervisor professor Jan Haavik, for feedback and guidance on the way.

For excellent guidance in the laboratory, for expertise and advice, I would like to thank Magnus Hole. Furthermore, I would like to thank my fellow student Gerd-Marie Eskerud Harris for insightful discussions and for sharing this experience with me. Thank to the Biorecognition group and to Erlend Hodnelnd for help with statistics.

Finally, I would like to thank my family and friends. For being loving and supportive I am deeply grateful to my father Kristoffer and my mother Wibeke. Special thanks also to my amazing colleagues and participants at my second home, Sissels Trening og Trivsel, for providing me with the energy needed for rainy days. A special thank to my partner Rune Åbø for always supporting me and for discussing my master thesis with full immersion even though he barely understood what we were talking about.

ñ*All growth is a leap in the dark, a spontaneous unpremeditated act without the benefit of experience.****ò***

- Henry Miller

Rikke Landsvik Berg

Bergen, May 2014

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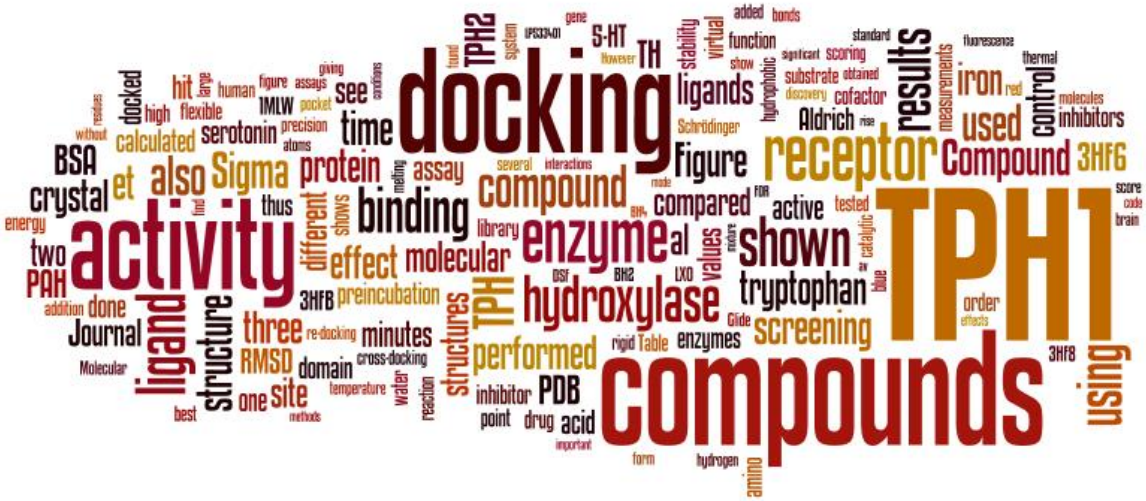


ABSTRACT

Background: In this study we use molecular docking to find compounds that interact with the enzyme tryptophan hydroxylase (TPH), followed by experimental validation. TPH is a tetrahydrobiopterin (BH_4) dependent enzyme that catalyze the conversion of L-tryptophan to 5-hydroxytryptophan (5-OH-Trp). The reaction is the first and also rate limiting step in the synthesis of serotonin (5-hydroxytryptamine, 5-HT) which is precursor for the biosynthesis of melatonin. It was long believed that TPH was encoded by a single gene, but in 2003 Walther et al. reported that TPH was encoded by two genes in mammals, encoding two different enzymes (TPH1 and TPH2). TPH2 is mostly expressed in the brain being responsible for physiological processes like sexual behaviour, appetite, mood and learning. TPH1 is most abundantly expressed in the gastrointestinal tract where it is involved in several biological functions and is the enzyme we have focused on in this study. Serotonin is an important regulator of GI function. Dysregulation of the serotonin production in this area is associated with symptoms of irritable bowel syndrome (IBS), such as abdominal pain and diarrhea, chemotherapy-induced nausea and vomiting and pulmonary hypertension. Direct inhibition of TPH1 is an approach that has been very little explored as a therapeutic approach, due to the importance of 5-HT in the brain and the challenges in selective reduction of 5-HT in the periphery. The discovery of the two distinct genes that encodes two distinct enzymes, TPH1 and TPH2, provides the possibility of selective inhibition of TPH1.

Method: In this thesis both virtual and experimental methods were used. In order to find potential inhibitors of TPH1, molecular docking of a large virtual database was performed, followed by activity measurements in the laboratory.

Results: From the molecular docking together with experimental validation we found 11 compounds that inhibited TPH1 activity significantly. 4 of these compounds also decreased the thermal stability of TPH1.

Conclusion: We have found one compound that is a potent inhibitor of TPH1 and could be a valuable starting point for hit-to-lead optimization.

SAMMENDRAG

Bakgrunn: I denne studien bruker vi datasimulering for å finne forbindelser som interagerer med enzymet tryptophan hydroxylase (TPH), fulgt opp av eksperimentell validering. TPH er et tetrahydrobiopterin (BH_4)- avhengig enzym som katalyserer omdannelsen av L-tryptophan til 5-hydroxytryptophan (5-OH-Trp). Reaksjonen er det første og hastighetsbestemmende trinnet i syntesen av serotonin (5-hydroxytryptamine, 5-HT), som er forløperen for biosyntesen av melatonin. Det var lenge trodd at TPH var kodet av et enkelt gen, men i 2003 rapporterte Walther et al. at TPH var kodet av to gener i pattedyr, som koder to forskjellige enzymer (TPH1 og TPH2). TPH2 er hovedsaklig uttrykt i hjernen og er der ansvarlig for fysiologiske prosesser som seksuell atferd, appetitt, humør og læring. TPH1 er mest uttrykt i mage-tarmkanalen, hvor den er involvert i flere biologiske funksjoner, og det er dette enzymet vi i all hovedsak har fokusert på i denne studien. Serotonin er en viktig regulator av mage-tarmfunksjonen, Feilregulering av serotoninproduksjonen i dette området er forbundet med symptomer på irritabel tarm, sik som magesmerter og diaré, kjemoterapi-indusert kvalme og oppkast og pulmonal hypertensjon. Direkte inhibering av TPH1 er en tilnærming som er blitt svært lite utforsket som en terapeutisk tilnærming, grunnet viktigheten av 5-HT i hjernen og utfordringene med selektiv reduksjon av 5-HT i periferien. Oppdagelsen av to distinkte gener som koder til to distinkte enzymer, TPH1 og TPH2, gir muligheten for selektiv inhibering av TPH1

Metode: I denne masteroppgaven ble både virtuelle og eksperimentelle metoder brukt. For å finne potensielle inhibitorer av TPH1 ble en stor virtuell database screenet ved hjelp av datasimulering, fulgt opp av aktivitetsmålinger i laboratoriet.

Resultat: Fra datasimulering sammen med eksperimentell validering fant vi flere forbindelser som inhiberer TPH1s aktivitet. 4 av disse forbindelsene reduserer også den termiske stabiliteten til TPH1.

Konklusjon: Vi har funnet en forbindelse som er en potent hemmer av TPH1 og som videre kan være et godt utgangspunkt for videre legemiddeloptimalisering.

ABBREVIATIONS

5-HT	Serotonin
5-HT3	5-hydroxytryptamine receptor 3
5-HIAA	5-hydroxyindole acetic acid
5-OH-Trp	5-hydroxytrypamine
AAAH	Aromatic amino acid hydroxylase
BH_4	Tetrahydrobiopterin
BH_2	Dihydrobiopterin
BSA	Bovine serum albumine
CNS	Central nervous system
DSF	Differential scanning fluorimetry
DMSO	Dimethyl sulforxide
DTT	Dithiothreitol
FDR	False discovery rate
GI	Gastro intestinal
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
hTPH	Human tryptophan hydroxylase
IBS	Irritable bowel syndrome
IBS-d	Diarrhea-predominant irritable bowel syndrome
LP-533401	(2*S*)-2-amino-3-(4-(2-amino-6-(2,2,2-trifluoro-1-(3_-fluorobiphenyl-4-yl)ethoxy)pyrimidin-4-yl)phenyl)propanoic acid
L-Trp	L-tryptophan
NMR	Nuclear magnetic resonance
PAH	Phenylalanine Hydroxylase
pCPA	*Para*-chlorophenylalanine
PDB	Protein Data Bank
PKU	Phenylketonuria
RMSD	Root-mean-squared deviation
TH	Tyrosine Hydroxylase
TPH1 and TPH 2	Tryptophan hydroxylase 1 and 2, respectively

1 Introduction

1.1 Tryptophan hydroxylase

Tryptophan hydroxylase (TPH, EC 1.14.16.4) is the enzyme that catalyse the conversion of L-tryptophan to 5-hydroxytryptophan (5-OH-Trp). The reaction requires (6-*R)*-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) as a cofactor, dioxygen (O_2) as additional substrate and non-heme iron (1). The reaction is the first and also rate limiting step in the synthesis of serotonin (5-HT) which is the precursor for the biosynthesis of melatonin in the pineal gland (2, 3). Serotonin most famously executes its effects as a neurotransmitter, and controls multiple biological functions in both the periphery and the central nervous system. The vast majority of 5-HT is found in the gut, where it modulates motility and initiates secretory and peristaltic reflexes (4, 5). Serotonin is also found in the brain, where it is a part of physiological processes like sexual behaviour, appetite, mood and learning (6, 7). Melatonin is mainly found in both the pineal gland, but also exist in the retina (8, 9) and is involved in the control of the circadian rhythm (10, 11). Equation 1.1 shows the reaction catalysed by TPH:

$$\textit{Equation 1: L-Trp} + BH_4 + O_2 + 2H \xrightarrow{TPH} \textit{5-OH-Trp} + \textit{4a-OH-}BH_4$$

It was long believed that TPH was encoded by a single gene, but in 2003 Walther et al. reported that TPH was encoded by two genes in mammals, encoding two different enzymes (TPH1 and TPH2) (12). The known isoform was called TPH1 and the newly discovered isoform was named TPH2. TPH1 is mainly expressed in non-neuronal tissues, e.g. the intestinal enterochromaffin cells, pineal gland and retina (13, 14). In the periphery, about 95% of the 5-HT is located in the gastrointestinal tract (4). Knockout of *Tph1* in mice has shown that TPH1 accounts for the majority of the 5-HT synthesis in the GI (12, 13). Enteric neurons are known to express *Tph2* but only a small percentage (0.7 % in the jejunum and 3.4 % in the duodenum) of the 5-HT content of the GI tract is a product of TPH2 activity (13). TPH2 is expressed in neuronal cells located in the myenteric plexus of the gut and the dorsal raphe

nucleus of the brain and is responsible for most of the 5-HT synthesis in the central nervous system (CNS) (12, 15, 16). TPH1 and TPH2 have a high sequence identity with 71 % amino acid identity in humans (17). The two isoforms also show different molecular properties, e.g. different phosphorylation sites, kinetic properties and TPH2 has a larger molecular weight (18). This can be utilized when searching for new pharmacological agents targeting only one of the distinct enzymes. TPH was extensively studied before the knowledge of two different TPH-genes. Thus, these findings leave a certain uncertainty regarding what is known about each enzyme. This has to be considered when reading papers published prior to 2003.

TPH is a tetramer, with each monomer being organized in three different domains; a regulatory N-terminal, a catalytic domain and a C-terminal oligomerization domain. Figure 1.1.1 shows the domain organization of TPH1 and TPH2. Figure 1.1.2 shows the crystal structure of the catalytic domain of TPH1. The structure of the regulatory domain and the motif of tetramerization domain are yet to be solved.

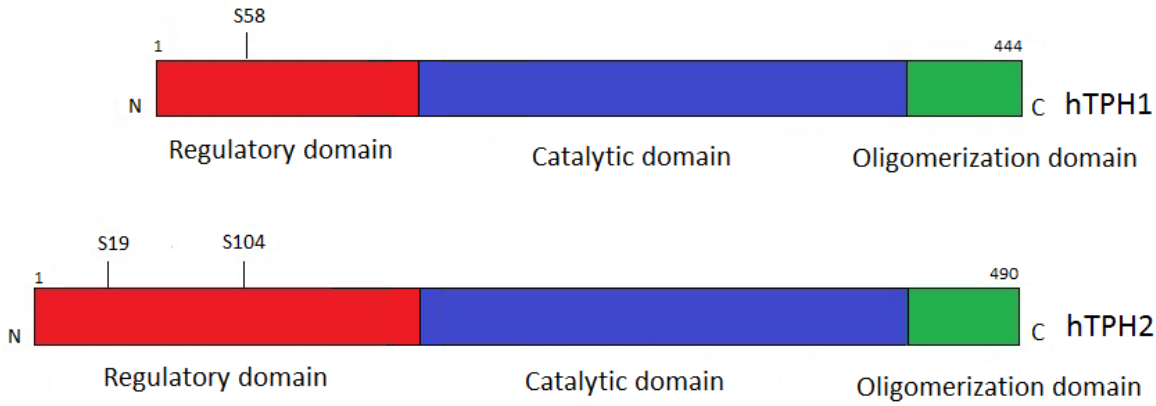
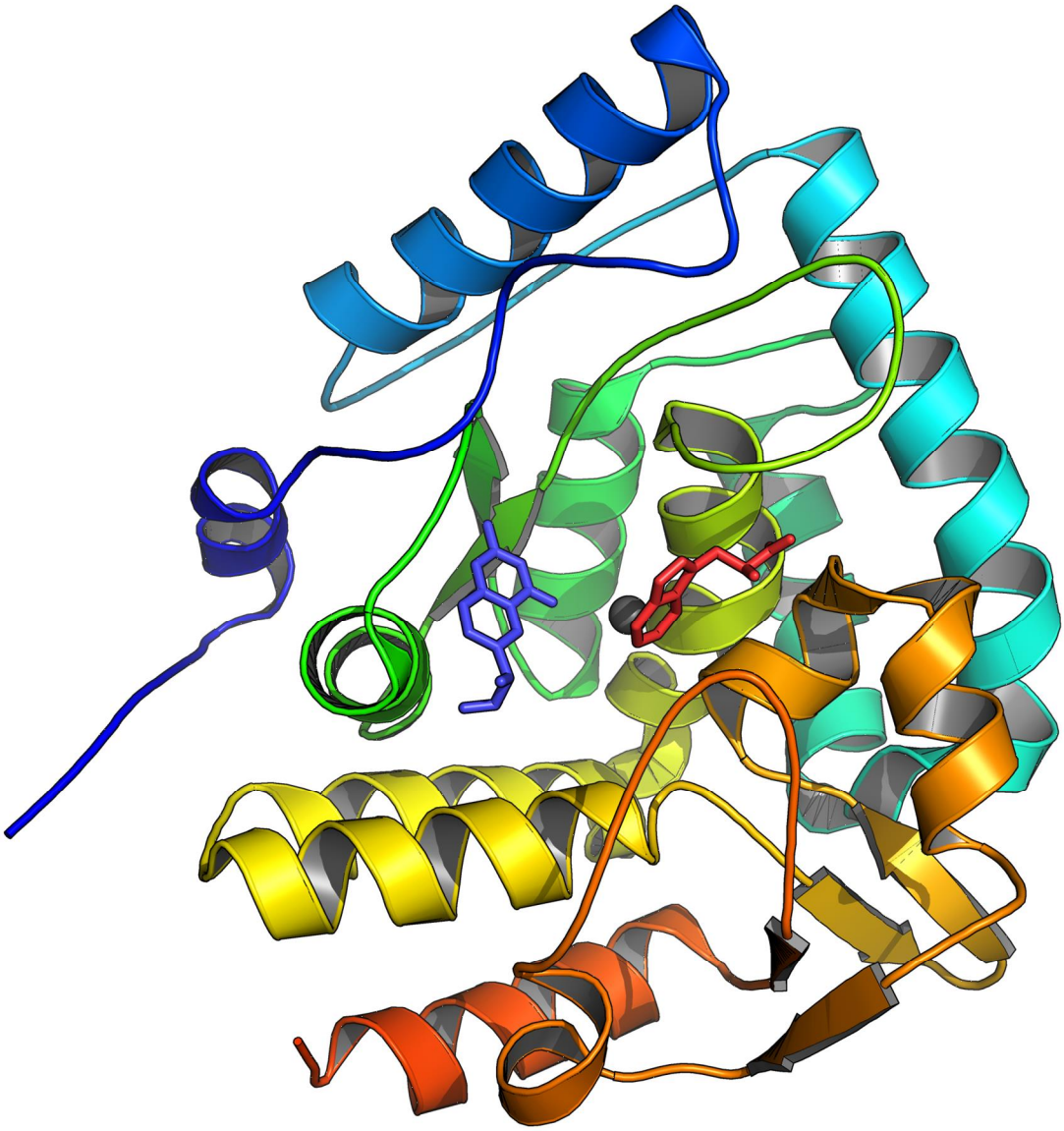


Figure 1.1.1: *The domain organization of the human TPH1 and TPH2:* There are three domains: an N-terminal regulatory domain (red) containing one ore more serine (S) phosphorylation sites, a catalytic domain (blue) and a C-terminal oligomerization domain (green).



Figue 1.1.2: *Crystal structure of the catalytic domain of TPH1:* The figure was prepared in PyMOL (19) by superimposing the structure of TPH1 in complex with BH_2 (pdb code 1MLW) onto the structure of TPH1 in complex with L-Trp (pdb 3E2T). The cofactor analogue (BH_2) is shown in blue sticks, the substrate (L-Trp) is shown in red sticks. The active site iron is shown as a grey sphere in between the substrate and the cofactor.

TPH activity is regulated by phosphorylation at different serine residues in the N-terminal domain; For TPH1 Ser^{260} (20, 21) and for TPH2 Ser^{19} and Ser^{104} (18, 22) (corresponding to Ser^{58} in TPH1). The catalytic domain is where the active site is situated and this is where substrate specificity is controlled (23). The oligomerization domain is responsible for

dimerization and tetramerization. The active site consist of a 13 Å deep and 10 Å wide pocket, including an iron (Fe^{2+}) atom (Fig. 1.1.3) and two binding pockets, one substrate binding site and one cofactor binding site (24).

Figure 1.1.4 shows the binding pocket of L-Trp in chicken TPH1. The polar interactions of L-Trp are with Thr266, Ile367 and Ser337 and a salt bridge to Arg258. Residues Tyr236, Ile367, Phe314, Phe319, Pro269 and Thr266 line up the hydrophobic part of the tryptophan binding pocket (25). As seen in figure 1.1.3, the iron is coordinated by three amino acids; His272, His277 and Glu317 (26). This structural motif is referred to as the 2-his-1-carboxylate facial triad, and is common among the family of non-heme iron (II) enzymes (25, 27-29).

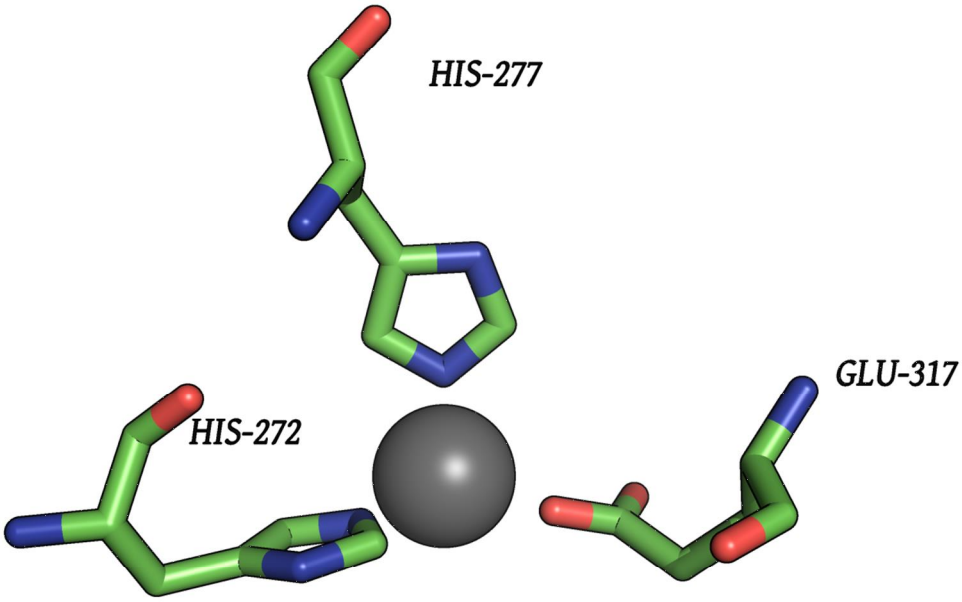


Figure 1.1.3: *Iron coordinated by the 2-his-1-carboxylate facial triad*: Iron is shown as a grey sphere coordinated by the three amino acids His272, His277 and Glu317 (shown in stick representation) also named the 2-his-1-carboxylate facial triad. This structural motif is found in other metal-binding enzymes. Iron is shown as a grey sphere, oxygen in red, hydrogen in green, nitrogen in blue. Prepared using PyMol (19) (PDB entry 1MLW).

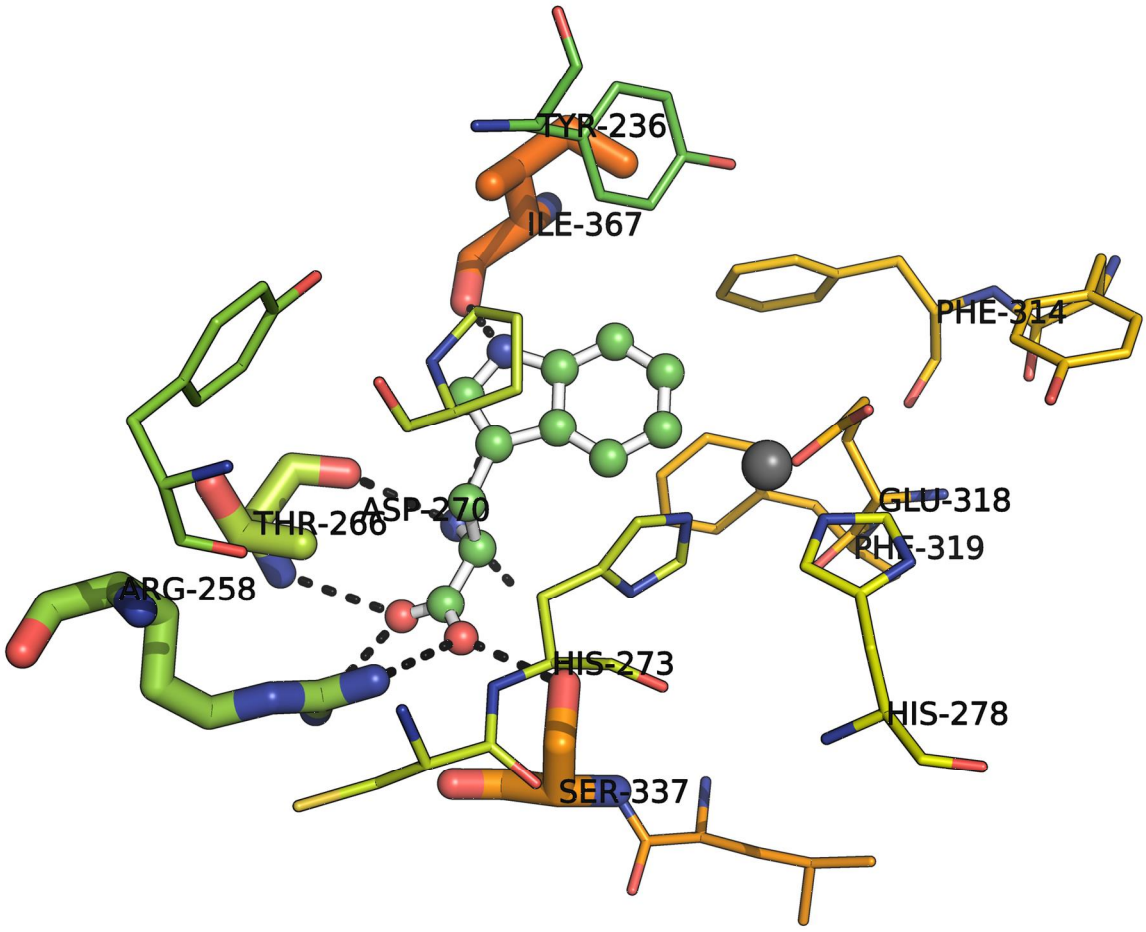


Figure 1.1.4: *The active site of chicken TPH1 (PDB 3E2T):* Polar contacts are shown as dashed lines and amino acids involved in these interactions are shown in sticks. Iron is shown as a grey sphere. L- Trp is shown in ball-and-stick with carbon in green, nitrogen in blue and oxygen in red. Amino acids that line up the hydrophobic part of the L-Trp binding pocket are shown as sticks.

Figure 1.1.5 shows the co-factor binding site of TPH1. In the oxidized cofactor analogue 7,8-dihydro-L-biopterin (BH2) form of TPH1, Tyr235 and Phe241 sandwich BH_2 in the form of ´-stacking interactions. The region Gly234-Pro238 is the region mainly responsible for pterin binding. Gly234 form hydrogen bonds to the cofactor, and Glu273 forms water-mediated hydrogen bonds (25).

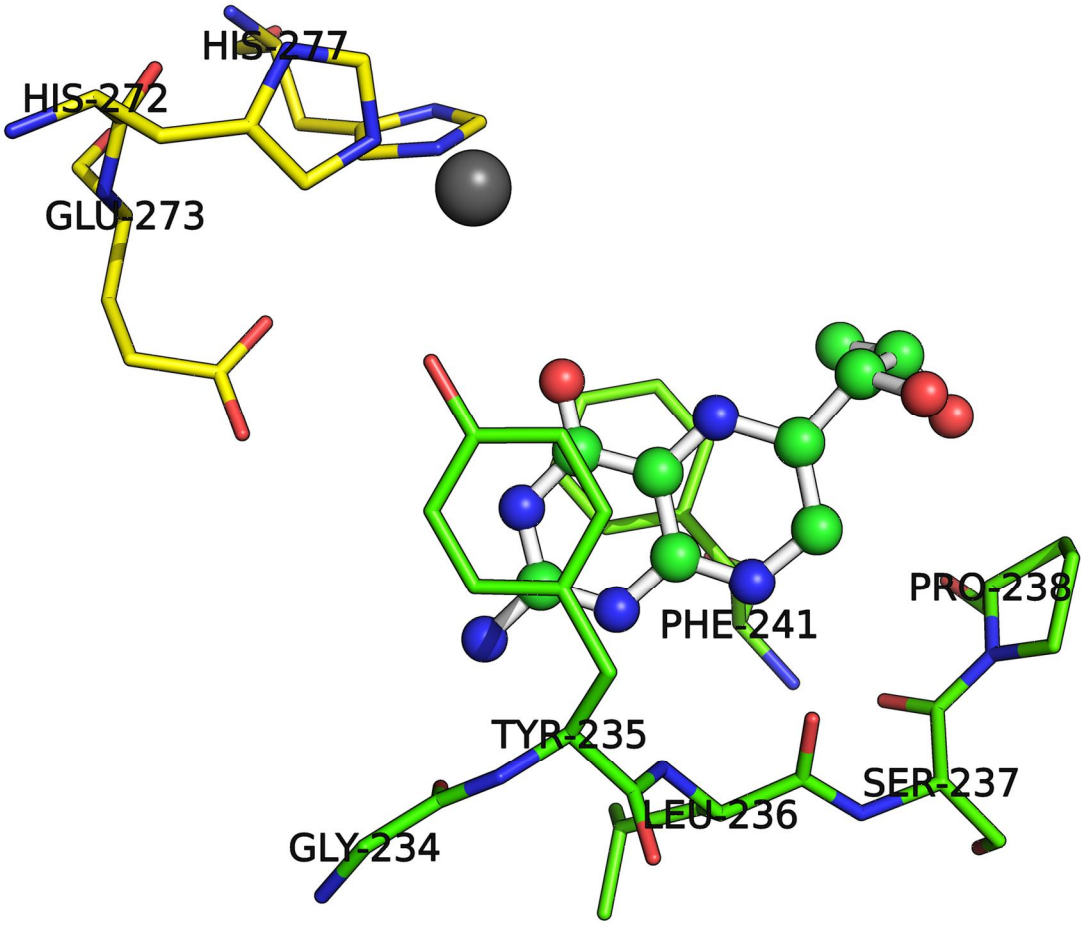


Figure 1.1.5: *The binding site of the oxidized cofactor 7,8-dihydro-L-biopterin analogue (BH2) in TPH1 (PDB 1MLW):* The cofactor is shown in ball-and-stick, with carbon in green, nitrogen in blue and oxygen in red. Iron is shown as a gray sphere, coordinated by the 2-his-1-carboxylate facial triad.

1.2 The aromatic amino acid hydroxylases (AAAH)

The tryptophan hydroxylases (TPH1 and TPH2), together with tyrosine hydroxylase (TH, EC 1.14.16.2) and phenylalanine hydroxylase (PAH, EC 1.14.16.1) make up the aromatic amino acid hydroxylase (AAAH) enzyme family. Mammals contain one TH gene one PAH gene and two TPH genes (TPH1 and TPH2). TH is an enzyme that catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxypenylalanine (L-DOPA), which is the rate limiting step in the biosynthesis of catecholamines (dopamine, norephedrine and epinephrine) (30). PAH catalyzes the hydroxylation of L-phenylalanine to L-tyrosine, which is the rate limiting step of the catabolic pathway of phenylalanine (1). Because of its instability in vivo and the scarcity of the enzyme in animal tissue, TPH is the enzyme we know least about. Figure 1.1.6 shows the hydroxylation reactions catalysed by the AAAH enzymes. TPH and PAH has a similar substrate specificity in that both L-Trp and L-Phe are good substrates for both enzymes. TH can hydroxylate all three aromatic amino acids and is the only enzyme capable of hydroxylating L-Tyr.

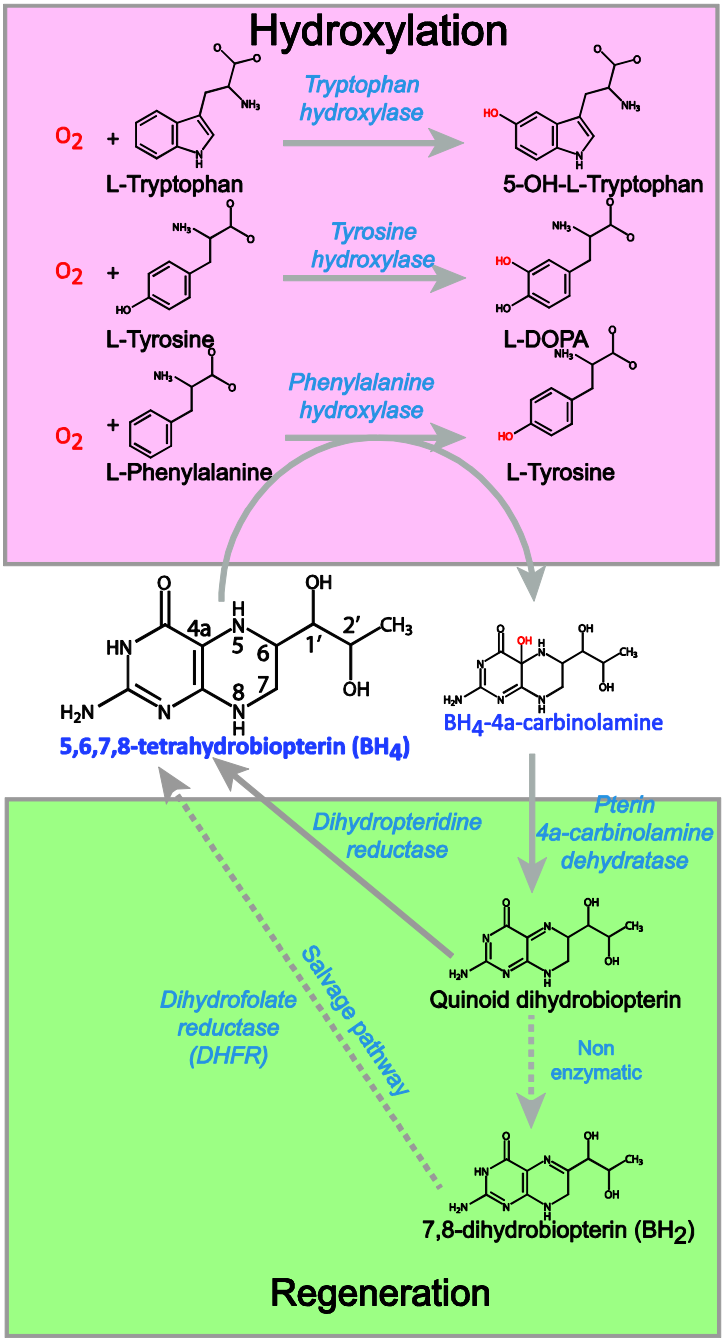


Figure 1.1.6: *Hydroxylation reaction catalysed by the AAAH enzymes on their respective substrates, using BH_4 as co-factor:* The cofactor is hydroxylated together with the substrate during catalysis and needs to be regenerated back to the active cofactor before another cycle of catalysis can be performed.

TH and PAH is worth mentioning because of their similarities to the TPHs. X-ray crystal structures has been determined for various forms of TPH1, TH and PAH (25, 26, 28, 31-34). The AAAH enzymes regulate amino acid metabolism and facilitate proper functions of the brain (1). All four members are dependent both on reduced pterin cofactor e.g. BH_4, iron and

dioxygen (O_2) to hydroxylate their respective aromatic amino acid substrates. The three enzymes also have a high structural and sequence similarity and it is believed that they have evolved from a common progenitor (35, 36). Both TPH and TH have been shown to exist as exclusively as tetramers, contrary to PAH, that has been shown to exist in a state of equilibrium between tetrameric and dimeric forms (37, 38). Each subunit consists of three domains: i) an N-terminal regulatory domain, where phosphorylatable Ser residues are located ii) a catalytic domain (sharing more than 80 % sequence identity among the AAAHs) and iii) a C-terminal oligomerization domain with dimerization and tetramerization motifs (36, 39-43). The catalytic domain contains a ferrous iron atom bound to one glutamate and two histidines. As shown in figure 1.1.3 in TPH1 these coordinating residues are His272, His277 and Glu317. All three iron coordinating residues are completely conserved in the enzymes. The iron is essential for enzyme activity of the AAAHs (44, 45). The iron needs to be in ferrous form (Fe(II)) in order for the enzymes to be active, and in both TH and PAH the cofactor has shown to reduce the ferric iron (Fe(III)) (46-48).

Although the enzymes show high sequence similarities, some non-conserved residues are localized in the binding sites of both the substrate and the cofactor. Chimeric hydroxylases indicate that substrate specificity is linked to only the catalytic domain (49). Phe313 in TPH correspond to a Trp residue in both TH and PAH (Trp326), thus seems to be facilitating substrate specificity in the enzyme family (24). Phenylalanine is a smaller amino acid than tryptophan, thus the substrate binding pocket in TPH is wider compared to TH and PAH. This makes room for bigger substrates, for example L-Trp. Figure 1.1.7 shows the catalytic domain of TPH1 (26), TH (28) and PAH (32). Using PyMOL the enzymes has been aligned. TPH2 has yet to be crystallized.

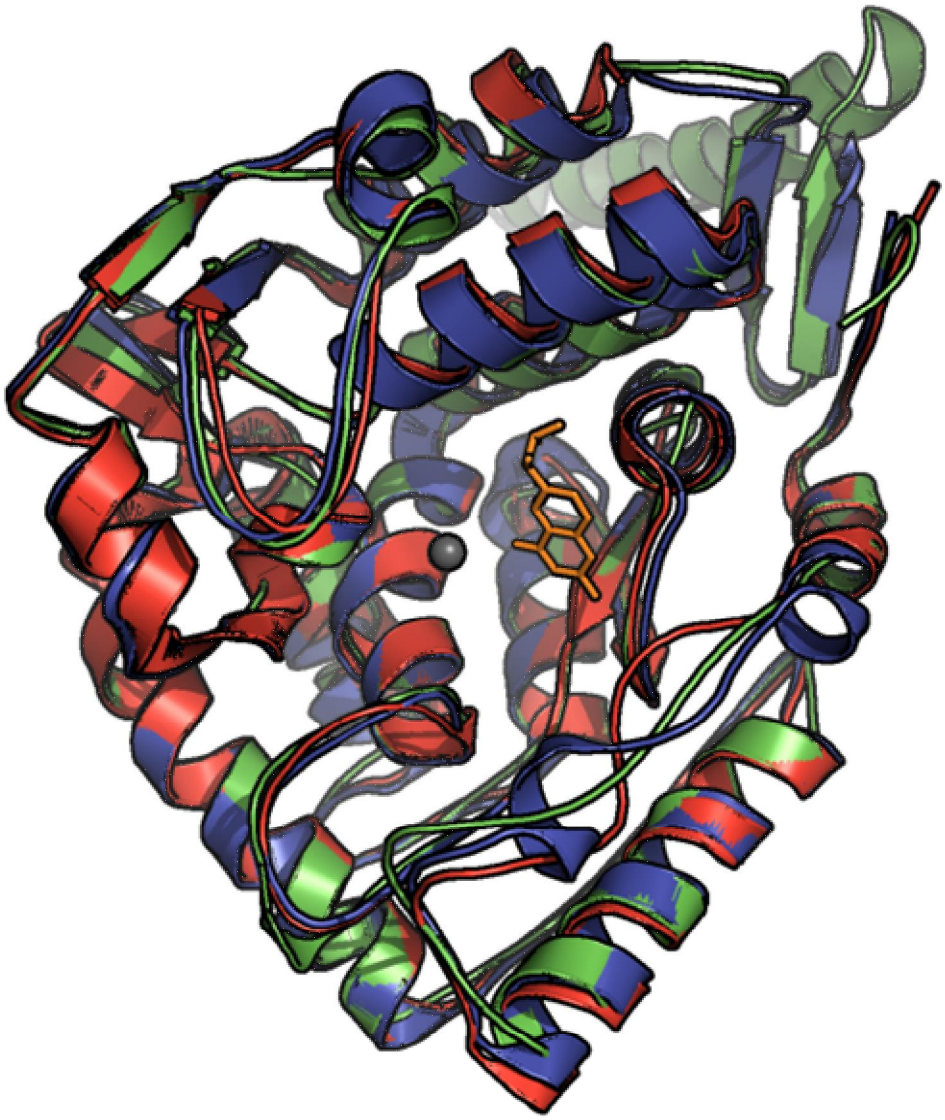


Figure 1.1.7: *The three members of the AAAH family superimposed by molecular overlay*: The catalytic domain of human TPH1 (red, pdb accession code 1MLW), human PAH (blue, pdb 1J8U) and rat TH (green, pdb 2TOH). The active site iron is shown as a grey sphere and cofactor as a stick-model in orange. Iron and cofactor (BH_4) are taken from PAH.

These crystal structures of the catalytic core show the structural similarities of this enzyme family. Especially the binding site for the co-factor BH_4 (Phe^{234} and Glu^{273} for TPH1) and Fe^{2+} (His^{272}, His^{277} and Glu^{317} for TPH1) is located in a highly conserved part of the structure. When doing molecular docking on TPH we are interested in the structure and location of the

active site of the enzyme. Figure 1.1.8 shows an outline of the binding site of the cofactor in TPH1, TH and PAH.

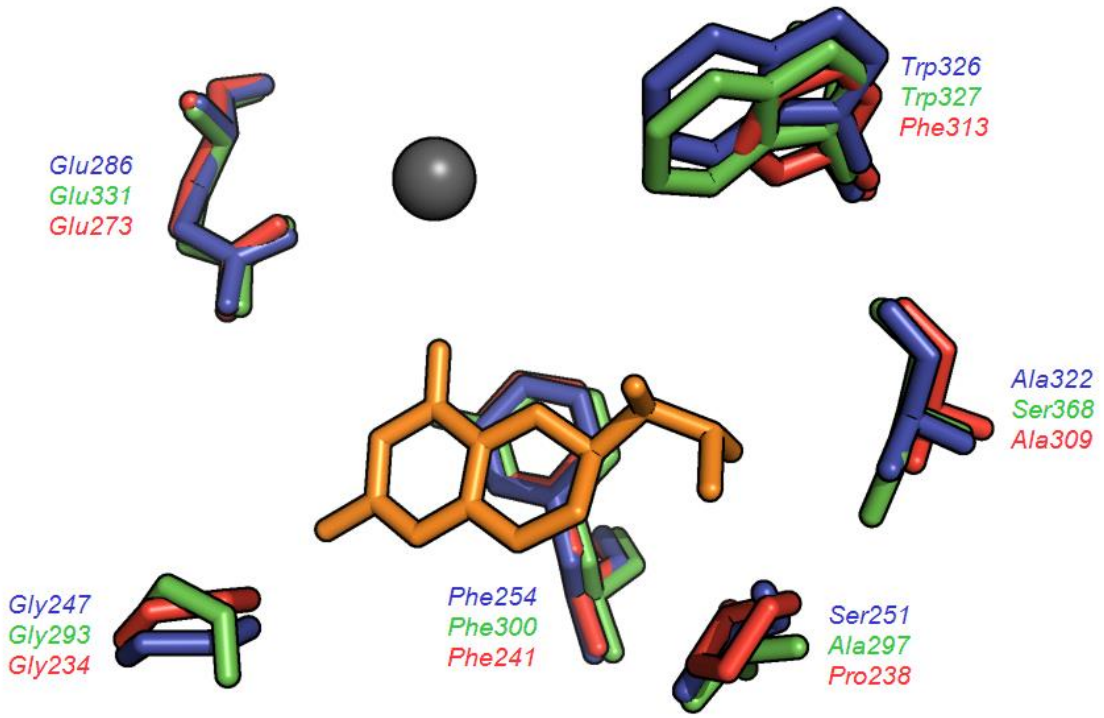


Figure 1.1.8: *The cofactor binding site in the PAH, TH and TPH1:* Human PAH (blue, PDB 1J8U), rat TH (green, PDB 2TOH) and TPH1 (red, PDB 1MLW). The enzymes are structurally aligned using PyMOL. BH_4 is shown in orange and is taken from PAH.

The AAAHs have been linked to several clinical conditions. For both TH and PAH several genetic variants associated with clinical syndromes have been described. Only a few coding region polymorphisms have been described for either TPH gene, possibly because of the challenge in diagnosing people with deviation in serotonin production. A common heterozygous missense variant in human TPH1 has been discovered *in vivo*. This polymorphism is in the catalytic domain, at a site near the tryptophan binding pocket, causing substitution of isoleucine for valine at codon 177 (V177I) (not at the active site) (50). TH mutations have been associated with L-DOPA responsive parkinsonism (51), Tyrosine Hydroxylase Deficiency (THD) (52) and dystonia (53). It is also suspected that dysfunctions in TH could be associated with different psychiatric and/or neurological symptoms. Mutations in

the PAH gene are associated with hyperphenylalaninemia or the more severe phenylketonuria (PKU).

In this Master thesis we are interested in finding new inhibitors of TPH1. When doing both docking as well as working with the enzyme in the laboratory, we used truncated forms of the TPH1 enzyme. In our laboratory work we used both truncated TPH1 (102-402) and the common variant TPH1 V177i (102-402). This was done because of enzyme availability. In *vivo* TPH exist in full length, but the truncated form is more stable and soluble, thus easier to work with in the laboratory. Inhibitors are expected to bind to the active site of the enzyme which is defined by the catalytic domain alone. As the truncated form contains the entire catalytic domain, we expect that the results found in this work are transferable to the full-length enzyme. The mutation in V177i is at a site outside the active region, and thereby is not likely to affect the results. When working with TPH2 we used full length enzyme.

Three crystal structures with bound inhibitors are resolved for TPH1, as shown in figure 1.1.9. These compounds were developed by Lexicon Pharmaceuticals Inc (http://www.lexgen.com/) and one of them, LP-533401 (LX1033), are currently in clinical trials for the treatment of diarrhea-predominant irritable bowel syndrome (d-IBS). The three inhibitors for which a structure in complex with TPH has been solved are shown in Fig 1.1.10. All three compounds share the same phenylalanine unit that is buried in the active site of TPH1. The compound LP-533401 4-{2-amino-6-[(1R)-2,2,2-trifluoro-1-(3'- fluorobiphenyl-4-yl)ethoxy]pyrimidin-4-yl}-L-phenylalanine is the only of the three that we have found to be commercially available, and used as a reference for the inhibitory effect on TPH in our study.

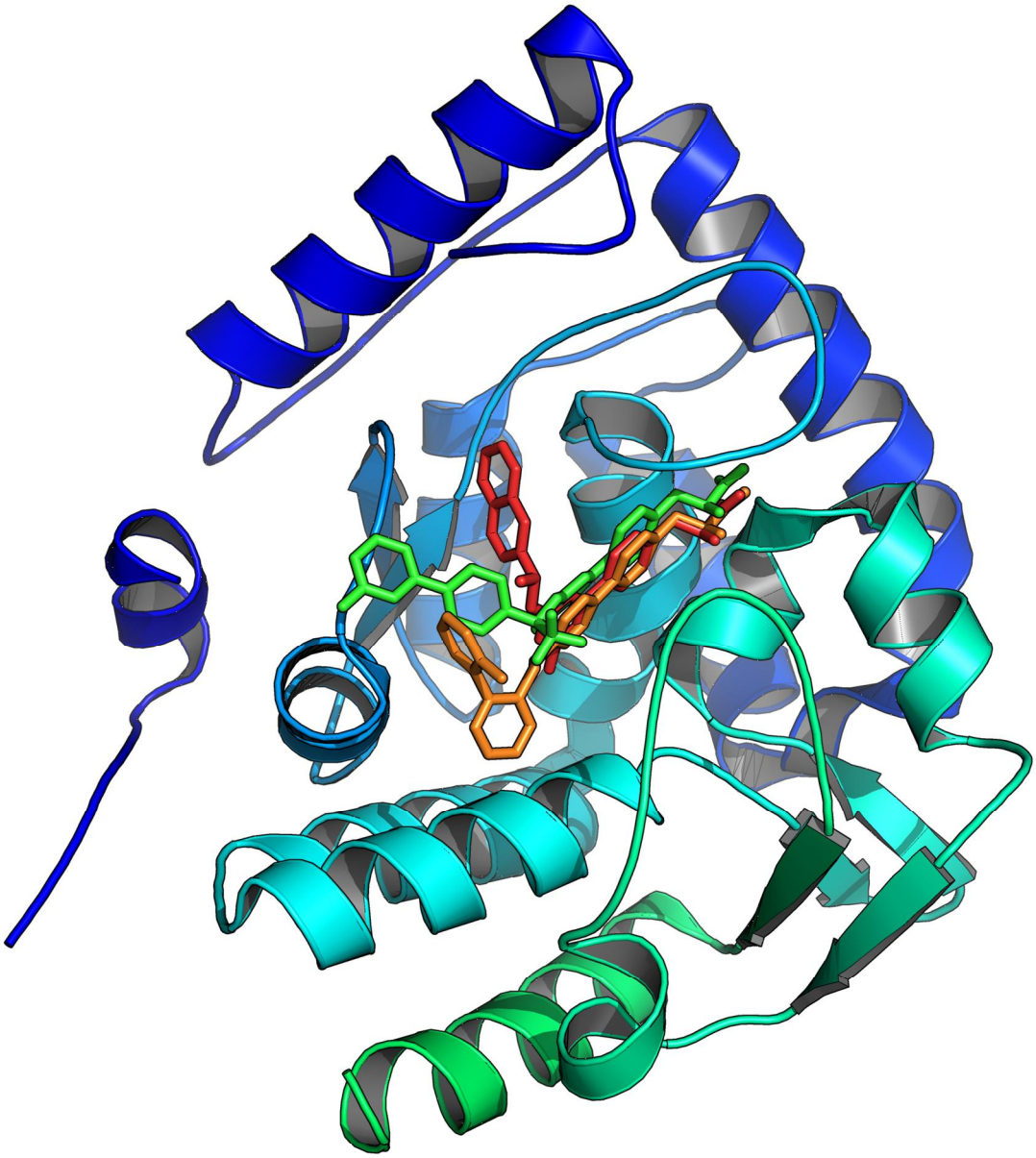


Figure 1.1.9: *Three inhibitors of TPH1 aligned*: LP-521834 in red (PDB: 3HF6, LX0), LP-533401 (LX1033) in green (PDB: 3HF8, ML0) and LP-534193 in orange (PDB: 3HFB, ML4). TPH1 is represented by PDB 3HF6. The loop corresponding to residues 119-128 in 3HF6 were not resolved (54). Note the L-Phe unit from all three compounds buried deep in the active site of TPH1.

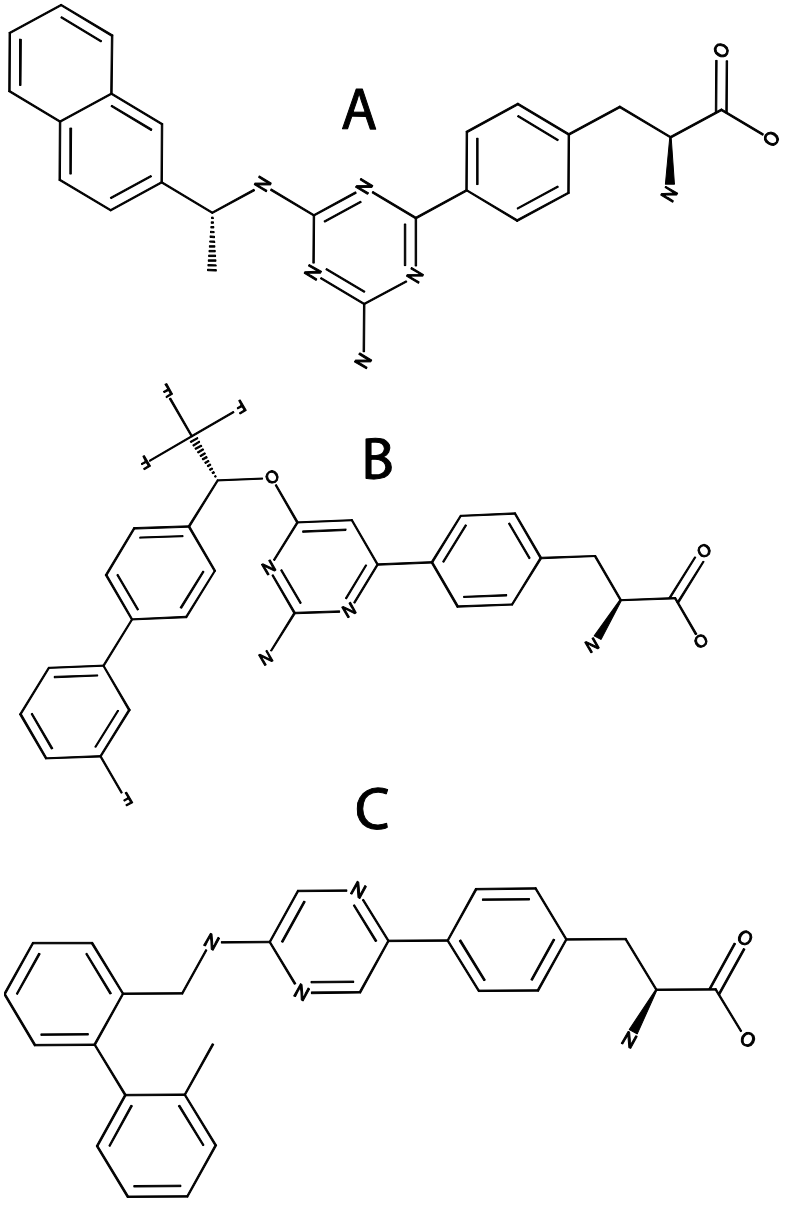


Figure 1.1.10: *Three inhibitors of TPH developed by Lexicon Pharmaceuticals Inc:* A) LP-521834. B) LP-533401 (LX1033), the inhibitor used as a reference for the inhibitory effect on TPH in our study. C) LP-534193. Note the L-Phe moiety in all three compounds.

1.3 Disorders related to TPH1 and TPH2

Serotonin is a neurotransmitter associated with the control and modulation of physiological processes. In the CNS, 5-HT regulates important behavioural functions. In the periphery 5-HT is involved in several very different responses, such as peristalsis, nausea and intestinal secretion. Dysregulation of the serotonin signalling system gives rise to different medical abnormalities. In the periphery dysregulation is linked to IBS (55), carcinoid tumors (56), pulmonary hypertension (57) and chemotherapy-induced emesis (58-60). In the case of IBS-d and chemotherapy-induced emesis, 5-HT3 antagonists are effective treatments (61, 62). This indicates that the modulation of 5-HT in the gut via inhibition of TPH1 is a possible treatment of these conditions. For the latter, 5-HT3 antagonists are effective in treating nausea and vomiting in the majority of patients. However, for patients receiving certain types of chemotherapy, delayed onset nausea and vomiting continue to be an issue (62).

In the CNS, several associations link dysfunction of the serotonin system to the pathogenesis of behavioural abnormalities. Thus, both of the TPH enzymes have been linked to conditions such as alcoholism, impulsive aggression, manic depressive illness, depression, attention defect disorder (ADHD), migraine and suicidal behaviour (7, 63-72). For several of these conditions genetic variations of the TPH gene has been thought of as playing an important role (63). In the CNS, too low amounts of 5-HT has been implicated in many disorders, which makes finding pharmacological chaperones of TPH2 of interest. Attempts on stabilizing TPH2 have been made, without any convincing results so far. BH_4 has previously been tested as an antidepressant without giving any consistent results, possibly explained by its lack of stability and poor blood-brain barrier (BBB) permeability (73).

1.4 Inhibition of TPH1

In this study we aimed to find new inhibitors of TPH1 that possibly could be used to treat diseases related dysregulation of the serotonin production in the gastro intestinal tract. Since TPH is involved in many physiological functions, making an inhibitor is challenging. The inhibitor can either be isoform selective, with low affinity to TPH2, or have a restricted tissue distribution, for example by limited blood-brain barrier permeability. In this way the inhibition of TPH1 could potentially exert a therapeutic effect on IBS or other functional gastrointestinal disorders related to serotonergic dysregulation, without exerting undesired CNS-effects. The brain levels of 5-HT have been shown not to be related to levels of 5-HT and its metabolites in the periphery (17, 74), thus inhibiting peripheral TPH1 should not effect the levels of 5-HT in the brain. At concentrations >0.2 mM, TPH is inhibited by its substrate L-Trp (75).

TPH is an intracellular enzyme and thereby effective inhibitors must be able to cross the plasma membranes in order to reduce 5-HT levels in vivo. In addition, serotonin produced in the periphery has many functions, making it important to develop a drug with low systemic exposure. Disruption of the tph1 gene in mice has shown to give abnormal cardiac activity ultimately leading to heart failure (13).

Several attempts on designing potent TPH1 inhibitors have been made. An inhibitor of TPH1, LP533401 (LX1033, figure 1.1.9), is a drug developed by Lexicon Pharmaceuticals. The compound inhibits both TPH1 and TPH2 *in vitro* but selectively lowers 5-HT levels in the GI tract. This is most likely explained by its inability to cross the blood-brain barrier (74). It is at the present time being tested in clinical phase 2 trial, for use in patients with diarrhoea-predominant irritable bowel syndrome (IBS-d). In a press release in December 2013, Lexicon Pharmaceuticals stated that LP533401 showed positive effects on abdominal pain compared to placebo. The biomarker for serotonin synthesis, 5-HIAA, was significantly more reduced by LP533401 compared to placebo (for further information, see lexgen.com). The drug has also given promising results when being tested in mice for prevention of osteoporosis (76).

Another compound named *para*-chlorophenylalanine (pCPA) has previously been tested in humans, proving to be effective in treating emesis induced by chemotherapy and diarrhoea in patients with carcinoid cancer (60, 77). However, side-effects like depression hindered it from becoming a drug for therapeutic use. The case of pCPA pins out one of the challenges when developing a drug affecting either of the two TPH isoforms. The presence of two different isoforms and their different functions in the body makes finding a drug without unwanted side-effects a challenge. In addition, TPHs similarity to both TH and PAH, also creates a challenge in enzyme specificity.

2 THEORETICAL BACKGROUND

2.1 Virtual screening

When the 3D structure of the target molecule (usually a protein receptor or an enzyme) is known, it is possible to visualize drug-target interactions *in silico*. Virtual high throughput screening (vHTS) is a computational technique used in the search for drug lead compounds from large chemical libraries of potentially active small molecules in order to find those that most likely bind to the known 3D structure of the target. The pharmaceutical industry often utilizes their own database of compounds when performing virtual screening. Also several freely accessible databases exist; among these is the Zink database (78). This database consists of over 35 million commercially available, ready-to-dock, 3D compounds, thus making it one of the largest databases of compounds for virtual screening. (May 2014). The Zink library consists among other compounds from the Sigma-Aldrich library, a compound library from the company with the same name. The latter library consists of 98 352 compounds (January 2014).

In this master thesis structure- based virtual screening was performed, in which compounds from the virtual Sigma library was docked into the active site of TPH1.

2.2 Molecular docking

Molecular docking, also called computer aided docking, can be an extremely useful tool when looking into ligand-protein interactions and the discovery of new drugs. Molecular docking is a procedure where the geometry of the receptor is used to position a ligand within the receptor of a protein, so that they interact favourably with one another. When the three dimensional structure of the receptor is known, virtual compounds can be docked into its active site. The search algorithm and the scoring algorithm are two important components of a docking program. In the process of docking the program computationally predict the conformational geometry and orientation of a ligand within the active site, using an automated computer algorithm. The scoring algorithm is then responsible for determining if the poses chosen by the search algorithm are the most energetically favourable. Different poses of the ligand are suggested and a scoring function evaluates the possibility of binding and how strongly the protein interacts with the compound, hence calculates its theoretical binding affinity.

The docking approach is based on some simplifying assumptions. Most docking programs can treat the ligand as flexible. However, this has been shown to be a great challenge for the receptor. At present, the great majority of docking algorithms treat the protein structure as a rigid body. Rigid docking is when the bond lengths, bond angles and torsion angles of the component is not modified. This is clearly a gross approximation given the intrinsic dynamic nature of the protein receptor with its large degree of freedom. Ignoring this ability may result in poor scoring and eventually discarding of compounds that in reality would be interesting hits.

In this project Glide (Grid-Based Ligand Docking with Energetics) was used for the molecular docking. This is a module of the Schrödinger package (79-81). Glide provides the possibility of a rational workflow for virtual screening. Glide provides three different searching algorithms with different speed and accuracy of precision. Starting with the high-throughput virtual screening (HTVS) mode and then the standard precision option (SP), it is possible to do virtual screening on large chemical libraries of millions of compounds. Accurate screening on a few remaining compounds is then done by using the extra precision (XP) mode (80).

Glide firstly generates a ligand pose. A *ligand pose* is the combination of orientation and position of the ligand relative to the receptor. Glide then uses a hierarchical series of filters in the search for promising locations of the ligand in the defined grid. The defined grid represents the shape and properties of the receptor. The scoring at this stage is done by using Schrödingerôs version of the ChemScore empirical scoring function (82). The algorithm score metal-ligand interactions, hydrogen-bonding and favourable hydrophobic interactions. Grid minimization is performed to minimize the energy of the poses by improving the geometry of the ligand poses. Final scoring is done on these energy-minimized poses, by using GlideScore. GlideScore is calculated from equation 2.

Equation 2:

GlideScore = 0.05*vdW + 0.15*Coul + Lipo + Hbond + Metal + Rewards + RotB + Site

The GlideScore is an empirical scoring function: In order to calculate the binding energy of a complex, GlideScore summarize the effects of multiple types of intermolecular interactions between the ligand and the receptor. The purpose of the scoring function is to estimate binding affinity for the given protein, ligand pose and conformation. Depending on atom type and which adjacent atoms are present, the atoms are identified with particular chemical labels. These labels are used to calculate the GlideScore; The Van der Waals term (vdW) calculates repulsion and attraction parameters between the atoms of the receptor and the ligand. The force is very significant if the contact surface is large and when the molecules are close. The Coulomb energy term (Coul) calculates electrostatic interactions between the atoms of the receptor and the ligand. This force draws molecules closer together or further apart according to their electrical charge. The two terms are calculated with reduced net ionic charges on groups with formal charges (e.g. carboxylates and metals). The lipophilic term (Lipo) is calculated for lipophilic receptor- and ligand atoms, rewarding favourable hydrophobic interactions. The Hydrogen bond term (Hbond) is calculated for all complementary possibilities of hydrogen bonds between the atoms of the receptor and the atoms of the ligand. Hydrogen bonds give a favourable contribution to affinity. If the ligand is a strong hydrogen bonder the solvent will attract it and make the receptor less favourable. If the receptor has hydrogen bonding qualities this can attract the ligand into binding. The term is separated into three different components depending on the donor and acceptor. Either both are neutral, one is charged and one is neutral or both are charged. The first of these contributions (both neutral) is

found to be the most stabilizing and the last (both charged) is the least important. The metal-ligand interaction term (Metal) is calculated for metal atoms in the receptor and all acceptor and acceptor/donor atoms in the ligand. Only the interactions with highly polar acceptor atoms or anionic atoms are included. Only if the net metal charge in the apoprotein (enzyme without its prosthetic group) is positive, the preferences for polar or anionic ligands are included. The Site term (Site) calculates polar interactions in the active site. Non-hydrogen-bonding polar atoms in a hydrophobic region are rewarded. The rewards term (Rewards) give rewards and penalties for different features, such as hydrophobic enclosure, buried polar groups and other terms that are not explicitly mentioned. In addition penalties for freezed rotatable bonds (RotB) are given. If the ligand is very flexible the equilibrium will be shifted against the solvent because the degree of disorder (qS) is greater here, thus causing a lower free energy. The free energy of the system should be as small as possible. A low (negative) energy indicates a stable system, and thus a likely binding interaction.

Finally, to select the correctly docked pose, a composite scoring function named Emodel is used. Emodel uses the combination of the GlideScore, the internal energy of the ligand and the non-bonded ligand-receptor interactions, and is used to pick the best-docked pose. The scoring function does not optimize the receptor to fit a ligand, which has shown to be a problem when docking (83). Even though the computation capacity has increased over the last years, dealing with receptor flexibility is still a huge challenge. The main reason for this is the large number of degrees of freedom in a protein. The challenge of induced fit of the receptor upon ligand binding is approached by scaling down the van der Waals radii of selected protein and/or ligand atoms. This to allow the docked compounds to partially overlap with atoms of the protein, allowing additional space in the binding pocket to be explored during pose generation, thus indirectly allowing moderate receptor flexibility (79).

Glide can be run in rigid or flexible docking mode, treating the ligand as a rigid body or generating conformations for the input ligand respectively. Different ligand chemotypes may induce different receptor conformations, thus potent ligands may score poorly against another receptor confirmation. Glide treats the receptor as a rigid body, but can perform ñgrid minimizationò when scoring different ligand poses. This function is intended as an approach to allow for further flexibility of the receptor. The function allows the generated grid to enclose around the docked ligand, before the calculation of docking score is performed. There are

several other approaches for dealing with the challenge of a flexible protein. One of these is ensemble docking. Here multiple available static receptor conformations can be used with the aim of mimicking a flexible receptor. The Virtual Screening Workflow module of the Schrödinger package is designed to do a sequence of jobs for screening large libraries against a target. Each ligand will be docked to each receptor, using Glide. The module also enables the use of progressive precision. Compared to other commonly used docking programs, Glide has proven to perform well (80, 84, 85).

2.3 Differential scanning fluorimetry (DSF)

Differential scanning fluorimetry (DSF), also called fluorescence thermal shift assay is a method often used when evaluating the effect of a ligand on a protein of interest. DSF was used in order to test if the hit compounds found by virtual screening had an effect on the experimental melting temperature of TPH1. The technique is used to identify low-molecular-weight ligands that bind and stabilise a desired protein. We were not primarely looking for stabilizers of TPH1, but inhibitors may as well bind strongly and by that stabilize the enzyme thermally. A fluorescent dye (SYPRO Orange) is added. This dye interacts with the hydrophobic areas of the protein. To find the temperature at which a protein unfolds, an increase in the fluorescence of the dye with affinity to hydrophobic area of the protein is measured. When the protein is folded the fluorescence dye is not able to interact with the protein. The protein will then be in solution where the fluorescent signal is quenched by water. As the temperature rise the protein unfolds and the hydrophobic parts are exposed to the hydrophobic dye. The fluorescence dye can now interact with the unfolded protein, thus giving rise to detectable fluorescence signal. The temperature of half-denaturation, at which 50 % of the protein is unfolded, is obtained. The difference in temperature of half-denaturation between presence and absence of ligand is related to the binding affinity of the ligand. The compound is stabilizing the protein if the temperature of half-denaturation is increased (86-88).

3 AIMS OF THE PROJECT

The aim of this project is to find small molecule compounds that inhibit the activity of human tryptophan hydroxylase 1. The motivation of this aim is that potent inhibitors of TPH1 can be further developed into drugs for treating dysfunction of TPH1, such as irritable bowel syndrome, chemotherapy-induced emesis and pulmonary hypertension as an extension of this project.

To accomplish the goals of the project several sub-projects will be conducted. Molecular docking will be performed, to find possible inhibitors of TPH1. The most promising compounds will be tested further in the lab. Here the compounds inhibitory effect in TPH1 will be validated by activity measurements.

4 MATERIALS

INSTRUMENTS

Method	Instrument	Provider
Concentration measurements	Nanodrop ND-1000	Saveen Werner
Centrifugation	Centrifuge 5430R	Eppendorf
Experimental screening	LightCycler 480	Roche Applier Science
HPLC	1200 Infinity series	Agilent Technologies
pH measurements	691 pH Meter	Metrohm
Weighing	Melter Toledo AB104-S	Bergman

CHEMICALS

Name	Provider
5000X SYORO Orange	Sigma Aldrich
Acetic acid (CH_3COOH)	Sigma Aldrich
Ammonium iron (II) sulphate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot H2O$)	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
Catalase	Sigma Aldrich
Compound IV (5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one)	Maybridge Ltd.
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Distilled water	Milli-Q
Ethanol	Sigma Aldrich
Dithiothreitol (DTT)	Sigma Aldrich
HEPES (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid	Sigma Aldrich
Hydrogen chloride (HCl)	Sigma Aldrich
Natrium chloride (NaCl)*	Sigma Aldrich

Propan-2-ol	Sigma Aldrich
Tetrahydrobiopterin (BH_4)	Schricks Laboratories
Sodium hydroxide (NaOH)	Sigma Aldrich
L-tryptophan	Sigma Aldrich
LP-533401 (2*S*)-2-amino-3-(4-(2-amino-6-(2,2,2-trifluoro-1-(3_-fluorobiphenyl-4-yl)ethoxy)pyrimidin-4-yl)phenyl)propanoic acid	Dalton Pharma Service
Sodium hydroxide (NaOH)	Sigma Aldrich
ZINC40566543 (2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(1H-pyrrolo[2,3-b]pyridin-3-yl)propanoic acid)	Sigma Aldrich
ZINC04262340 (2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid)	Sigma Aldrich
ZINC02529153 ((R)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-5-phenylpentanoic acid)	Sigma Aldrich
ZINC00080832 ((2S)-2-{[(benzyloxy)carbonyl]amino}-3-(1H-indol-3-yl)propanoic acid)	Sigma Aldrich
ZINC02583087 (Fmoc-5-fluoro-DL-tryptophan)	Sigma Aldrich
ZINC02539225 ((2S)-2-[(fluoren-9-ylmethoxy)carbonylamino]f-4-[benzyloxycarbonyl6butanoic acid)	Sigma Aldrich
ZINC02567256 (Fmoc-L-9-Anthrylalanine)	Sigma Aldrich
ZINC29319828 ((E)-2-Butyl-1-(p-carboxybenzyl)-alpha-2-thenylimidazole-5-acrylic acid, monomethanesulfonate)	Sigma Aldrich
ZINC02517148 ((2S)-2-[(fluoren-9-ylmethoxy)carbonylamino]-3-[4-(phenylmethoxy)phenyl]propano ic acid)	Sigma Aldrich
ZINC02539235 ((2S)-2-[(fluoren-9-ylmethoxy)carbonylamino]-3-[4-(phenylmethoxy)phenyl]propano ic acid)	Sigma Aldrich
ZINC05010475 (7-amino-4-hydroxy-3-[(5-hydroxy-7-sulfo-2-naphthyl)azo]naphthalene-2-sulfonic)	Sigma Aldrich
ZINC04545850 (4-amino-5-[1-[(1-carboxy-2-phenyl-ethyl)carbamoyl]-2-methyl-propyl]amino-5-oxo- pentanoic)	Sigma Aldrich

ENZYME

Trucated TPH1 (amino acids 102-402), TPH1 V177i (amino acids 102-402) and full length TPH2 was provided by Jan Haavik, professor at The Department of Biomedicine, in the Neurotargeting Research group. The truncated TPH1 enzyme preparation used in this project was from the same preparation that was used for crystallization of TPH and is thus the exact same enzyme we used when working *in silico (26).*

BUFFERS AND SOLUTIONS

FPLC buffer

Concentration	Chemical	Mm (g/mol)	For 1 L
20 mmol	HEPES	238.3	4.77 g
200 mmol	NaCl	58.44	11.69 g

The pH is adjusted to 7.0 with 2 M NaOH before all the water is added. The buffer is cooled down to 4 ℃.

HEPES buffer

Concentration	Chemical	Mm (g/mol)	For 0,5 dL
400 mmol	HEPES	238.3	4.77 g

The pH is adjusted to 7.0 with 2M NaOH before all the water is added.

HPLC buffer

Concentration	Chemical	Initial concentration	For 1 L
2 % (v/v)	2-propanol	< 99.8 %	20 ml
2.5 mmol	Acetic acid	< 99.8 %	2.5 ml

Stop solution

Concentration	Chemical	Initial concentration	For 1 dL
98 % (v/v)	Ethanol	Absolute	98 ml
2 % (v/v)	Acetiv acid	< 99.8 %	2 ml

The stop solution is cooled down to ï 20 °C.

SOFTWARE

Program	Version	Provider
Maestro	9.6	Schrödinger LLC
Canvas	1.8	Schrödinger LLC
Epik	2.6	Schrödinger LLC
Glide	6.1	Schrödinger LLC
LigPrep	2.8	Schrödinger LLC
Protein Preparation Wizard	2.6	Schrödinger LLC
PyMOL	1.5.0.4	Schrödinger LLC
Discovery studio	3.5	Schrödinger LLC
Virtual Screening Workflow	Schrödinger release 3-2013	Schrödinger LLC

Discovery studio is used for visualization and editing PDB-files.

PyMOL is used for visualization.

Other software is mentioned in methods.

5 METHODS

5.1 Molecular docking

5.1.1 Preparation of crystal structure

The crystal structures of TPH1 were found in the Protein Data Bank (PDB: 1MLW, 3HF6, 3HF8, 3HFB). For the initial docking we chose to work with the crystal structure with PDB code 3HF6. When deciding which PDB structure to work with, several factors had to be considered; the enzyme should be human, not be missing any important residues and have a high resolution. This decision was made after careful evaluation of the four available crystal structures of human TPH1. 1MLW had a more ñclosedò binding site compared to the three others. When docking the protein will be treated as rigid, and therefore a more open binding site is to prefer. When taking a closer look at the three remaining structures, all of them had missing residues (3HF6; Cys118 to Leu129, 3HFB and 3HF8; Arg121 to Asp138). When looking at the area with the missing residues it did not look like it was a part of the active site. None of the missing residues is mentioned as important parts of the enzyme activity (24, 25). From the three remaining structures 3HF6 had the highest resolution (3HF6; 180 Å, 3HF8; 185 Å, 3HFB; 192Å), and was therefore the structure of choice.

Before the docking some careful preparations of the receptor had to be done. Optimization and preparation of the receptor was carried out using the Protein Preparation Wizard, an application incorporated in the Schrödinger suit of programs (89, 90). Default options were used. All water molecules were removed to ensure an optimal hydrogen network. If active site water molecules are decided to be kept, careful analysis has to be done to avoid including water molecules that can be replaced by a ligand (83). In Glide SP and XP scoring functions both include terms designed to account for solvation of the active site. Thus, it is not necessary to add water molecules in order of estimating desolvation effects. For example when hydrophobic ligand groups are fully enclosed by hydrophobic receptor residues, an extra reward term is incurred. Missing hydrogen atoms were added. Terminal amino acids were caped to avoid charged residues near the active site. The formal charge of the iron in the active site was set to 2+ and low energy states were generated.

In the preparation of the crystal structures for the docking of the Sigma library, the PDBs 1MLW, 3HF6, 3HF8, 3HFB were aligned using molecular overlay in Discovery Studio. The ligands were deleted followed by adding of the ligand of 3HF6 (LX0) into each receptor. The proteins were prepared according to the previous description, but were not minimized, as this has been shown to bias the receptor binding pocket to preferentially given high docking scores to ligands similar to the one it is in complex with. To confine the mass centre of the docked ligand, a grid was generated defined by a 12 Å x 10 Å x 10 Å box centred on LX0 ligand in the active site. The grid was investigated to confirm that it included both the 3 available ligands and BH_2.

5.1.1 Validation of method

Validation tests were performed to ensure that the parameters being used were giving acceptable results. Re-docking (self-docking), where a ligand is docked to the protein from which it was removed was performed. Initially the re-docking was performed on the prepared structure of TPH1 with one of the three ligands bound (PDB code 3HF6). Secondly re-docking and cross-docking of 3 ligands and one co-factor (BH_2) with known binding mode was done using 3HF6. Cross-docking, where a complex containing a different ligand is used, provides a more realistic evaluation of the docking programs ability to reproduce the correct binding mode. The ligands were taken from PDB 3HF6 (LX0), 3HF8 (ML0), 3HFB (ML4) and 1MLW (BH_2). Different low energy sates of the ligands were prepared and docked. Both flexible and rigid docking of the ligands were performed. HTVS, SP and XP precision were applied. Based on the results cross-docking was performed on all available crystal structures of human TPH1. Now the 4 structures were both docked into their respective receptors (re-docking) and additionally into the other 3 receptors (cross docking). The latter resembles the conditions when docking the Sigma library and was done in order to measure how well the docking program performed when docking ligands into receptors that are not adapted to the ligand structure. For this flexible docking and standard precision was used. Finally ensemble docking was performed using all 4 crystal structures, enabled by Virtual Screening Workflow (VSW) from the Schrödinger suit of programs. Flexible ligand docking and HTVS and SP were applied. The docked ligands were superimposed with the reference ligands using molecular overlay in Discovery Studio. The accuracy of the docking was assessed based on the calculated

root-mean-square deviation (RMSD) between the coordinates of the heavy atoms of the ligand in the top docking pose and of the atoms of the ligand in the crystal structure.

5.1.3 Docking of the Sigma library

The Sigma library was obtained form the ZINC database (78, 91). The database is a non commercial collection of commercially available chemical compounds. It consists of a collection of filtered compounds. Filtering is based on physiochemical properties, in order to make the compounds relevant. As an example, molecules with an greater formula weight than 700, calculated logP greater than 6 and less than -4 and molecules containing an atom other than H, C, N, O, F, S, P, Si, Cl, Br or I are removed. The databases consist of compounds ready to dock. The compounds are in 3D and have low energy states at pH 7. The compounds are prepared especially for virtual screening. In addition we included ñmetal statesò, tautomers at high pH (up to pH 9). Deprotonation may be necessary when binding to metal-containing enzymes like TPH.

All four PDB structures (1MLW, 3HF6, 3HF8, 3HFB) were superimposed, using Discovery Studio. The ligand of 3HF6 was kept in all structures, and the other ligands were deleted. Each PDB was prepared separately, in the same way as in section 5.2.1 Minimization was not performed because we did not want all 4 receptors to adapt to the ligand of 3HF6. The crystal structure has a high resolution, which is preferable when omitting the minimization step. A grid with 12 Å, 10 Å and 10 Å around the centre was generated for 3HF6. Coordinates from this was obtained and used for generating grids for the other three PDBs. We made sure that all four compounds were included in the grid.

We performed docking of the 66 374 compounds available at the time (pr. Nov 2013) from the Sigma virtual library. The ligands were first docket in flexible mode, using HTVS. The 35 000 compounds with the highest docking score were taken further for standard precision docking All options were default.

5.1.4 Testing virtual compounds experimentally

The best compounds form the docking were acquired and tested experimentally by DSF and activity measurements.

5.2 Experimental methods

5.2.1 Experimental screening by DSF

The screening was performed using a modification of the method described by Pey et al. (92). A LightCycler 480 Real Time PCR System from Roche Applied Science was used for the experiment. The enzyme was diluted to 0.2 g/L in FPLC-buffer (20 mmol HEPES and 200mM NaCl). 5X SYPRO and the compounds dissolved in DMSO were added, to a concentration of 0.04 g/L. The compounds used were the 12 chosen hits from the molecular docking. In addition to TPH1 the hit compounds were tested in PAH and TH. When developing a potential drug affecting TPH it is also important to investigate if the compound has an effect on the other enzymes of the AAAH enzyme family. All of the compounds were provided by Sigma Aldrich. The assays were done on 96-well microplates provided from Roche Applied Science. The total volume was 25 εL. Further, the samples were loaded into the PCR-machine. Controls with 2 % DMSO were performed. The average melting point of TPH1 alone was calculated from 3 controls. Increase in SYPRO Orange fluorescence is associated with the protein unfolding. The thermal denaturation was monitored by following the SYPRO Orange fluorescence. The unfolding curves were registered from 20 °C to 95 °C with a scan rate of 0.04 °C/min. 4 measurements were made for each increasing degree. Temperatures for half-denaturation ($T_{0.5}$) were detected, both in the presence and absence of compounds. The shifts ($\Delta T_{0.5}$) from the control in the absence of compound were calculated.

5.2.2 Standard assay of enzyme activity

The TPH assay protocol was provided by Jan Haavik, but modified according to the protocol of standard assay of TH provided by Magnus Hole. This was done in order to enable comparison between data from activity assays done on TH and PAH. When using different protocols differences in results can be due to other factors than the enzyme or compound tested. Due to

familiarity and previously good results with the TH protocol, this was used as a template for the development of a new common protocol of TH, PAH and TPH.

TPH1 activity was assayed at 37 °C. The incubation mixture contained 400 mM NaHEPES pH 7.0, 10 mg/ml catalase, 10 mM ferrous ammonium sulphate (degassed), 1 mM L-Trp in 10 mM HCL and Milli Q water. The enzyme was kept on ice and diluted to 0.2 mg/ml using 0.1 mg/ml BSA, in FPLC buffer (20mM Hepes pH 7.0, 200 mM NaCl). The enzyme was added to the mixture with a final concentration of 0.02 mg/ml. After the enzyme was added to the mixture it was preincubated for 5 minutes at 37 °C. By adding 2 mM BH_4 in 20 mM DTT and 10 mM HCL the reaction was started, this also at 37 °C. The final volume was 50 εL. The reaction was stopped after 10 minutes, by adding 50 εL cold 2% (v/v) acetic acid in ethanol. The enzyme was precipitated at -20 °C for at least 30 minutes before it was centrifuged at 14000 rpm for 10 minutes to remove enzyme. Using high performance liquid chromatography (HPLC), L-Trp and 5-OH-Trp were separated. A cation exchanger functioned as a stationary phase and 2.5 mM acetic acid with 2% 2-propanol as a mobile phase. A fluorescence detector was used to determine the amount of 5-OH-Trp produced, using excitation at 290 nm and emission at 340 nm. Blank samples without enzyme added were also tested, to check for non-enzymatic conversion of L-Trp to 5-OH-Trp.

5.2.3 Linearity of the specific activity of TPH1

In order to see over how long time the activity of the enzyme remained constant, the reaction time was varied between 1 and 10 minutes.

5.2.4 Effect of bovine serum albumin and iron on enzyme activity

- Optimization of activity assay

A protocol is often a product of doing what is known to work, without any knowledge of how many of the factors are needed for the assay to work. In order to optimize and simplify the activity assay, the effect of both bovine serum albumin (BSA) and iron was tested. TPH is known to be unstable and difficult to study in the laboratory. TPH thrives poorly in dilute solutions, thus BSA is added to increase the concentration of protein in the sample. BSA is known to stabilize the enzyme and at the same time not affecting other properties of the enzyme. Ferrous iron, Fe^{2+} , is required for enzymatic activity (43). TPH1 activity was assayed

at 37 °C with iron and BSA, with only iron, with only BSA and with neither of the two. The enzyme was preincubated for 5 minutes. The iron may produce free radicals that theoretically can destroy the enzyme. To avoid this, we added the iron 4 minutes into the preincubation time. Reaction time was 5 minutes. The results were then compared.

5.2.5 Determination of optimal conditions for activity loss

When TPH is preincubated at 37 °C the enzyme looses activity. This can either be because of denaturation/unfolding or because of inactivation. The preincubation time was varied from 1 to 40 minutes to see how the activity decreased with time. As BSA has been shown to stimulate activity of the AAAHs, enzyme assays with and without BSA in the reaction mixture was performed (93, 94). The reaction time was 5 minutes. Both activity loss of TPH1 and TPH2 was assayed.

5.2.6 Testing of hit compounds

Some of the top-hit-compounds from the docking of the Sigma library were acquired and tested experimentally. In addition, the inhibitor LP533401 from Lexicon Pharmaceuticals and compound IV were tested. LP533401 was tested to se how well it performed compared to our potential inhibitors. Compound IV has shown promising results in being a pharmacological chaperone for PAH (95), thus it was interesting to see what effect is had on TPH1. Outliers were detected using Dixons Q-test. P-values were calculated to find significance. For controlling the false discovery rate (FDR) the Benjamin-Hochberg procedure was applied (96). FDR used to limit false positives. In a famous report Bennett et al. showed the importance of controlling the FDR when they detected brain activity in dead Atlantic Salmon (97). False discovery rate were set at < 0.05, meaning that at most 5 % of the detected results are expected to be false positives.

Effect of activity

In order to find out whether the compounds interacted with TPH1 or not, we performed activity measurements with 5 minutes preincubation and 10 minutes reaction time. Enzyme was diluted to 0.1 mg/ml, using FPLC-buffer. The enzyme was added to the mixture with a final concentration of 0.01 mg/ml. Compounds were added to the assay in 0.4 mg/ml and had a final

concentration of 0.04 mg/ml (This is equivalent to 81,2-118,6 εM depending on molecular weight of the compounds) in the mixture. Controls were added the same amount of DMSO as in the tests with compounds. The assay was carried out at 37 °C. Compounds giving rise to a decreased activity is inhibitors. Compounds giving rise to larger activity compared to the controls may either be stabilizers or activators.

Effect of stability

In order to determine whether or not the compounds giving rice to high activity in the previous assay are stabilizers or activators the effect of stability assay was performed. The assay was carried out in the same way as previously, but this time the compounds had 10 minutes of preincubation together with the enzyme at 37 °C. This way we could se if the compounds could protect TPH1 from loosing activity during these 10 minutes.

6 RESULTS

6.1 Molecular docking

6.1.1 Validation of method

Re-docking and cross-docking of the 4 ligands with known binding mode was performed in order to validate our docking procedure. Initially the re-docking and cross-docking were done using 3HF6, its associated ligand LX0 and BH_2 acquired from the PDB 1MLW. The first results gave rise to quite large RMSD values when doing flexible docking of the ligand. Based on this different low energy states were created for the ligands and all were included in the docking. Table 6.1 and 6.2 show the results form this re-docking and cross-docking to 3HF6. Both rigid docking and flexible docking were performed, using HTVS, SP and XP docking. RMSD values under 2 Å were considered acceptable results.

Table 6.1: *Results from the rigid re-docking and cross-docking using the PDB 3HF6 as a receptor.* Different energy states were created for the ligands. All three precision modes were applied. Manual inspection was used to find the best predicted pose. Both docking score and calculated RMSD are shown.

Rigid docking						
Compound	**HTVS**		**SP**		**XP**	
	RMSD [Å]	**Docking score [kcal/mol]**	**RMSD [Å]**	**Docking score [kcal/mol]**	**RMSD [Å]**	**Docking score [kcal/mol]**
LX0	0.2543	-8.810	0.2976	-8.919	0.3076	-8.2850
ML0	8.4220	-4.237	10.1558	-6.769	3.8402	-10.6060
ML4	0.8835	-7.663	1.0660	-8.381	0.9495	-7.8860
BH2	1.3259	-5.935	1.4215	-6.019	1.2952	-4.7530

Table 6.2: *Results from the flexible re-docking using the PDB 3HF6 as a receptor.* Different energy states were created for the ligands. All three precision modes were applied. Manual inspection was used to find the best predicted pose. Both docking score and calculated RMSD are shown.

Flexible docking						
Compound	**HTVS**		**SP**		**XP**	
	RMSD [Å]	**Docking score [kcal/mol]**	**RMSD [Å]**	**Docking score [kcal/mol]**	**RMSD [Å]**	**Docking score [kcal/mol]**
LX0	5.4799	-4.112	4.9398	-9.208	3.840	-6.868
ML0	6.8465	-7.261	5.381	-10.550	4.6377	-10.321
ML4	3.378	-5.567	6.5307	-7.905	4.9061	-9.023
BH4	1.8055	-4.871	1.5113	-5.636	9.225	-8.857

From the tables we see that best results were obtained by docking LX0 into its own receptor (re-docking). Based on these results we did re-docking and cross-docking of the 4 different ligands into the 4 different crystal structures; PDB 3HF6, 3HFB, 3HFB and 1MLW. Standard precision with both rigid and flexible docking was performed. Two differently prepared receptors were generated for each PDB, one were energy minimized and were without energy minimization. Table 6.3 and 6.4 show results from the docking when using a protein that was not minimized. Table 6.5 and 6.6 show results from the docking when using a protein that was minimized

Table 6.3: *The calculated RMSD values from the re-docking and cross-docking using a crystal structure prepared without energy minimization.* The best result for each receptor is highlighted in grey. Rigid docking and standard precision were applied. Manual inspection was used to find the best predicted pose.

Rigid docking

Ligand (PDB code)	**Receptor PDB code**			
	3HF6	3HF8	3HFB	1MLW
LX0 (3HF6)	0.3782	3.4397	3.2216	5.2784
ML0 (3HF8)	2.3957	0.3389	0.4379	2.0148
ML4 (3HFB)	1.1254	0.4306	0.3341	4.0553
BH2 (1MLW)	1.7617	1.5369	3.2216	1.8748

Table 6.4 *The calculated RMSD values from the re-docking and cross-docking using a crystal structure prepared without energy minimization.* The best result for each receptor is highlighted in grey. Flexible docking and standard precision were applied. Manual inspection was used to find the best predicted pose.

Flexible docking

Ligand (PDB code)	**Receptor PDB code**			
	3HF6	3HF8	3HFB	1MLW
LX0 (3HF6)	7.1650	3.9122	5.1328	6.7640
ML0 (3HF8)	7.1440	1.0758	7.1448	7.8255
ML4 (3HFB)	5.7183	2.6720	1.2899	5.1603
BH2 (1MLW)	1.8412	1.8633	1.7203	2.0768

Table 6.5 *The calculated RMSD values from the re-docking and cross-docking using a crystal structure prepared with energy minimization.* The best result for each receptor is highlighted in grey. Rigid docking and standard precision were applied. Manual inspection was used to find the best predicted pose.

Rigid docking

Ligand (PDB code)	**Receptor PDB code**			
	3HF6	3HF8	3HFB	1MLW
LX0 (3HF6)	0.4793	3.3138	7.4933	5.8656
ML0 (3HF8)	2.5464	0.5371	0.5840	2.4022
ML4 (3HFB)	6.7876	0.5908	0.3932	2.7868
BH2 (1MLW)	2.6175	1.8893	1.5805	1.3025

Table 6.6: *The calculated RMSD values from the re-docking and cross-docking using a crystal structure prepared with energy minimization.* The best result for each receptor is highlighted in grey. Flexible docking and standard precision were applied. Manual inspection was used to find the best predicted pose.

Flexible docking

Ligand (PDB code)	Receptor PDB code			
	3HF6	3HF8	3HFB	1MLW
LX0 (3HF6)	7.3334	4.8241	5.0184	5.3885
ML0 (3HF8)	7.0285	6.1109	6.9344	5.7243
ML4 (3HFB)	5.9032	6.2021	3.0155	4.9533
BH2 (1MLW)	1.9947	1.7750	1.8177	1.5208

Further on we decided to use multiple receptor docking using the 4 available crystal structures of human TPH1. This was performed using the Virtual Screening Workflow (VSW) module of the Schrödinger package. Results are shown in table 6.7. Flexible docking using HTVS and SP precision mode were performed. When preparing the receptor the minimization step was skipped.

Table 6.7*: Results of the cross-docking and re-docking of the four different ligands using VSW.* Which receptor the ligands were best docket into is shown together with glide score and the calculated RMSD.

Ligand (PDB code)	**Ensemble receptor PDB code**	**Glide score**	**RMSD**
LX0 (3HF6)	3HF6	-11.332	7.6148
ML4 (3HFB)	1MLW	-10.668	6.5418
ML0 (3HF8)	3HFB	-10.263	7.3804
BH_2 (1MLW)	1MLW	-6.556	2.4127

6.1.2 Docking of the Sigma library

The 66 374 compounds obtained form the Sigma library were docked into TPH1 using Glide. The ligands of the complete library were initially flexibly docked using the fast HTVS mode. 35 000 of the highest ranked ligands were then re-docked using the standard precision (SP) mode. The top 1000 scoring compounds were saved for further analysis. Two of the top hits

were L-Trp analogues and two were L-Phe analogues. All of the compounds docked (to different extends) to both the co-factor and the tryptophan binding site. A large proportion of the best-docking compounds (80 % of the 30 best docked compounds) had the 3 ring system observed in the compounds of row 1. Canvas (98, 99) was used to examine whether or not this had to do with an overrepresentation of these structures in the Sigma library, or if it was a result of high affinity for the TPH1 receptor. Results showed that only 1.2 % (800 of 66 374) of the compounds in the Sigma library had this feature. Figure 6.1 shows compound ZINC02583087 docked into 3HFB; 8 of the 12 acquired structures had this three-ring scaffold. When taking a closer look, all of them t-stacked with Phe318 in the tryptophan binding pocket. Phe318 has been shown to be essential L-Trp binding in TPH (24). The two tryptophan analogues stacked with Phe241, a residue involved in co-factor binding (24). Figure 6.1 and 6.2 show Compound 5 (ZINC0501475) docked into 3HF8. The latter shows the binding mode predicted by Glide. Figure 6.3 and 6.4 show Compound 12 (ZINC02567256) docked into 3HFB and figure 6.5 and 6.6 show Compound 7 (ZINC02567256) docked into 3HFB.

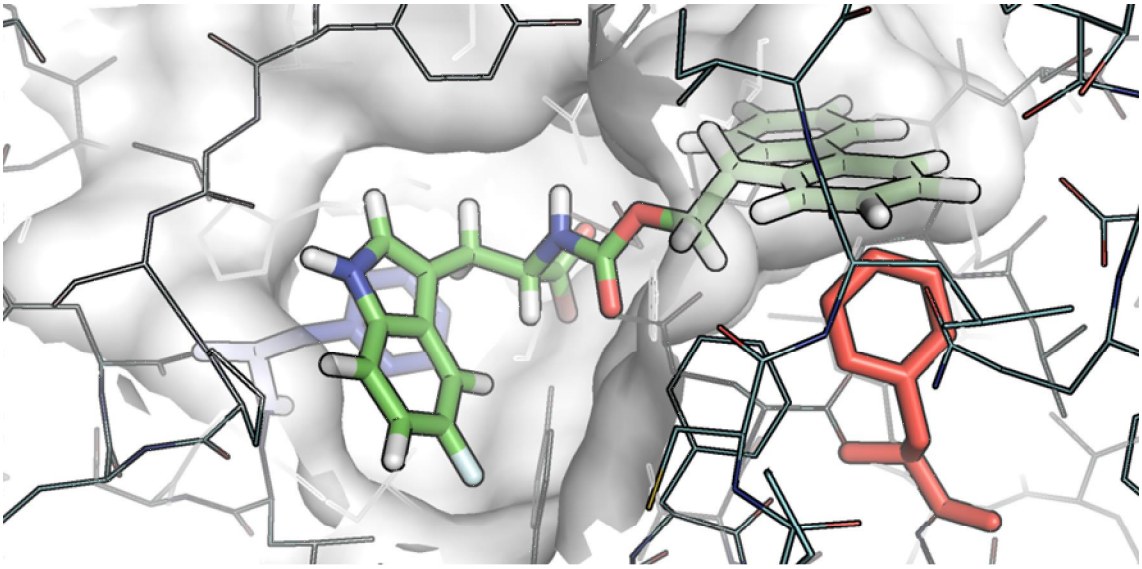


Figure 6.1: *Compound 5 (ZINC02583087) docked into the receptor of TPH1:* The three-ring system is t-stacking with Phe318 (shown in red) in the tryptophan binding pocket. The tryptophan analogue is stacking with Phe241 (shown in blue) in the co-factor binding pocket. (PDB: 3HFB)

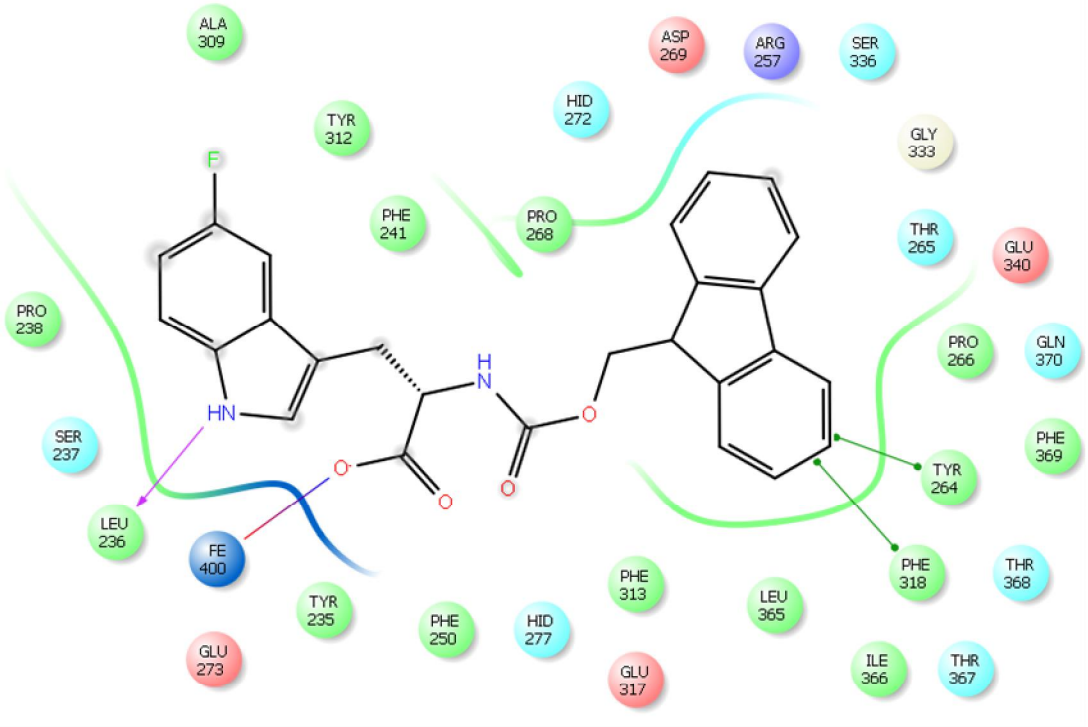


Figure 6.2 : *The 2D structure of Compound ZINC02583087 interacting with the receptor of TPH1 (PDB:3HFB*): Amino acids coloured red have a positive charge, purple have a positive charge, blue are polar, green are hydrophobic. Metal is shown in blue. Green lines are showing ´-´ stacking, purple lines shows hydrogen bonding and blue/ red lines shows salt bridges. Grey circles indicate solvent exposure.

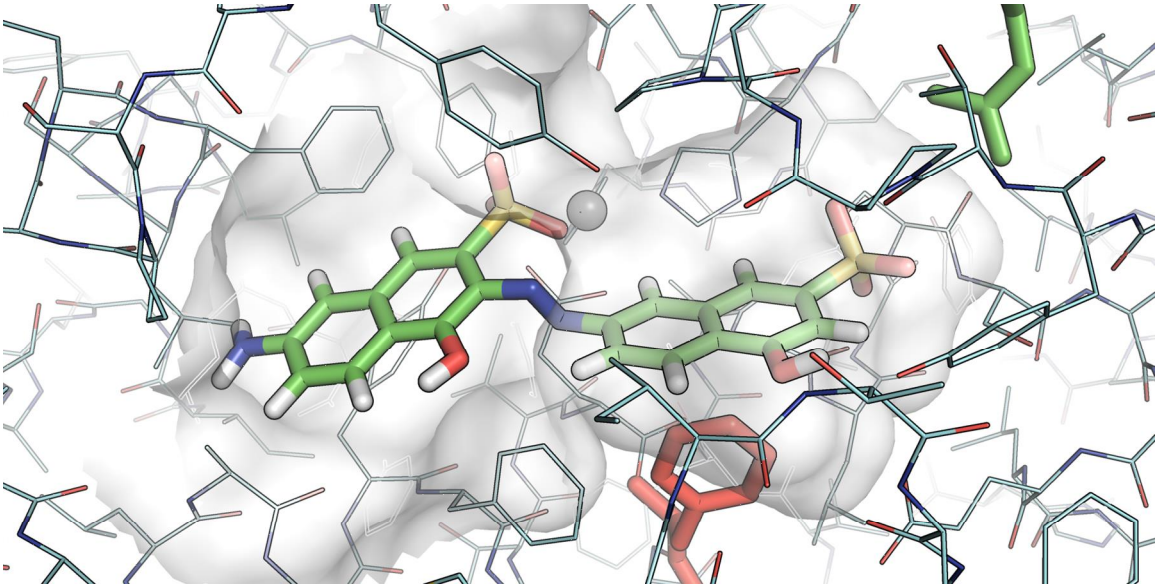


Figure 6.3*: Compound 12 (ZINC0501475) placed into the receptor of TPH1:* The ring system is t-stacking with Phe318 (shown in red) in the tryptophan binding pocket. Oxygen also interacts with Arg257 (shown in green) (PDB: 3HF8)

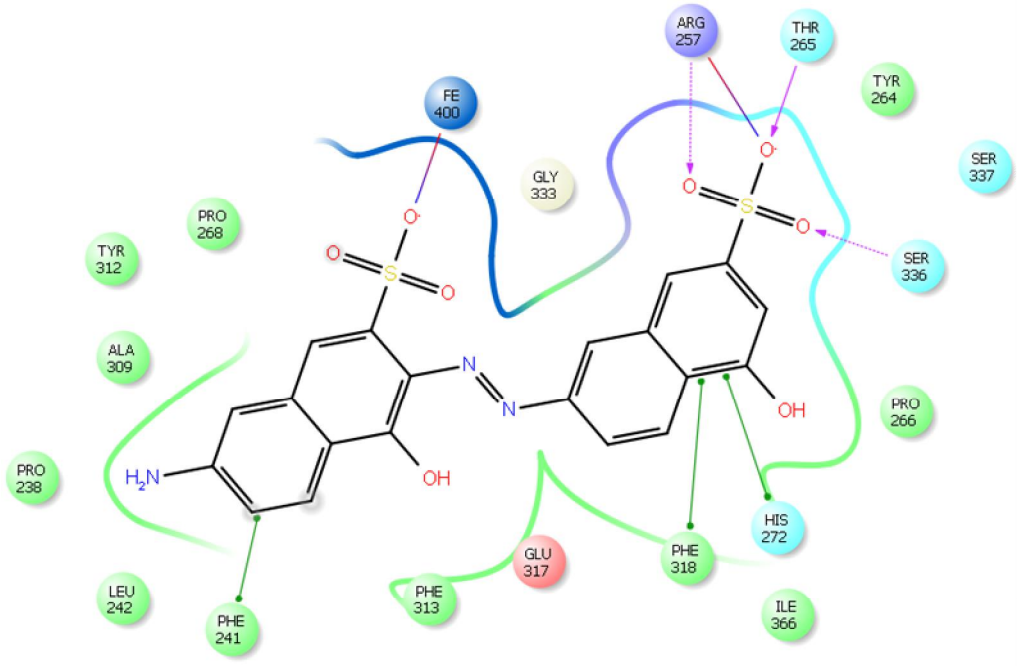


Figure 6.4: *The 2D structure of Compound 12 (ZINC0501475) interacting with the receptor of TPH1 (PDB 3HF8):* Amino acids coloured red have a positive charge, purple have a positive charge, blue are polar, green are hydrophobic. Metal is shown in blue. Green lines are showing ´-´ stacking, purple lines shows hydrogen bonding and blue/ red lines shows salt bridges. Grey circles indicate solvent exposure.

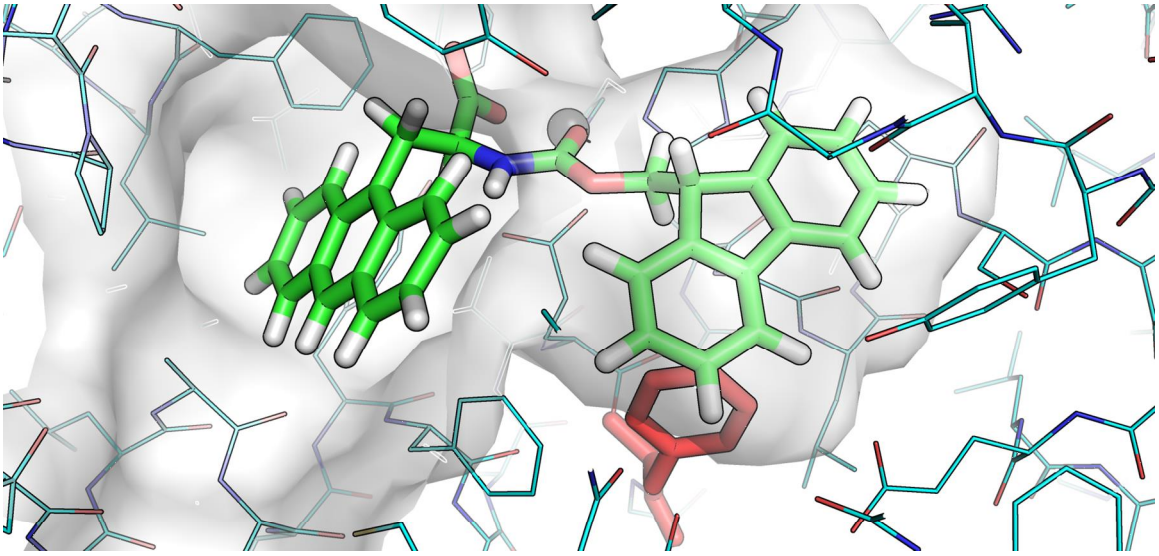


Figure 6.5: *Compound 7 (ZINC02567256) docked into the receptor of TPH1*: The three-ring system is t-stacking with Phe318 (shown in red) in the tryptophan binding pocket. (PDB: 3HFB)

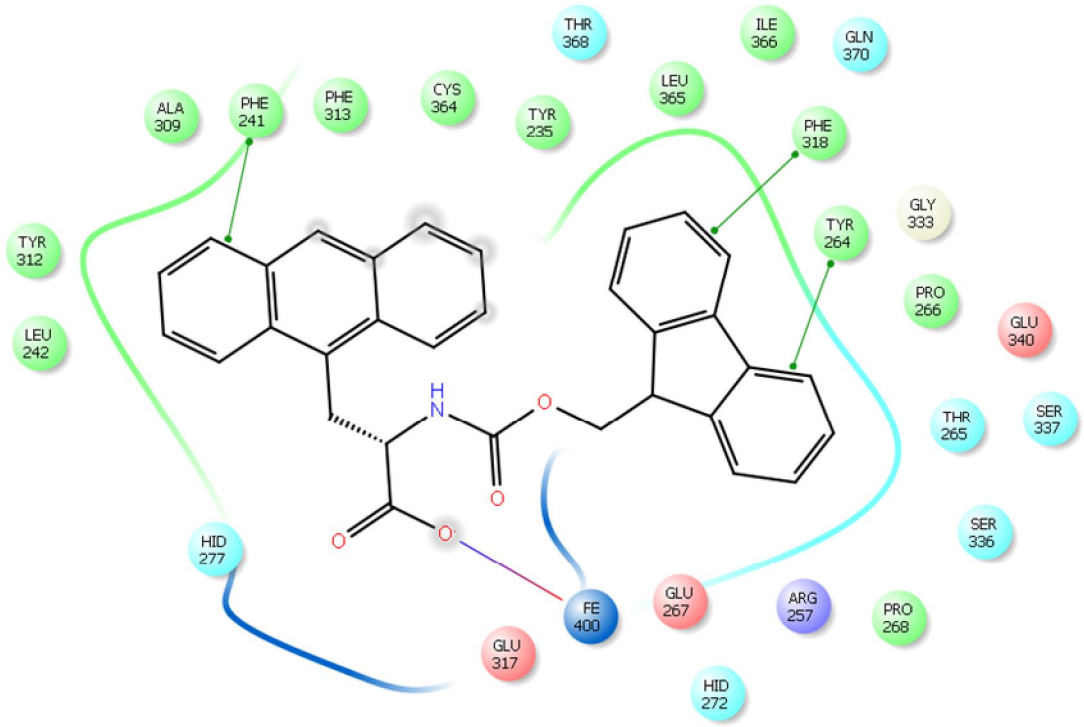


Figure 6.6: *The 2D structure of Compound 7 (ZINC02567256) interacting with the receptor of TPH1 (PDB 3HFB):* Amino acids coloured red have a positive charge, purple have a positive charge, blue are polar, green are hydrophobic. Metal is shown in blue. Green lines are showing ´-´ stacking, purple lines shows hydrogen bonding and blue/ red lines shows salt bridges. Grey circles indicate solvent exposure.

For practical and economical reasons we could not proceed with all of the best-docking compounds. The docking score, together with manual inspection of binding mode, were used to decide which compounds to obtain for the experimental validation. As many of the structures were quite similar we also tried to pick structures that did not resemble each other. All 12 compounds acquired were within the top 31 out of the top 1000 scored compounds, with docking scores ranging from -14.09 to -13.04. Figure 6.7 show the 2D structure of the 12 acquired compounds form Sigma Aldrich.

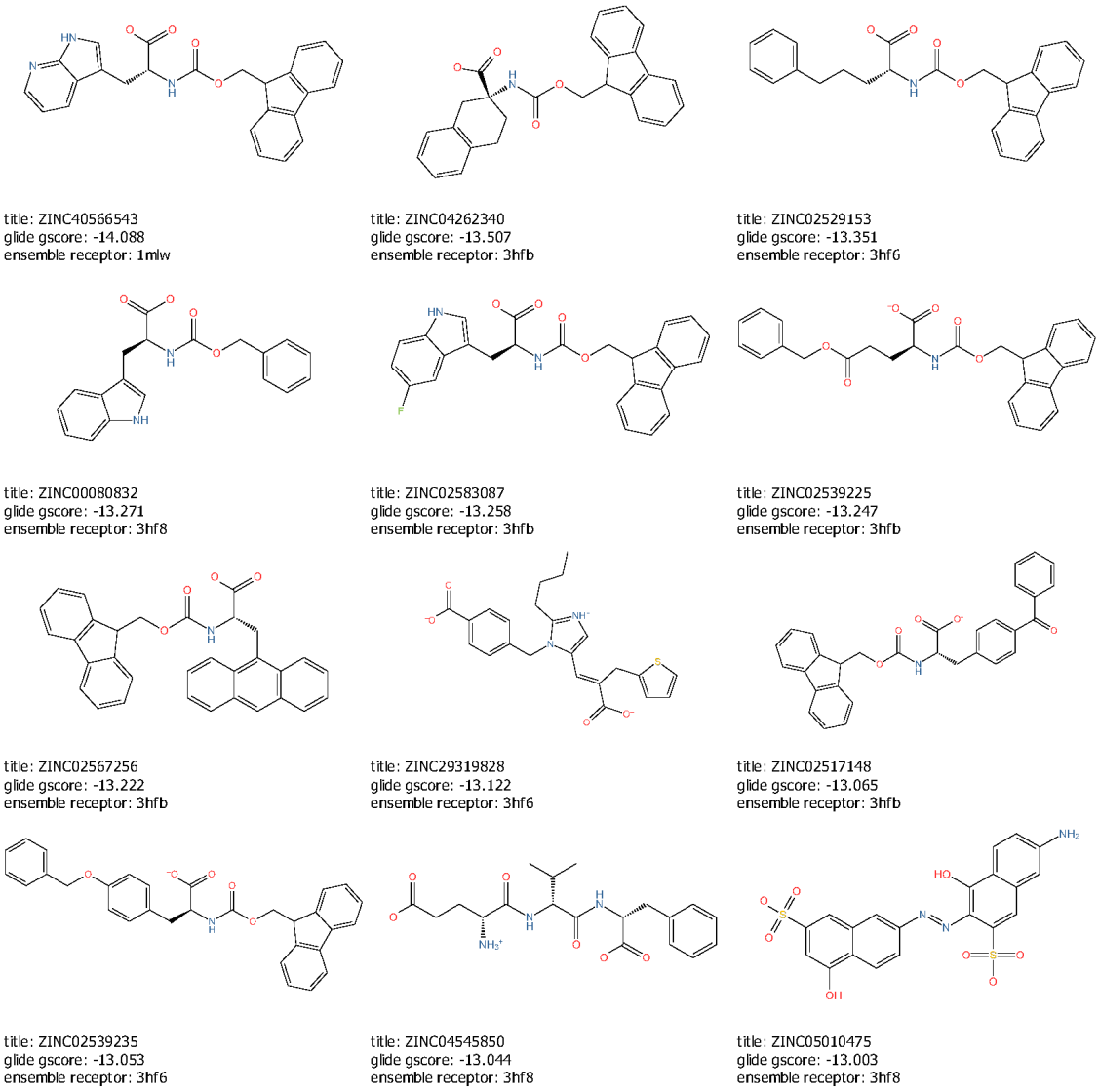


Figure 6.7: *2D structures of the compounds acquired from Sigma Aldrich*: This was based on the docking score as well as manual inspection of the binding mode of the compounds. The ñglide gscoreò and which of the 4 receptor it was docked into is shown.

6.2 Experimental screening by DSF

Experimental screening by DSF was done on TPH1, PAH and TH, to test the hit compounds from the molecular docking on the enzymes. Figure 6.8 shows the results from the screening. Fluorescence is shown on the y-axis. 1 is the highest fluorescent signal, and at this point all of the protein has unfolded. At fluorescence of 0.5 ($T_{0.5}$) 50% of the protein is unfolded. This is referred to as the melting point of TPH1 and is where we compare temperature values. For the control (TPH1 in of 2% DMSO in FPLC-buffer) $T_{0.5}$ = 56.2 ±0.2 °C (calculated from the mean of 3 parallels). When the line of TPH1 in the presence of a compound follows the line of the control, the compound does not have any effect on the melting point of TPH1. The melting point ($T_{0.5}$) of TPH1 in presence of the hit compounds was compared to the melting point of TPH1 in the control. The changes in $T_{0.5}$ were calculated as $\Delta T_{0.5.}$

From Fig. 6.8 we see the compounds having the greatest effect on TPH1s melting temperature. Three compounds, ZINC02539225 (A06), ZINC40566543 (D05) and ZINC04262340 (E05) (the dark blue, the red and the light blue line on the left of the control in black) lower the melting point of TPH1 by 3.1, 0.7 and 1.8 °C relative to the control, making TPH1 unfold at a lower temperature. One compound, ZINC04545850 (green line to the right of the control), increase the thermal stability of TPH1 by 0.5 °C.

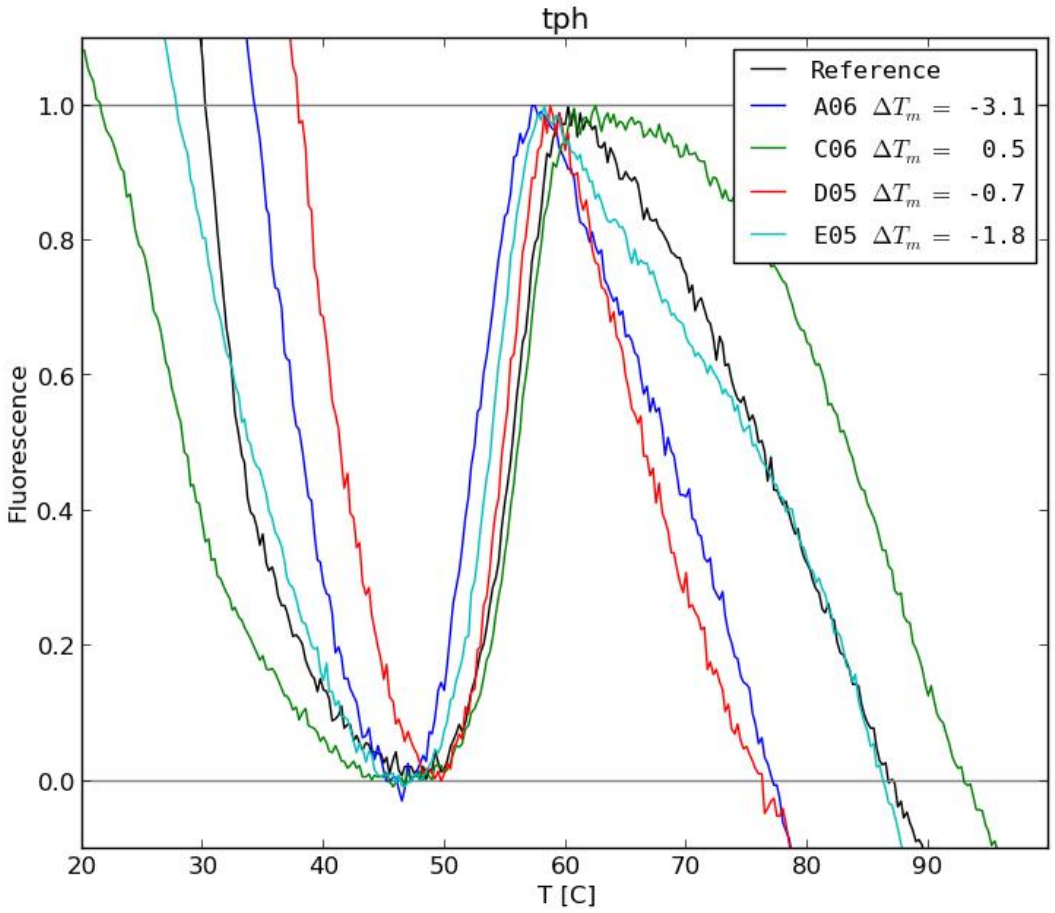


Figure 6.8: *Results from the DSF screening on TPH1:* 4 of the hit compounds giving rise to the larges change in melting point ($T_{0.5}$) of TPH1. Fluorescence is shown as a function of temperature. The control is shown in black, and is containing only TPH1 and DMSO.

The 12 compounds were also tested on PAH and TH. Three of the compounds, ZINC02539225 (A06), ZINC40566543 (D05) and ZINC04262340 (E05), are shown in figure 6.9 and 6.10. These compounds decreased the thermal stability of PAH by 3.3 °C, 2.5 °C and 3.3 °C, respectively. The same trend was seen in TH, as the thermal stability was decreased by 3.1 °C, 2.0 °C and 2.4 °C, respectively. As shown in Table 6.8 6 of the compounds had a destabilizing effect on PAH, and 6 on TH.

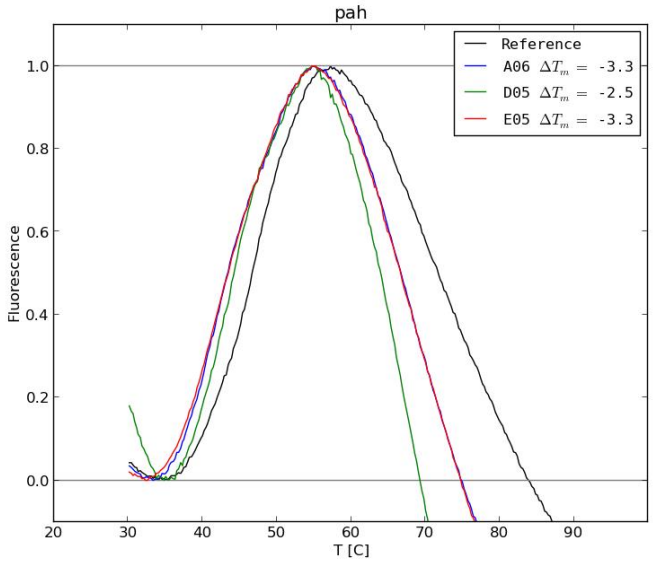


Figure 6.9: *Results from the DSF screening on PAH:* Results of three of the hit compounds giving rise to change in melting point ($T_{0.5}$) of PAH. Fluorescence is shown as a function of temperature. The control is shown in black, and is containing only TPH1 and DMSO. Compound ZINC02539225 (A06) is shown in blue, ZINC40566543 (D05) in green and ZINC04262340 (E05) in red.

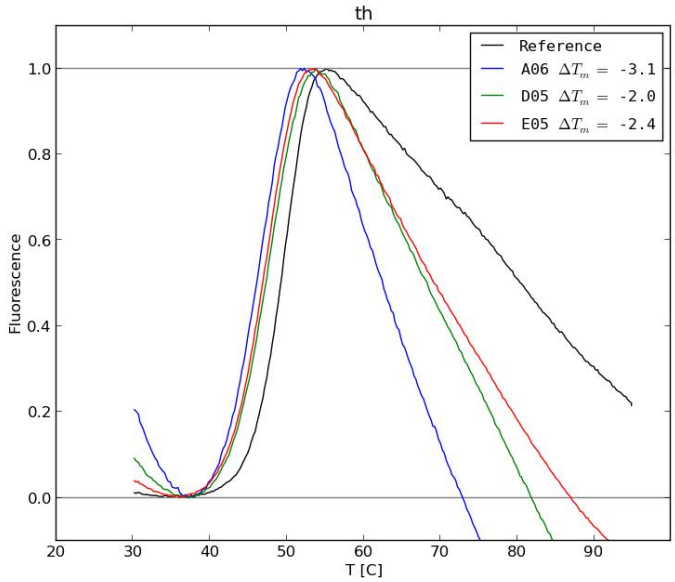


Figure 6.10: *Results from the DSF screening on TH:* Results of three of the hit compounds giving rise to change in melting point ($T_{0.5}$) of TH. Fluorescence is shown as a function of temperature. The control is shown in black, and is containing only TPH1 and DMSO. Compound ZINC02539225 (A06) is shown in blue, ZINC40566543 (D05) in green and ZINC04262340 (E05) in red.

Table 6.8: *Difference in melting point ($\varphi T_{0.5}$) for TPH1, PAH and TH in the presence of the hit compounds compared to the controls (with 2 % DMSO).* Final concentrations of the compounds were 0.04 g/L (This is equivalent to 81-118 εM depending on molecular weight of the compounds). The $T_{0.5}$ for the control was 56.2 °C. The compounds with no value did not cause any change in $T_{0.5}$.

Compound	**TPH1** $\varphi T_{0.5}$ (°C)	**PAH** $\varphi T_{0.5}$ (°C)	**TH** $\varphi T_{0.5}$ (°C)
Compound 1 (ZINC40566543)	-0.7	-2.7	-2.0
Compound 2 (ZINC04262340)	-1.8	-3.5	-2.4
Compound 3 ZINC02529153	-	-4.4	-2.3
Compound 4 ZINC00080832	-1.8	-1.0	
Compound 5 ZINC02583087	-3.2	-6.0	-2.1
Compound 6 ZINC02539225*	-	-3.6	--3.1
Compound 7 ZINC02567256*	-	-	-4.3
Compound 8 ZINC29319828*	-	-	-
Compound 9 ZINC02517148*	-	-	-
Compound 10 ZINC02539235*	-	-	-
Compound 11 ZINC05010475 *			
Compound 12 ZINC04545850	0.5		

*For some of the compounds $\varphi T_{0.5}$ was difficult to find, thus giving no results (marked -). These compounds most likely have a fluorescence of their own, resulting in error signals.

6.3 Activity measurements with TPH1

The data presented in this work is given as percent (%). Even though we put large efforts into standardizing the conditions between days and experiments, the absolute activity of TPH varied somewhat between days and experiments. One error source difficult to overcome is the varying time from when the enzyme is taken out of the Nitrogen-tank and to the assay is started.

6.3.1 Linearity of the specific activity of TPH1

In order to see if the activity remains constant after 10 minutes, an activity assay was performed using varying incubation time. The enzyme was preincubeted for 5 minutes. Fig. 6.11 shows how much 5-OH-Trp is produced after 1 minute, 5 minutes and 10 minutes of incubation. From the figure we see that the product formation is approximately linear when plotted against time. Results are calculated from the mean of three parallels. From these results the reaction time of 5 min were used in the following experiments and 10 min were used when testing the hit compounds.

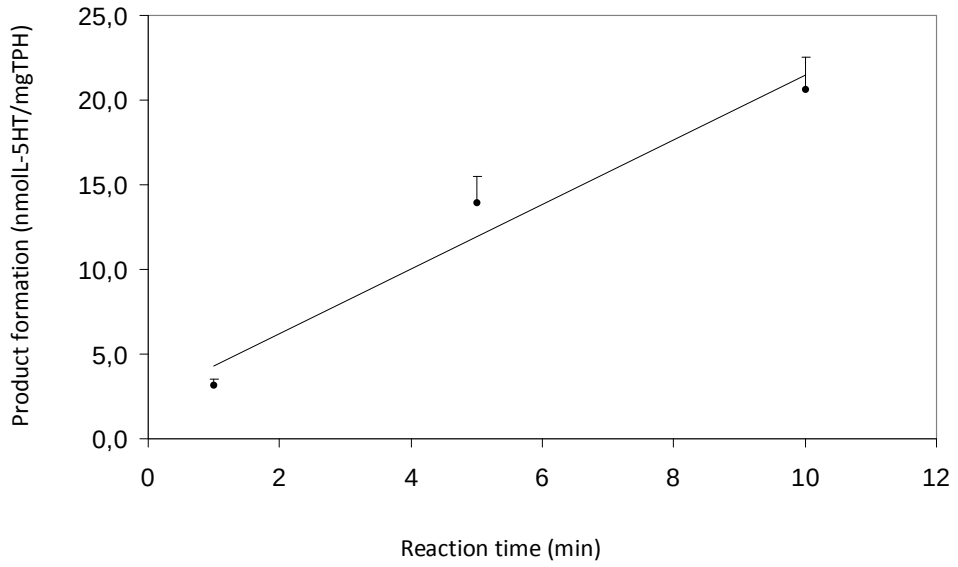


Figure 6.11: *Product formation ($nmol_{5\text{-}OH\text{-}Trp}/mg_{TPH}$) as a function of reaction time.*

6.3.2 Effect of iron and bovine serum albumin on enzyme activity

We investigated the effects of both bovine serum albumin (BSA) and iron on the assay. Figure 6.12 shows the results from the assays. The measurements were done on the same day. The results are calculated as a mean of three parallels. The control, with both BSA and iron, was defined as 100 %.

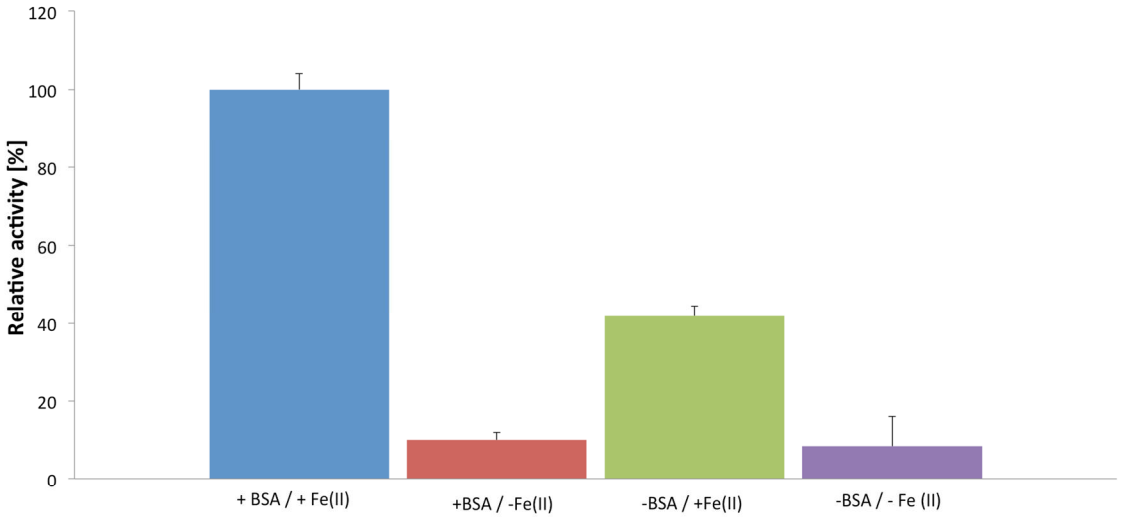


Figure 6.12: *Effect of BSA and iron on TPH1 activity*: The values representing mean of triplicate measurements, including standard deviation.

TPH1 activity was reduced to approximately 10 % of the maximal when removing iron. When only removing BSA the enzyme activity dropped down to 42 %. From table 6.9 we see that each of the varying conditions had a significant effect on TPH1 activity. The difference in activity between the assays with BSA and no iron was not significantly different from the assay with neither (P-value=0.697).

Table 6.9: *Relative specific activity of TPH1, under varying essay conditions.* P-values (compared to the assay with both BSA and iron) are shown.

	Relative specific activity %				
	P1*	**P2***	**P3***	**Average**	**p-value**
$^{+ BSA}/_{+ Fe(II)}$	100	100	100	100	
$^{+ BSA}/_{- Fe(II)}$	12.2	12.2	8.7	11.1	≤0.001
$^{- BSA}/_{+ Fe(II)}$	42.8	43.8	38.7	41.8	≤0.001
$^{- BSA}/_{- Fe(II)}$	15.3	9.7	0	8.33	≤0.001

* (P=parallel)

Based on these results we decided to keep both BSA and iron in the following activity assays.

6.3.3 Determination of optimal conditions for activity loss

When the temperature is kept at 37 ℃, a loss of enzyme activity is observed over time. To be able to see whether a compound has a protective and stabilizing effect on TPH we needed to find an optimal time of preincubation. At this time the enzyme activity must be considerably reduced, but still there has to be an ongoing reduction of activity. Figures 6.13-6.16 show how the activities decrease with the preincubation time. The first measurement was set to 100 % and had a preincubation time of 1 minute. The following measurements were done at 4, 12, 20, 32 and 43 minutes. The activity assays were performed on both truncated TPH1 and full length TPH2. Activity assays were also performed with and without BSA in the reaction mixture in order to obtain the best conditions for the destabilization. Some of the measurements gave no activity, most possibly because of an unstable enzyme.

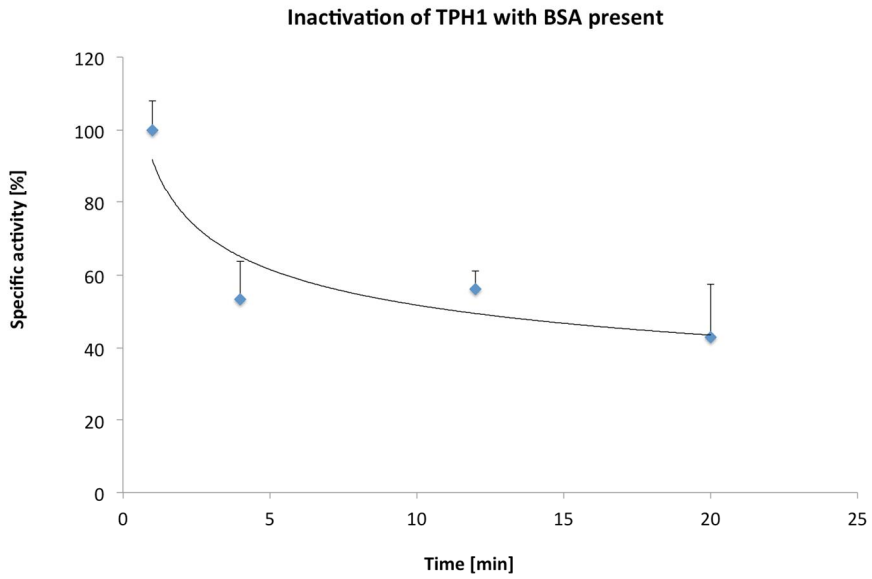


Figure 6.13*: Specific activity of TPH1 (nmol$_{5\text{-}OH\text{-}Trp}$/(mg$_{TPH1}$ x min)) as a function of preincubation time at 37 °C:* BSA is added to the reaction mixture. The time is the length of the preincubation time. The values represent the mean of triplicate measurements including standard deviation. The curve is fitted to a logarithmic decay equation.

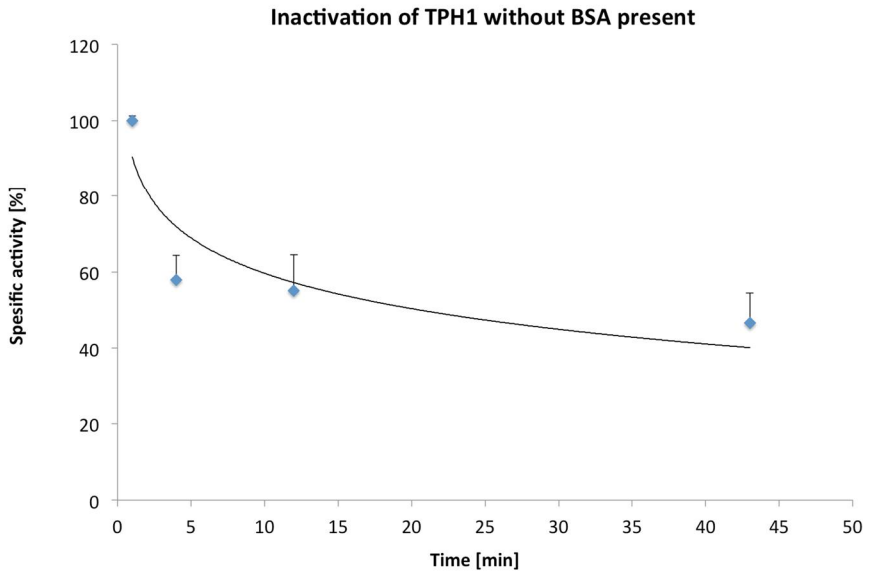


Figure 6.14: *Specific activity of TPH1 (nmol$_{5\text{-}OH\text{-}Trp}$/(mg$_{TPH1}$ x min)) as a function of preincubation time at 37 °C*: BSA was not added to the reaction mixture. The time is the length of the preincubation time. The values represent the mean of triplicate measurements including standard deviation. The curve was fitted to a logarithmic decay equation.

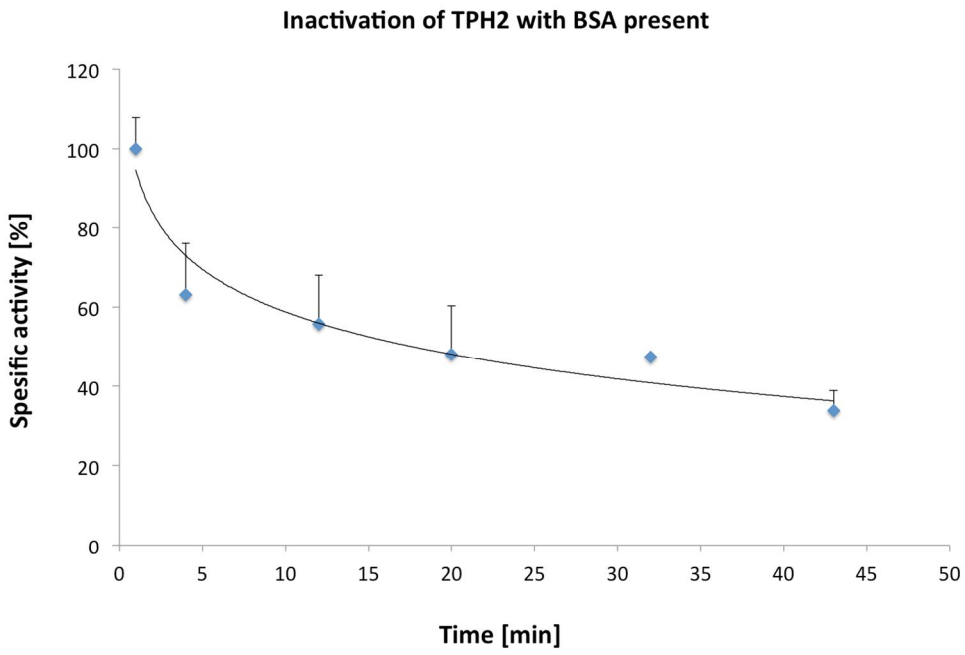


Figure 6.15: Specific *activity of TPH2 ($nmol_{5\text{-}OH\text{-}Trp}/(mg_{TPH1}$ x min)) as a function of preincubation time at 37 °C:* BSA is added to the reaction mixture. The time is the length of the preincubation time. The values represent the mean of triplicate measurements including standard deviation. The curve was fitted to a logarithmic decay equation.

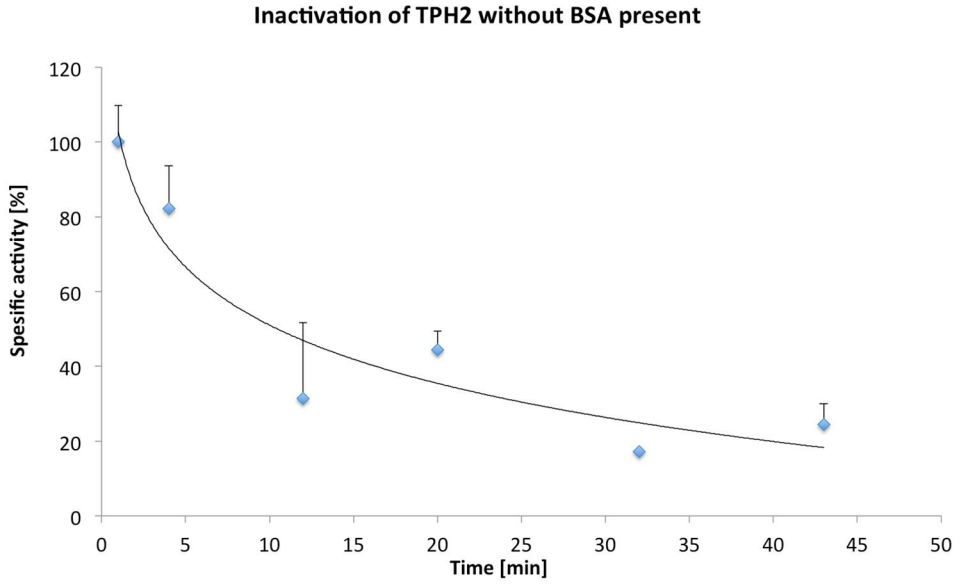


Figure 6.16: *Specific activity of TPH2 ($nmol_{5\text{-}OH\text{-}Trp}/(mg_{TPH1}$ x min)) as a function of preincubation time at 37 °C:* BSA was not added to the reaction mixture. The time is the length of the preincubation time. The values represent the mean of triplicate measurements including standard deviation. The curve was fitted to a logarithmic decay equation.

We observed that after approximately 10 minutes the activity was about 60-50 % compared to the activity after 1 minute of preincubation. Thus, a preincubation of 10 minutes was considered optimal for testing the hit compounds effect on the enzyme activity. Comparing the graphs of the assays performed with and without BSA, there is no huge difference between them. Based on this BSA was kept in the reaction mixture in the following research. By testing the activity of the enzyme after 10 minutes preincubation in the presence of the compounds, we can detect stabilizers of TPH1 and TPH2.

6.3.4 Testing of the hit compounds

Figure 6.17 shows specific activity of the hit compounds tested on TPH1. In addition Compound IV and the inhibitor LP533401 obtained from Dalton Pharma Service was included. Values are mean of 6 parallels. The control is a mean of 6 parallels. Enzyme was diluted to a concentration of 0.1 g/L and had a final concentration of 0.005 g/L. Dixons Q-test was applied on measurements differing considerably from the majority of the rest. Values judged to be outliers were discarded.

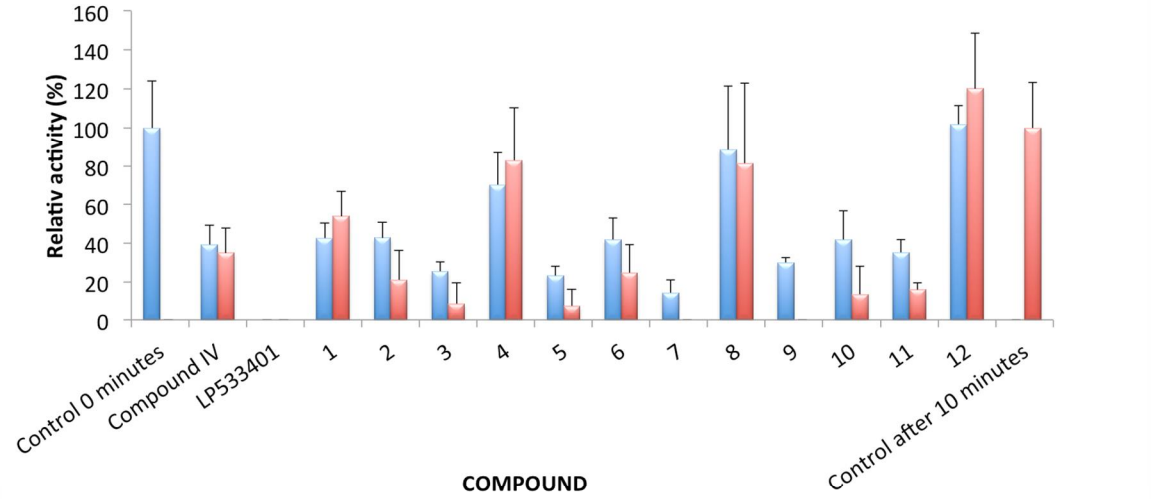


Figure 6.17: *Relative activity of TPH1 in the presence of compounds 1-12 and compound IV and inhibitor LP533401:* Final concentrations of the compounds were 0.04 g/L (equivalent to 0.8 mM ï 1.2 mM). Relative activities of TPH1 from the assays performed with no preincubation time are shown in blue (bars to the left). Relative activities of TPH1 from the assay performed with 10 minutes preincubation are shown in red (bars to the right). In order to compare control with activity results obtained with compound both controls were set to 100 %. In reality the control from the assay with 10 minutes preincubation time had an activity of 64 % compared to the one without preincubation time. The values represent the means of 6 parallels including standard deviation. Experiments were performed on the same day.

Effect on activity

Table 6.10 shows the results from the assay. Activities of TPH1 in the presence of compounds are compared with the control (only DMSO). P-values are calculated from absolute values by a

two sided paired t-test. FDR values are calculated using the Benjaminiï Hochberg procedure (96).

Table 6.10: *Relative specific activity (%) of TPH1 in the presence of the hit compounds.* Final concentrations of the compounds were 0.04 g/L (equivalent to 0.8 mM ï 1.2 mM). P-values compared to the control are also shown. FDR values are calculated with an uncertainty level of 0.05, thus compounds with p-values below 0.05 are significant results. FDR was adjusted for using the Benjaminiï Hochberg procedure. For compounds with P-values exceeding the limit of the FDR value results are not significant.

Compound	P1	P2	P3	P4	P5	P6	Average	P-value	FDR
Contol 0 minutes	92.1	100.0	100.0	100.0	100.0	100.0	100.0		
Compound IV	49.1	40.5	53.3	31.5	33.4	27.1	39.2	Ò0.001	0.0038
LP533401	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Ò0.001	0.0077
1 (ZINC40566543)	41.2	52.7	2.1	45.9	46.1	41.2	42.7	0.003	0.0115
2 (ZINC04262340)	48.3	50.8	50.0	41.1	33.6	32.9	42.8	Ò0.001	0.0154
3 (ZINC02529153)	31.4	28.1	28.5	25.3	19.2	19.9	25.4	Ò0.001	0.0192
4 (ZINC00080832)	89.1	75.3	86.2	59.5	46.1	65.6	70.3	0.006	0.0192
5 (ZINC02583087)	27.6	30.9	22.0	22.0	19.1	18.4	23.3	Ò0.001	0.0231
6 (ZINC02539225)	59.2	49.2	43.7	37.3	29.6	32.4	41.9	0.002	0.0269
7 (ZINC02567256)	11.7	15.5	10.8	15.0	19.4	0.0	12.1	Ò0.001	0.0308
8 (ZINC29319828)	60.8	92.4	135.7	44.6	86.5	110.5	88.4	0.002	0.0346
9 (ZINC02517148)	34.1	30.9	30.9	28.5	25.9	0.0	25.1	0.002	0.0385
10 (ZINC02539235)	51.5	60.4	53.0	31.8	28.7	26.2	41.9	0.002	0.0423
11 (ZINC05010475)	39.3	41.3	38.4	32.9	36.6	23.3	35.3	0.002	0.0462
12 (ZINC04545850)	88.8	90.2	150.3*	86.5	86.3	109.0	92.2	0.0685	0.0500

**Parallel was detected as an outliner using Dixonôs Q-test.*

When choosing a significance level of 0.05 we see that compounds 1-11, compound IV and LP533401 significantly reduces the activity of TPH1 compared to the control. When applying the Benjaminiï Hochberg procedure, also with a significance level of 0.05, we see the same results.

Effect on stability

Table 6.11 shows the results from the assay. Activities of TPH1 in the presence of compounds are compared with the control (only DMSO). P-values are calculated from absolute values by a two sided paired t-test. FDR values are calculated using the Benjaminiï Hochberg procedure.

Table 6.11: *Relative specific activity (%) of TPH1 in the presence of the compounds.* Final concentrations of the compounds were 0.04 g/L (equivalent to 0.8 mM ï 1.2 mM). P-values compared with the control are also shown. FDR values are calculated with an uncertainty level of 0.05, thus compounds with p-values below 0.05 are significant results. FDR was adjusted for using the Benjaminiï Hochberg procedure. For compounds with P-values exceeding the limit of the FDR value results are not significant.

Compound	P1	P2	P3	P4	P5	P6	Average	P-value	FDR
Control 10 minutes	100.0	100.0	100.0	100.0	100.0	100.0	100.0		
Compound IV	51.7	43.8	28.0	19.6	31.8	95.8*	35.0	0.003	0.0045
LP533401	0.0	0,0	0.0	0.0	0.0	0.0	0.0	Ò0.001	0.0045
1 (ZINC40566543)	73.6	59.1	51.4	42.8	43.0	0*	54.0	0.019	0.0091
2 (ZINC04262340)	50.2	38.4	0.0	20.6	17.9	0.0	21.2	0.004	0.0091
3 (ZINC02529153)	16.6	0.0	0.0	10.3	0.0	25.9	8.8	Ò0.001	0.0136
4 (ZINC00080832)	112.0	101.4	91.4	90.3	65.5	36.6	82.9	0.1198	0.0182
5 (ZINC02583087)	19.6	15.7	10.1	0.0	0.0	0.0	7.6	Ò0.001	0.0182
6 (ZINC02539225)	41.0	23.6	16.9	18.0	0**	49.4	29.8	0.002	0.0227
7 (ZINC02567256)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Ò0.001	0.0273
8 (ZINC29319828)	116.1	80.2	108.7	121.0	25.9	36.1	81.3	0.1640	0.0318
9 (ZINC02517148)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Ò0.001	0.0364
10 (ZINC02539235)	37.9	21.1	23.0	0.0	0.0	0.0	13.7	Ò0.001	0.0409
11 (ZINC05010475)	16.5	25.2	19.5	19.2	17.4	0**	19.5	Ò0.001	0.0455
12 (ZINC04545850)	115.1	116.2	171.0	129.1	98.2	91.2	120.1	0.8258	0.0500

**Parallel was detected as an outliner using Dixonôs Q-test.*

***Parallel was excluded because of observed experimental error.*

When choosing a significance level of 0.05 we see that compound all of the compounds except compound 4, 8 and 12 significantly reduces the activity of TPH1 compared to the control. When applying the Benjaminiï Hochberg procedure, also with a significance level of 0.05, we see the same results. From the results we see that compound 12 gives promising results for stabilization, with the activity of TPH1 being 120.1 % compared to the control. Even though it is not significant according to the p-value, this is a positive trend.

7 DISCUSSION

The aim of our project is to find a compound that can be further developed into a drug used in the treatment of dysregulation of serotonin in the GI tract, such as IBS, chemotherapy-induced nausea and vomiting and pulmonary hypertension. The wide range of medical disorders linked to dysregulation of 5-HT is reflected by the large number of available pharmaceuticals and by the many different 5-HT receptors now being targeted (100). Direct inhibition of TPH1 is an approach that has been very little explored as a therapeutic approach, due to the importance of 5-HT in the brain and the challenges in selective reduction of 5-HT in the periphery. The discovery of the two distinct genes, TPH1 and TPH2 provides the possibility of selective inhibition of TPH1. Liu et al. did a study on mice using inhibitors of TPH, showing that GI-specific TPH inhibitors may provide novel treatments for various disorders associated with 5-HT dysregulation. When administering the inhibitor the mice showed a reduced emetic response. Additionally the mice had normal levels of brain 5-HT while having reduced levels in the GI-tract (74). This suggests that developing a selective inhibitor of TPH1 can be of large therapeutic interest for a number of diseases linked to dysregulation of the serotonin peripheral pathways, either alone or in combination with existing therapies.

Virtual screening is considered as a valuable method in the search and development of new potential drugs. Over the last decade molecular docking has become a frequently used tool in structure-based rational drug design. The use of virtual screening methods has led to the discovery of several drugs and drug candidates, for example lead compounds for treating Alzheimerôs disease and rheumatoid arthritis (101).

In our project we have combined the method of virtual screening with experimental procedures in the laboratory. Both methods share a common goal: to find a new lead compound. There are clear advantages of combining these two methods. Virtual screening can be used for screening large libraries assessing millions of compounds, in the search of a potential hit. This task would often be too costly and time-consuming done in the laboratory. Another advantage of virtual screening is that compounds difficult to synthesise can also be included. In addition virtual screening can be used to exclude compounds that exhibit unfavourable pharmacokinetic properties (ADMET). However, no drugs are created solely *in silico.* As computational approaches are based on assumptions and results are only predictions, the latter can be used for validation of the hit compoundsô *real* effect on the target.

7.1 Molecular docking

Re-docking and cross-docking were performed in order to validate our docking method. This is necessary to achieve best possible results when docking the Sigma library. The first validation performed was re-docking of LX0 into its associate receptor 3HF6. The calculated RMSD values for both flexible and rigid docking were quite low for rigid docking (varying between 0.25 to 0.3 for all three precision modes), thus matching the orientation of the inhibitor as described in the crystal structure by Ciancetta et al. (54). The RMSD values obtained from the flexible docking were quite large (varying from 3.8 to 5.4 for all three precision modes). However, re-docking is not the most relevant way of validation our method for docking of the Sigma library. The receptor of LX0 is already adapted to the ligand, which is not the case when docking the Sigma library. This is nevertheless a good starting point for further validation. With this in mind we decided to perform a cross-docking validation including the other two ligands, ML0 and ML4, and the cofactor analogue, BH_2, from the other three crystal structures. Low energy conformations of the ligands were generated for the cross-docking.

From Table 6.1 and 6.2 we can see the root-mean-square deviations (RMSD) and the docking scores obtained from the initial docking. The confirmation and orientation of the ligand of 3HF6 (LX0) was best predicted. Surprisingly, also ML4 obtained quite low RMSD values in rigid docking. We see that the RMSD values are quite high for the cross-docked ligands. This was not surprising as the crystal structure is shaped for the interaction with one particular ligand giving rise to bad predictions of the rest (102). We can also see that between flexible and

rigid ligand docking, best RMSD values are obtained when using rigid docking. This is because the input conformation only can be rigidly rotated relative to the receptor. The high RMSD values from the flexible docking is not surprising as the ligands are quite large in addition to having a relative high number of rotatable bonds (LX0:7, ML0; 8, 3HFB4; 8). Because of the size of the conformational space to be sampled, accuracy of docking decreases with the number of rotatable bonds in a ligand. From comparing table 6.1 and 6.2 we can see that the smaller sized co-factor, with only two rotatable bonds, is docked well both when using flexible and rigid docking. Glide has been shown to provide good results with increasing numbers of rotatable bonds with a relative small decrease in accuracy compared to other programs (85). The results shown are from the best predicted pose, based on a manual inspection. Even though Glide often had good pose predictions, these poses were often given a lower docking score than other poses that differed more from the reference ligand.

As the re-docking gave best results we proceeded with re-docking and cross-docking using all 4 available crystal structures of human TPH1; PDB 3HF6, 3HFB, 3HFB and 1MLW. This in order to see if one of these would be better representations of the TPH1 receptor in general, thus giving rise to lower RMSD values for a greater proportion of the ligands. We generated two sets of grids for each crystal structure, one minimized and one not. This was because minimization of the ligand receptor complex before docking has been shown to bias the receptor binding pocket to preferentially give high docking scores to ligands similar to the one it is complex with (83). In this process there is a chance of loosing a novel interaction. Table 6.3-6.6 shows the RMSD values obtained from the cross-docking. From the results where the minimization step was skipped we could se that in most cases (7 out of 8) the ligands were best docked into their associated receptor. We can also see that the three inhibitors obtained lower RMSD values when being docked into each others crystal structures compared to the one of the cofactor analogue. This was not surprising as we in the manually inspection of the crystal structures saw that in the crystal structure of 1MLW the active site was more enclosed than in the other three crystal structures. Additionally the three inhibitors binding mode in the substrate binding pocket are quite similar, thus we can expect better predictions when docking these ligands into each others crystal structure. Using a minimized protein resulted in all the ligands being best docked in their associate receptors in rigid docking. When doing flexible docking two of the ligands obtained best RMSD values in another crystal structure than its own. As expected, BH_2 obtained the lowest RMSD scores in flexible docking mode since this is the smallest and most rigid ligand.

To summarize we observed a trend of ligands being best docked into their associating receptors. Here the challenge of protein flexibility is made obvious. When using static crystal structures adapted to certain ligand conformations we risk discarding compounds that in reality would be novel hits. The binding site area of two different PDB structures of the same protein does not necessarily have the same features. In the search for inhibitors of TPH1 Cianchetta et al. showed that binding of three promising inhibitors of TPH1 induces major conformational changes of the enzyme (54). Thus, relying on only one PDB structure can have a bad effect on the docking performance (103). A way to address protein flexibility is to use multiple static protein structures to emulate receptor flexibility (83, 103). Teodoro et al. propose the procedure of using a combination of several flexible receptor methods, among these ensemble docking (104), but this is a time consuming strategy. Based on this knowledge and the results from the validation we decided to use ensemble docking when performing the docking of the Sigma library. Table 6.7 shows the docking score and the RMSD values from the ensemble docking. Interestingly, two of the ligands were docked into other receptors than their own.

The water molecules are removed prior to docking. Incorporation of active site water has been shown to decrease docking accuracy, thus water should be kept only after careful analysis (83). In the crystal structures all three inhibitors interact with water, all with one of the iron-coordinating water and additionally LX0 and ML0 form hydrogen bonds with second water (54). In the crystal structure of 1MLW the cofactor analogue also forms hydrogen bonds to two of the iron coordinating water molecules and some of the interactions with amino acids in the active site are bridged by water (26). This can be one of the factors explaining the discrepancy between the orientations of the docked ligands and the placement in the crystal structures. In chicken TPH L-Trp interact with two water molecules through hydrogen bonds. When removing water we thus risk discarding compounds that would have this interaction. However, keeping water molecules in the binding pocket effectively exclude this volume for compounds to dock. As we would like to explore as much of the active site as possible we decided to remove all water molecules prior to docking.

Figure 6.7 shows the chosen compounds from the molecular docking. A large majority of the best-docked compounds had the three ring system observed in many of the compounds (8 out of 12 compounds). A scan done by Canvas revealed that this did not have to do with an overrepresentation of this scaffold in the Sigma library (only 1.2 % of the compounds had this

scaffold). Thus, this trend probably has to do with the property of the receptor. From table 6.10 we see that the 8 compounds with the three-ring system all inhibited the effect of TPH1 down 50 % or lower, compared with the control, with compound 7 having the best inhibitory effect (12 % activity compared to control). This trend suggests that this scaffold could be an interesting starting point for hit-to-lead optimization.

Some of the compounds have already been studied in the development of drugs for other conditions. Compound 4 (ZINC00080832) has reported to inhibit gastrin-induced acid secretion and gastrin binding to cell receptors in rat (105). Compound 9 (ZINC02517148) is reported to be a potential precursor for therapies fighting trypanosomatid pathogens, including human African trypanosomiasis (106). It is worth mentioning that this compound inhibited TPH1 activity down to 25 % compared to the control in our *in vitro* assay. This may not be the case *in vivo*, but if the compound does affect either TPH1 or TPH2 it could potentially lead to side effects. Compound 8 (ZINC29319828) is currently a drug, Eprosartan, working as an angiotensin II type 1 receptor blocker (107). Interestingly, blockade of 5-HTT and 5 HT_{2B} receptors has shown to prevent or reverse experimentally induced pulmonary hypertension (PM) (108), thus inhibiting TPH1 may also have a similar effect. In fact, Abd et al. tested the inhibitor LP-533401 on mice with overexpressed 5-HTT in smooth muscle cells and PM. 250 mg/kg per day LP533401 reduced the lung and blood 5-HT levels and right vascular systolic pressure. Treatment partially prevented PH development in wild-type mice and together with the 5-HT transporter inhibitor, citalopram, the effects were even better (109). From the activity assay we can se that compound 8 has a mild inhibitory effect on TPH1, as TPH1 has an activity of 88.4 % in the presence of the compound compared to the control.

Two of the acquired compounds were L-Trp analogues, compound 1, 4 and 5. Surprisingly they were all placed into the cofactor binding pocket. For the two L-Phe analogues, compound 8 and 11, the L-Phe part also were placed in the cofactor binding pocket. Figure 6.1-6.6 shows the binding mode of 3 of the top hits, compound 5, 7 and 12. When inspecting the docking results we see that all compounds are placed overlapping with the substrate. In addition, all of them were placed overlapping (to different extents) with the cofactor. This is contrary to the inhibitors found by Chianchetta et al. which did not occupy the pterin binding pocket in the crystal structure (54). Compound 7, 12 and 9 are placed just slightly overlapping with the cofactor. From table 6.8 and table 6.10-6.11 we see the effects of the hit compounds on TPH1s thermal stability and activity, respectively. 11 (91.7 %) of the compounds have a significant

inhibitory effect on TPH1 activity, while 5 also had an effect on thermal stability, both stabilizing and destabilizing. The most interesting inhibitor is compound 7. From Fig. 6.6 we see that compound 7 ´-´ stacks with Tyr264, Phe318 and Phe241. In the crystal structure of chicken TPH, Phe241 is ´-´ stacking with L-Trp (25). Compound 7 also form a salt bridge to the metal atom. The second best inhibitor, compound 5, also ´-´ stack with Tyr264 and Phe318. Compound 12 showed a stabilizing trend in the activity assay. From Fig. 6.4 we see that compound 12, in addition to forming a salt bridge to Arg257, forms hydrogen bonds with Thr265, Ser336, Arg257, Phe318, His272 and Phe241. This is quite similar to the three inhibitors (LX0, ML0 and ML4), which all have hydrogen bonds to Thr265, Ser336 and Arg257 in the crystal structure (54). NMR and docking has shown Arg257, Ser336, and Phe318 to be important residues involved with L-Trp binding (24).

Even though docking is an extremely useful tool in drug discovery and development, the method also has its limitations. Some inhibitors form covalent bonds with metals. When docking the Sigma library we chose to include ñmetal statesò. TPH1 is a metalloenzyme capable to form metal-interactions with the ligands. However, ligand interaction with metals is a function that is not yet fully developed in docking programs (110). As covalent bonds are much stronger than non-covalent bonds, this can cause a significant error in estimating the binding affinity. Thus, the docking programs inability to include the chemical properties of iron hinders the identification of iron-coordinating compounds.

The use of an ensemble of rigid receptors with different conformations provided us with an opportunity of mimicking a flexible receptor. On the other hand, by using the available crystal structures of TPH1, we are most possibly just scratching the surface of the possible conformational changes taking place upon substrate interaction. Thus this method is only a simplified approach to a real flexible receptor. In addition, the conformational changes occurring in the receptor before and upon association with other molecules are not being accounted for. However, the use of this technique has given good results when using an extensive set of crystal structures (104). This requires not only the formation of a representative amount of three-dimensional structures of macromolecules, but also manual selection of the most relevant conformations of bound and unbound receptor. However, this is outside the scope of this master thesis.

7.2 Experimental screening by DSF

Experimental screening by DSF was performed in order to see if the hit compounds chosen from the molecular docking had an effect on TPH1 thermal stability. The hit compounds were also tested on PAH and TH. When working with developing a drug effecting one of the enzymes in the AAAH family, it is also important to evaluate its effect on the other enzymes of this enzyme superfamily. This technique was chosen because it allows us to assess the different compounds effectively. An advantage of DSF is that it requires very small amounts of both protein and ligand.

From Fig. 6.8 we see the effects of the hit compounds on TPH1s thermal stability. Compound 12 increase the melting point of TPH1 by 0.5 °C, while compound 2, 4 and 5 destabilize TPH1 by 1.8 °C, 1.8 °C and 3.2 °C respectively. The decrease in thermal stability is not obvious. We expect an increase in melting point of a ligand binds strongly to the native state of the enzyme, somewhat surprising the compounds seems to lower the thermal stability of TPH1. Results from the activity assays show that the same compounds inhibit TPH1 activity, with an activity of 42.8 %, 70.3 % and 23.3 % compared to the control, respectively. In the assay where we tested stability the activity of TPH together with the compounds were 54.0 %, 82.9 % and 7.6 % compared to the control, respectively. Two of the compounds seem to inhibit TPH1 under incubation, but also stabilize under preincubation, though the differences in activity were not significant.

As seen in Fig. 6.8 there was high background fluorescence, which is the product of the fluorescence of unbound dye and dye bound to the folded protein. This trend was only seen in the screening with TPH1. This can be explained by the fact that TPH1 is a rather hydrophobic protein compared to TH and PAH. The hydrophobic parts of TPH1 when at its native state may stick to the hydrophobic fluorescence dye, giving rise to the large background signal. However, the transition was well defined, so the SYPRO orange could still be used.

From Fig. 6.9 and 6.10 we see the effects of the hit compounds on PAH and TH activity. Compound 1, 2 and 6 showed the same trends in both enzymes, by decreasing the thermal stability. Fifty eight percent (7 out of 12) of the hit compounds from the molecular docking

using TPH1 thus had an effect on the other two enzymes in the hydroxylase family. This emphasises the importance of examining all three enzymes when developing drugs targeting an enzyme of this family.

A disadvantage of screening by DSF is that compounds with intrinsic fluorescence interfere with optical detection of reported fluorescence. This meant that we did not get any results in 13 of the measurements (6 of the compounds). In addition, when looking for an inhibitor DSF cannot be used alone. Even though a compound does not change the melting temperature of TPH1 that does not necessarily mean that it is not an inhibitor of TPH1 activity. Thus, all compounds were taken further for activity measurements. Additionally, if a compound does stabilize the enzyme at assay temperature (37 °C) it does not necessarily increase the melting point temperature. The technique also only allows us to find compounds that affect thermal stability of TPH1, and not those who inhibit the activity. However, the method is highly useful when doing experimental high throughput screening (eHTS).

7.3 Activity measurements with TPH1

Before assaying the hit compounds, we tested the impact of iron and BSA on TPH1 activity. This was done in order to optimize our protocol. It has been suggested that TPH activity is inhibited by hydroxyl radicals produced by iron at the active site, which in turn attack and destroy the enzyme (111). Based on this, we decided to ad iron after 4 minutes of preincubation. From figure 6.12 we see that the activity of TPH1 is highest when both BSA and iron is added. Removing iron results in a great reduction of activity. This is not surprising since both truncated and native forms of TPH have been shown to require Fe(II) for enzyme activity (45, 112-114). The small remaining activity indicates that only a small proportion of the purified enzyme contains iron, thus iron is needed when assaying this batch of enzyme. Removal of BSA also resulted in a reduced activity. This is consistent with the early findings of Jequier, et al., demonstrating that BSA stimulates TPH activity (94). From table 6.9 we see that all the varying assay conditions gave significantly lower enzyme activity compared with the control. Interestingly, there was no significant difference in the enzyme activity when only iron was removed compared with the activity when they were both removed. Thus, BSA had no effect on TPH1 activity when not present with iron. When assaying BSAs effect on PAH, Gurof et al. found that BSA only had an effect on the activation with iron and not in the

hydroxylation step (93). However, we added iron after 4 minutes of activation, so BSA and iron was only present together for 1 minutes of activation and 5 minutes of incubation. Based on these results we decided to keep both BSA and iron in the following activity assays. BSAs effect was also assayed when determining optimal conditions for activity loss.

Before assaying the hit compounds we had to determine the optimal conditions for activity loss. We choose both to perform this on TPH1 and TPH2. From the results we chose to use a preincubation time of 10 minutes, as the enzyme activity were decreased to about 60 % at this time. Additionally, from the results we saw that the activity of TPH2 was a bit lower than TPH1, even though they were subjected to the same assay conditions. Time from when the enzyme was taken out of the nitrogen-tank and until it was assayed was also very similar. This difference in enzyme activity can be explained by Jequire et al. (94) findings, showing that TPH from rat brain had a lower affinity towards L-Trp than bovine pineal TPH. This was later also confirmed by Nakata and Fujisawa (115).

Activity measurements were performed in order to see what effect the hit compounds had on TPH1 activity. The hit compounds effect on stability of TPH1 was also tested. When TPH1 was preincubated at 37 °C, the rate of product formation was reduced with time. Compounds that preserved the activity during preincubation are considered potential pharmacological chaperones for TPH1. The effect of the compounds on TPH1 activity is shown in figure 6.17 together with the effect on stability.

Table 6.10 shows the results from testing of the effect on TPH1 activity. For 83 % (10 of the 12) of compounds the activity of TPH1 was reduced to below 50 % compared to the control. The inhibitor LP533401 inhibited the enzyme completely. Compound 7 led to an activity of 12 % compared to the control, thus this is considered to be an extra interesting compound. It does not inhibit TPH1 to the same extent as LP533401, but the compound can be a viable starting point for hit-to-lead optimization. The compound needs to be characterised further to determine type of inhibition and to find its affinity to TPH1. The compounds effect on TPH2 must also be assayed. Liu et al. found LP533401 to inhibit both TPH1 and TPH2 *in vitro*, but when assaying the compound *in vivo* they found that it selectively inhibited 5-HT production in the GI tract. Another inhibitor of TPH, pCPA, was found to inhibit 5-HT production both in the brain and in the GI tract. It was speculated that the great molecular mass of LP533401 compared to pCPA (526.5 Da for LP533401 and 199.6 Da for pCPA) was the reason for this difference in

selectivity (74). Compound 7 has a quite similar molecular mass compared to LP533401 (486.547 Da). LP533401 is at the moment in clinical trial stage 2. However, there are still many obstacles on its way into becoming a drug. If LP533401 turns out not overcoming all of these, the scaffold of compound 7 can be used as a template in the further search of a potent inhibitor.

Compounds 1-11 together with Compound IV and LP533401 were all giving significantly lower activity of TPH1 compared to the control. The effect of compound IV is worth mentioning. The compound significantly reduces the activity of TPH1, down to approximately 40 % activity compared to the control. This is contrary to the predictions of Torreblanca et al. saying that compound IV is ñspecific for PAH, not significantly influencing the stability of the closely related enzymes, such as tyrosine and tryptophan hydroxylaseò. Interestingly, research done by Calvo et al. showed no effect on TPH2s activity *in vitro*, but they only used an activity assay with preincubation time of 1 minute in the presence of the compounds (compared to 10 minutes here) (95). However, different analyzes done by differential scanning fluorimetry showed a slight destabilization of hTPH2 and studies done with mice showed a decrease in the serotonergic metabolite -HIAA in the brain after 12 days of treatment with 5 mg/kg/day of this compound. Even though this was assays performed on TPH2 the sequence identity between TPH1 and TPH2 is so high that these findings probably can be transferred to TPH1.

Table 6.11 shows the results from testing of the hit compounds effect on TPH1 stability. The activity of the control with 10 minutes preincubation did drop down to 63 % compared to the control without preincubation from the previous assay. We see that compound 12 shows a stabilizing trend giving rise to a 20 % higher activity compared to the control. Even though the result is not significant, it is still an interesting compound that should be tested further. As TPH1 is an unstable enzyme, finding a compound that stabilizes it is in great interest. Additionally as TPH1 and TPH2 have such a high sequence similarity, there is the huge possibility that of compound 12 will stabilize TPH2 as well, which in turn is very clinically interesting. Several approaches are used in the treatment of abnormalities in the serotonin system, but little research has been done on developing drugs that can stabilise TPH2. Calvo et al. has done research on finding pharmacological chaperones of TPH2, but the chaperone did not have any effect in vivo (95). As we also have determined optimal conditions for activity loss of TPH2 (Fig 6.15 and 6.16), these results can be used in further work with this particular enzyme. The same protocol used for testing the hit-compounds on TPH1 could be used for

testing compound 12 on TPH2. Compound 1 and 4 had inhibitory effects on TPH1 in the previous assay. This is also the case in this assay, but the activity does not drop as much compared to the control as in the first assay. The compounds may have a protective effect of inactivation of TPH1.

In the assays testing the hit compounds we used a 96 well plate. This is necessary when doing screening of more than a few compounds. However, the use has its disadvantages. When adding enzyme or BH_4 to the reaction mixture we did not have the time to properly mix it, like we could when using reactions tubes. This is a source of systematic error and thus contributing to uncertainties in the data. Additionally centrifugation was not possible to do. TPH1 is difficult to study as it is a quite unstable enzyme. This is one of the reasons to why TPH is the enzyme we know the least about in the AAAH family. TPH easily aggregates because of misfolding of the protein. *In vivo* molecular chaperones help the protein to find its right conformation, but this is not the case when working *in vitro*. This can explain the standard deviation in the different experiments. Based on this we chose to use 6 parallels when assaying the hit compounds, so we would obtain solid data.

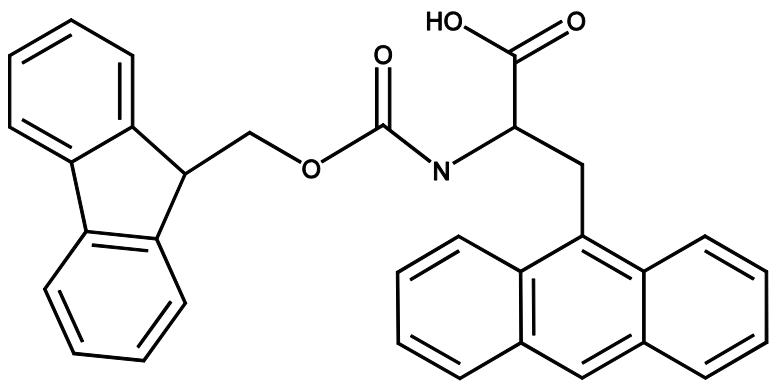
From the molecular docking together with experimental methods we have found several compounds having different effects on TPH1. As there is a high sequence similarity between the AAAHs is it plausible that these compounds also have an effect on the other enzymes of the family. It is therefore important to test the effects of the active compounds on PAH and TH activity. Although there are mostly interests in finding stabilizers of these two family members, research has been done to se if inhibition of TH could be a possible treatment of schizophrenia (116, 117).

Summarizing we have proved that molecular docking is useful for the discovery of hit leads for drug targets. From the docking we obtained one interesting inhibitor of TPH1 and one potential stabilizer. In addition several other compounds had significant effects on TPH1, thus proving the value of a computational approach. This validates the use of molecular docking in the search for active compounds, though all this has to be done with respect of the limitations of molecular docking. In addition docking is just the starting point in developing a new drug. Even though it can be an effective tool in the discovery of novel compound with potent activity, molecular docking does not take into account important aspects like toxicity,

bioavailability, distribution and other factors in the body. Safety issues derail many drugs late in development, so identifying such issues could save time and money.

8 CONCLUDING REMARKS

By combining molecular docking with experimental methods in the laboratory, we found several compounds that significantly inhibited the activity of TPH1. Several of the hit compounds from the docking shared a common scaffold; a three-ring system. One of these compounds, compound 7, showed very interesting results when being experimentally tested. This compound is a viable starting point for hit-to-lead optimization and can be a potential candidate for the treatment of irritable bowel syndrome, chemotherapy-induced emesis or other diseases associated with serotonin dysregulation in the periphery. Further on we found compound IV to have a significant destabilizing effect on TPH1.



9 FUTURE PERSPECTIVES

Compound 7 has proven to be an interesting inhibitor of TPH1 in the work of this Master thesis. The compound is a viable starting point for hit-to-lead optimization, however it needs to be characterised further to determine type of inhibition and its affinity to TPH1. The next step in this project is testing its affect on the other AAAHs. If the compound has an inhibitory effect on TPH2 modifications need to be done to ensure low blood-brain-barrier permeability. The next step is to find out if the compound is active in cells. Toxicity should also be examined, firstly by doing experiments in cells and then eventually in animal models. To increase the probability that the hit is a sensible starting point, an ADME predictions should be carried out.

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