Title
Insights Into Toxin Binding to Tubulin as a Potential Trigger in Parkinson's Disease Using Molecular Modeling

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Insights into Toxin Binding to Tubulin as a Potential Trigger in Parkinson’s Disease Using Molecular Modeling

A Thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science
in
Bioengineering
by
Debbie J. Hlava
December 2012

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__________________________________________________________________
__________________________________________________________________
__________________________________________________________________

Committee Chairperson

University of California, Riverside
Acknowledgements

I would like to express my gratitude to Professor Dimitrios Morikis for his guidance, and to also thank Tyler Backman, Professor Valentine Vullev, Professor Hyle Park, and Chris Kieslich for their support.
Dedication

To my beautiful mother who passed away in 1999 from complications of Parkinson’s disease. This thesis is partial fulfillment of my promise that I would one day strive to find the cause and potential cure for this devastating disease.
ABSTRACT OF THE THESIS

Insights into Toxin Binding to Tubulin as a Potential Trigger in Parkinson’s Disease Using Molecular Modeling

by

Debbie J. Hlava

Master of Science, Graduate Program in Bioengineering
University of California, Riverside, December 2012
Dr. Dimitrios Morikis, Chairperson

Despite years of research, little is known about the specific cause of Parkinson’s disease (PD), though genetic and environmental factors have been implicated. Evidence suggests that microtubule disruption may be involved; however this mechanism is not yet fully elucidated, and factors implicated in PD have not been conclusively related to microtubule function. This is the framework of this thesis.

We have expanded on a suggested PD cascade involving microtubule disruption triggering oxidization of dopamine, production of reactive oxygen species (ROS), aggregation of alpha-synuclein, and death of neuronal cells. We have provided computational and relational evidence demonstrating how factors implicated in PD may relate to microtubule disruption and reactive oxygen species (ROS). We selected 6 potentially PD-related compounds: rotenone, MPTP/MPP+, toluene, saccharin, and aspartame, and compared them to 4 tubulin inhibitors: colchicine, vinblastine, soblidotin, and taxol; identifying ~62% chemical similarity between rotenone and colchicine and ~78% similarity between aspartame and soblidotin. We performed molecular docking
calculations for the potentially PD-related compounds at the binding sites of the tubulin inhibitors on tubulin dimers and found that all potentially PD-related molecules demonstrated lowest (strongest) binding affinities on the colchicine site of tubulin. Rotenone and aspartame demonstrated significant binding affinities of -10.7 and -8.7 kcal/mole, comparable to affinities of compounds on their known receptors (-15.0 to -9.2 kcal/mole).

As compounds would need to enter the bloodstream and cross the blood-brain barrier (BBB) to enact damage that could trigger PD, we illustrated that all potentially PD-related compounds evaluated meet criteria for potential to cross the BBB, and that all but one (aspartame) have been proven to enter the bloodstream. We hypothesized that aspartame, believed to not enter the bloodstream after digestion, may potentially be absorbed sublingually. We compared aspartame to known sublingual drugs and noted comparable logP and molecular weight values.

Lastly, we demonstrated via molecular docking probable methods of aggregation of alpha synuclein (α-syn) fibrils and probable mechanisms of two α-syn aggregation inhibitors, curcumin and geldanamycin. We have thus provided a collective body of evidence to help substantiate the hypothesis that PD may be triggered by tubulin inhibition leading to excessive ROS production, triggering a PD cascade.
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Chapter 1 – Introduction

Though years of intensive research have been devoted to evaluating the pathology of Parkinson’s disease (PD), to date there is still no definite cause or accepted treatment for the disorder. We can assume that PD is triggered through a series of complex biochemical processes that can be elusive to researchers and physicians. Consequently, our approach was to compile and evaluate all current understanding and theories regarding not only PD, but also the chemicals, genes, and cellular insults proven (or even suspected) to be involved in PD, thereby elucidating “clues” and commonalities. This approach led us to convergence on two potential modes of cellular damage: tubulin inhibition and the production of reactive oxygen species (ROS). We evaluated a suggested PD cascade and related the causal factors in PD (genes, compounds, and cellular insults/injuries) to various points within this cascade, specifically those involving tubulin inhibition and/or ROS production, providing insights and relationships that we have not seen previously identified.

The proposed cascade involves a complex pathway of tubulin inhibition leading to the inability of vesicular monoamine transporter 2 (VMAT2) to sequester the neurotransmitter dopamine into vesicles, causing excess generation of ROS and aggregation of alpha-synuclein (α-syn, a protein involved in regulation of dopamine production), eventually leading to death of neuronal cells. We expanded on and evaluated this cascade through a tri-fold approach of molecular modeling, chemical clustering analyses, and relational evidence of chemical structure, function, and known biochemical effects of potentially PD-related compounds and known tubulin inhibitors.
We have provided computational and theoretical evidence to support the hypothesis that over-exposure to PD-related compounds, or the inability of the body to effectively counteract detrimental effects of such compounds, may be a trigger for a PD cascade. It is known that drugs and other ligands act by binding preferentially to their target proteins. Physiological compounds prevent undesirable binding to proteins such as tubulin[1], and neurotrophic factors protect against the selective toxicity of chemicals to neurons [2]. However, high concentrations of potentially toxic chemicals or compromised defense mechanisms may render these defenses ineffective [1]. We have provided evidence to suggest that, in PD, these chemicals include the potentially PD-related compounds that we have evaluated. The ability of the body to defend against these compounds may be driven by genetic mutations of the known PD-related genes, accumulation of excessive ROS within cells due to aging or other factors, or other cellular damage.
1.1 Background:

1.1.1 Overview of Parkinson’s disease

PD affects more than 1 million people in the U.S. and over 4 million world-wide[3]. PD is a progressive disorder of the central nervous system causing debilitating loss of motor functions. To date there is no preventive treatment, cure, or definitive cause of the disease, though it is theorized that PD is initiated through a combination of genetic and environmental factors. Certain genetic mutations, such as that of the LRRK2 (leucine-rich repeat kinase 2), PARK2 (Protein 2, E3 ubiquitin protein ligase; parkin), PARK7 (Parkinson protein 7), PINK1 (phosphatase and tensin homolog-induced putative kinase 1), and SNCA (α-syn-producing) genes, have been associated with early-onset PD [4]. Environmental factors have been associated with the more common late-onset PD [5]. Compounds that may be associated with PD include: pesticides [6, 7], solvents [8], the drug by-product MPTP and its metabolite MPP+[7], certain artificial sweeteners [9], and heavy metals[8].

PD progressively destroys dopamine-producing (DA) neurons. Dopamine is an important neurotransmitter involved in the control of movement and memory. DA neurons are clustered within the midbrain in the substantia nigra and are believed to be particularly susceptible to oxidative stress. Alpha-synuclein, a protein involved in dopamine regulation present in neurons in the substantia nigra, is generally in a soluble form in healthy neurons; however, within PD-affected neurons, α-syn forms aggregates of spherical clusters of proteins (Lewy Bodies, LB), which can cause neuronal cell death through restriction of cell function [10]. It is theorized that soluble α-syn decreases the
amount of dopamine transporter (DAT) at the plasma membrane, limiting the amount of dopamine entering nerve terminals. Controlled levels of dopamine can be moved to synaptic vesicles through vesicular monoamine transporter 2 (VMAT2) [11]. If cytoskeleton integrity is impaired through microtubule dysfunction, high levels of DAT can accumulate at the plasma membrane. Dopamine can then enter into cellular cytosol and form reactive oxygen species (ROS), triggering aggregation of α-syn [11] (Figure 1.1). Without transmission of signals across neurons via dopamine, motor functions are lost and PD symptoms can arise.

**Figure 1.1 Proposed PD cascade.**
In healthy cells, controlled levels of dopamine can be moved to synaptic vesicles via VMAT2. Through a cellular insult involving microtubule disruption, high levels of DAT can accumulate at the cellular membrane with loss of function of VMAT2. The cell is thus unable to effectively capture all of the excess DAT into vesicles, and oxidized dopamine is produced, leading to α-syn aggregation, Lewy Body (LB) formation, and cell death. (Modified from [11].)
Studies have confirmed that α-syn forms aggregates in the presence of ROS from dopamine oxidants [12, 13], though the “initial insult” that causes cytoskeleton damage leading to the intracellular accumulation of dopamine has not been fully elucidated. We suggest that cytoskeleton damage can be caused by PD-related compounds binding to tubulin, and we demonstrate this through molecular docking simulations of potentially PD-related compounds with tubulin.

1.1.2 Microtubules and Tubulin Inhibitors

Microtubules (MT’s) are involved in multiple cellular functions including maintenance of cell shape, cellular movement, cell signaling, cell division, and mitosis, and are crucial for cell survival [14]. MT’s are hollow cylindrical structures formed by oligomerization of α and β tubulin dimers into protofilaments which form protofilament sheets that close into tubular shapes (Figure 1.2).

Figure 1.2 Microtubule formation and tubulin inhibition.
Microtubules are formed from (1) oligomerization of tubulin dimers,( 2) formation of tubulin dimers into protofilaments,( 3) formation of protofilaments into sheets, (4) “rolling” of the protofilament sheet into a tube formation,( 5) further elongation of the microtubule through addition of more alpha-beta dimers (Modified from [15]).
As microtubules are dynamic structures that perform their cellular work through constant growing and shrinking (polymerization and depolymerization), interruption of either of these processes can lead to cell death [16]. Compounds that interrupt microtubule dynamics are known as tubulin inhibitors [17]. Tubulin inhibitors are classified as either tubulin stabilizers or tubulin destabilizers. Tubulin destabilizers such as colchicine, vinblastine, and soblidotin work by binding to unpolymerized tubulin dimers and prohibiting further polymerization (Figure 1.3a). Tubulin stabilizers such as taxol work by binding to polymerized tubulin and inhibiting depolymerization (Fig 1.3b).

![a. Method of action of colchicine](image1.png) ![b. Method of action of taxol](image2.png)

**Figure 1.3 Method of action of tubulin inhibitors – destabilizer colchicine and stabilizer taxol.**
a) Colchicine inhibits growth of microtubules by binding to unpolymerized tubulin (red). b) Taxol prevents depolymerization by binding to polymerized tubulin (blue).

There are three known binding sites for tubulin inhibitors as shown in Figure 1.4: the colchicine, vinblastine, and taxol site. The majority of known tubulin inhibitors bind to one of these three sites. Soblidotin binds in an area that overlaps that vinblastine site and thus is not considered to have its own defined binding site. [18] Depolymerization of tubulin has been implicated in PD [2]. Thus we would expect PD-related compounds to
bind to areas of tubulin that have been identified as binding sites of tubulin destabilizers (the colchicine or vinblastine/soblidotin sites).

**Figure 1.4 Binding sites of tubulin inhibitors.**
Note: “α” indicates alpha-tubulin; “β” indicates beta-tubulin. “1” and “2” indicate the number of the tubulin heterodimer in sequence from the N-terminus. Tubulin inhibitors can bind to any tubulin heterodimer in the configuration shown (i.e., colchicine can bind between α1 and β1, between α2 and β2, etc.; vinblastine can bind between β1 and α2, between β2 and α3, etc. Boxes indicate the binding sites of each inhibitor. The taxol binding site is on the “back” (or inside) of the microtubule (thus is ‘dashed’).
Chapter 2 – Materials and Methods

2.1 Retrieval of molecular structures

The 3-dimensional molecular structure of the 4 known tubulin inhibitors (colchicine, vinblastine, soblidotin, and taxol) on their respective tubulin heterodimers were obtained from the Protein Data Bank (PDB) [19]. The PDB codes for each of these structures are, respectively: 1SA0, 1Z2B, 3E22, and 1JFF (bovine tubulin with colchicine; bovine tubulin with colchicine and vinblastine, bovine tubulin with colchicine and soblidotin, and bovine tubulin with taxol, respectively). The chemical structures of the potentially PD-related compounds that we evaluated (rotenone, MPTP/MPP+, toluene, saccharin, and aspartame) were obtained from the PubChem Database [20]. PubChem chemical ID (CID) numbers for the 6 potentially PD-related compounds evaluated are, respectively: 6758, 1388/39484, 1140, 5143, and 134601. PubChem CIDs for colchicine, vinblastine, soblidotin, and taxol are, respectively, 6167, 13342, 656607, and 6314.

The chemical structure of our control molecules on their receptors were also obtained from the PDB. Structures utilized were: i) ibuprofen on COX-1 receptor, ii) quinazolinedione sulfonamide on GluR2 (glutamate receptor 2), iii) cholesterol on the Neimann-Pick receptor, iv) topomax on carbonic anhydrase, v) doxepin on the histamine receptor, and vi) retinol on retinol binding protein (RBP). PDB ID numbers are, respectively, 1EQG, 3R7X, 3GKI, 3LXE, 3RZE, and 1BRP. The PDB structures of quinazolinedione, cholesterol, topomax, doxepin, and retinol were bound to human receptors. The PDB structure of ibuprofen was not available on human receptor, thus a
model of ibuprofen on an ovis aries COX-1 receptor was used. A BLAST (Basic Logic Alignment Search Tool) [21] search identified 92% identity, 96% positives, and 0% gaps between the human and bovine COX-1 receptor; (UniProt Knowledge Database) [22] ID numbers used for human and ovis aries COX-1 receptors used were, respectively, P23219 and P05979. The control ligands were isolated from their receptors using Chimera for re-docking on tubulin dimers. A 2-dimensional representation of quinazolinedione sulfonamide was unavailable within ChemSpider, thus a representation was used from LookChem (CAS ID number 875153-98-3) [23].

The chemical structure of the two compounds that we evaluated for potential therapeutic effect in PD (curcumin and geldanamycin) were obtained from PubChem. PubChem ID numbers are, respectively, 969516 and 5288382.

The ChemSpider Database [24] was utilized for 2-dimensional molecular representations. ChemSpider ID’s for rotenone, MPTP, MPP+, toluene, saccharin, and aspartame are, respectively, 6500, 1346, 36101, 1108, 4959, and 118630. ChemSpider ID’s for ibuprofen, sulfonamide, cholesterol, topomax, doxepin, and retinol are, respectively: 3544, 5775, 4447672, 3046, and 393012. Chem Spider IDs for curcumin and geldanamycin are 839564 and 10272739, respectively.

As a PDB model utilizing human tubulin was not available within the PDB, we utilized bovine tubulin models as listed above. To understand the similarity between human and bovine tubulin, we performed homology modeling using the UniProtKB for sequences and BLAST searches for homology modeling. Both alpha and beta bovine tubulin had at least 99% identity, 99% positives, and 0% gaps to human alpha and beta
tubulin. UniProtKB ID numbers for human and bovine alpha tubulin utilized were, respectively Q71U36 and P81947, and UniProtKB ID numbers for human and bovine beta tubulin used were, respectively, Q13885 and Q6B856.

Chimera Molecular Modeling System [25] was used to retrieve all molecules from PubMed and databases. We isolated each of the 4 tubulin inhibitors from their respective tubulin heterodimers for our controlled docking studies using Chimera. We used Chimera post-docking to determine physicochemical contacts (hydrogen bonds and Van der Waals contacts) between the compounds evaluated and tubulin dimers, as well as between the known tubulin inhibitors and tubulin.

2.2 Chemical similarity clustering

Chemical similarity clustering was performed using PubChem Clustering analysis [20]. The PubChem structure similarity function utilizes either the Tanimoto score calculated from the 2D structure fingerprint, or the 3D shape/feature similarity.

The Tanimoto search function utilizes the following coefficient equation:

\[ TS = \left( \frac{N_{AB}}{N_A} \right) + \left( \frac{N_B}{N_A} \right) - \frac{N_{AB}}{N_A} \]  

(Equation 1)

TS is the Tanimoto coefficient; A and B are the two molecules being compared; \( N_A \) is the number of features in A, \( N_B \) is the number of features in B, \( N_{AB} \) is the number of features common to both A and B.
2.3 Drug-like properties evaluation

Evaluation of drug-like properties of compounds was conducted through comparison to the Lipinski “drug-like” compound criteria [26]. All pertinent molecular properties were obtained from PubChem.

2.4 Electrostatic potential of alpha-beta tubulin

We utilized AESOP [27] to calculate the electrostatic potential of alpha-beta tubulin dimers and heterodimers.

2.5 Docking calculations

AutoDock Vina molecular docking program [28] was used to perform docking calculations of compounds. All docking calculations were performed three times each, with nearly identical values obtained each time (within +/- 0.1 kcal/mole). Results were averaged to 1 decimal place.

AutoDock Vina utilizes the following scoring function:

\[ c = \sum_{i<j} f_{t_i t_j}(r_{ij}) \]  

(Equation 2)

The summation is the sum of all inter- and intra-molecular forces “c” summed over all of the pairs of atoms that can move relative to each other. Each atom i is assigned a type \( t_i \) and a symmetric set of interaction functions \( f_{t_i t_j}(r_{ij}) \) of the inter-atomic distance \( r_{ij} \) is defined.

The optimization function finds the global minimum of c and other low-scoring functions, which are ranked. Predicted free energy of binding is calculated from the intermolecular part of the lowest scoring function as follows:

\[ s_1 = g(c_1 - c_{\text{intra}}) = g(c_{\text{inter}}) \]  

(Equation 3)
s = free energy of binding and \( l = \) lowest scoring conformation, \( c_{\text{intra1}} = \) sum of all intra-molecular forces of the lowest scoring conformation, \( c_{\text{inter1}} = \) sum of all inter-molecular forces of the lowest scoring conformation.

The interaction functions \( (f_{ij}) \) are defined relative to the surface distance \( d_{ij} \) as follows:

\[
d_{ij} = r_{ij} - R_{t},
\]

(Equation 4)

\( R_t \) is the van der Waals radius of atom type \( t \). Additionally, \( f_{ij} (r_{ij}) \equiv h_{ij} (d_{ij}) \), \( h_{ij} \) is a weighted sum of steric interactions. The conformation-independent function \( g \) was chosen to be:

\[
g(c_{\text{inter}}) = c_{\text{inter}}/ (1 + wN_{rot})
\]

(Equation 5)

\( N_{rot} \) is the \# of active rotatable bonds between heavy atoms and \( w \) is the associated weight.

2.6 Binding preparation

Ligand-binding coordinates for each of the tubulin inhibitors were extracted from the PubChem structures of each inhibitor within their binding sites on tubulin heterodimers. Ligand-binding coordinates for our control molecules were extracted from the PubChem structures of each compound on its known receptor. A search space grid of \( 20\AA \times 20\AA \times 20\AA \) was used in our docking calculations. Polar hydrogens were added to each tubulin dimer and the Gasteiger charge function \([29]\) was utilized to add appropriate charges to each ligand.
2.7 Physicochemical properties

Physicochemical properties of compounds evaluated were found using Chimera structure analysis tools. Hydrogen bond cut-off criteria was 3.5 Å (relaxed by 0.4 Å), with an angle relaxed to 180° for inclusion of all hydrogen bonds. Hydrophobicity surfaces were generated using the Chimera “Preset Interactive 3 hydrophobicity” viewing tool. Van der Waals interaction cut-off was an overlap of 0.4 Å. Coulombic surfaces were evaluated using the Chimera Surface/Binding analysis/Coulombic surface coloring tool.
Chapter 3 – Results

3.1 Chemical similarity evaluation

Figure 3.1 is a 2-dimensional representation of the chemical structures of the 6 potentially PD-related compounds and the 4 tubulin inhibitors that we evaluated. As illustrated in Figure 3.1, the potentially PD-related compounds that we investigated share structural characteristics with tubulin inhibitors: all contain aromatic groups, all but one (saccharin) contain alkyl groups, and 2 of the 6 (saccharin and aspartame) contain amine groups. All 4 tubulin inhibitors contain all of these 3 functional groups. These groups function in tubulin binding, as illustrated within the “Physicochemical Interactions” section.

![Chemical structures](image)

a. Chemical structures of the 6 potentially PD-related compounds evaluated
b. Chemical structure of the 4 tubulin inhibitors evaluated

Figure 3.1 Chemical structure of potentially PD-related compounds and tubulin inhibitors.
Figure 3.1a illustrates the chemical structure of potentially PD-related compounds which we identified for our study. Figure 3.1b illustrates the chemical structure of the 4 known tubulin inhibitors used in our study.

Lipinski’s “Rule of Five” estimation predicts that the majority of all oral drugs exhibit the following chemical properties: (i) ≤ 5 H-bond donors, (ii) ≤ 10 H-bond acceptors, (iii) molecular weight ≤ 500 g/mol, (iv) calculated LogP (CLogP) ≤ 5; where P (a lipophilicity measure), is oil-to-water partition coefficient, and v) polar surface area ≤ 150Å² [26]. (Note that the use of polar surface area as a criteria was added after the initial “Rule of Five” was proposed as an optimization of the estimation [30].)

Table 3.1 illustrates these pertinent properties for the potentially PD-related compounds that we evaluated as well as for the tubulin inhibitors. Note that all of the potentially PD-related compounds that we evaluated meet the Rule of 5 “drug-like” property criteria. The only tubulin inhibitor that meets the Rule of 5 criteria is colchicine, which is consistent with literature results [31]. Tubulin inhibitors are not generally used as oral drugs but are either directly injected a tumor or given intravenously [32].

<table>
<thead>
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<th>Compound</th>
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<tr>
<td>taxol</td>
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Table 3.1. “Drug-like” properties for potentially PD-related compounds and tubulin inhibitors.

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<th>PubChem CID#</th>
<th>Log P</th>
<th>MW (g/mol)</th>
<th>H bond donors</th>
<th>H bond acceptors</th>
<th>Polar surface area (Å²)</th>
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<td>221.29</td>
</tr>
</tbody>
</table>

Italics indicates compound does not meet “Rule of 5” criteria

We discuss Lipinski’s Rule of Five as it pertains to the 6 potentially PD-related compounds that we evaluated to illustrate that, while not all of these 6 compounds are classified as “drugs”, they all have the potential to behave as such (i.e. potential for entry into the bloodstream and chemical binding).

3.1.1 Chemical similarity clustering: Potentially PD-related compounds and tubulin inhibitors

To further evaluate similarities between the potentially PD-related compounds and the known tubulin inhibitors that we evaluated, we screened each of the potentially PD-related compounds for structural similarity to the tubulin inhibitors using the PubChem chemical similarity clustering function. Results are shown in Figure 3.2. The clustering analysis identified a similarity of ~78% between soblidotin and aspartame, ~76% similarity between MPTP and toluene, ~62% similarity between colchicine and
rotenone, and at least a 50% similarity between all potentially PD-related compounds and tubulin inhibitors.

**Figure 3.2 Clustering diagram of potentially PD-related compounds and tubulin inhibitors.**
Results of the PubChem clustering analysis of the 6 potentially PD-related compounds and the 4 tubulin inhibitors. The strongest similarity was demonstrated between soblidotin and aspartame (~78% similarity); followed by ~76% similarity between MPTP and toluene, and ~62% similarity between colchicine and rotenone. All molecules clustered together at ~50% similarity.

Our analysis of the potentially PD-related compounds (rotenone, MPTP, MPP+, toluene, saccharin, and aspartame) has suggested that all have the potential to exhibit drug-like behavior and all share functional groups with and exhibit at least a 50% chemical similarity to the tubulin inhibitors. The calculated chemical similarity between aspartame and soblidotin was the highest at ~78% (≥ 90% Tanimoto similarity is considered statistically significant in a PubChem search [33], though we illustrate in Figure 3.4 that similarly functioning compounds generally range in similarity from ~40% - 85%). Though the PubChem search identified only a ~62% similarity between colchicine and rotenone, it has been demonstrated in binding experiments that rotenone binds to the colchicine site of tubulin and acts to depolymerize microtubules [2].
Additionally, it has been demonstrated in binding experiments that MPP+ acts to depolymerize microtubules [34], though the PubChem clustering analysis identified only a ~55% similarity between MPP+ and colchicine.

To illustrate the level of discrimination within a PubChem clustering analysis, we added two common molecules to those we evaluated in Figure 3.2: water (PubChem ID 962) and glucose (PubChem ID 5793). Figure 3.3 details the results. Calculated similarity between the tubulin inhibitors and potentially PD-related compounds and glucose was only 20%, and between water and the same set of compounds was only 5%.

![Figure 3.3 Clustering diagram of potentially PD-related compounds and tubulin inhibitors with glucose and water added for comparison.](image)

Categorization of chemical behavior through similarity screening can exhibit difficulties (i.e., certain chemicals may perform the same function but may not exhibit a significant similarity within a clustering analysis). We suggest that a similarity criteria of ≥90% may be too conservative. To illustrate, we performed clustering analyses of 3 common drugs families (penicillin derivatives, pain relievers, and antifungal drugs; Figures 3.4a-c, respectively). Of the similarly-functioning drugs that we evaluated, only
ampicillin and penicillin exhibited ≥90% similarity; the second highest similarity demonstrated was ~85%, with the majority of similarly functioning compounds exhibiting between 40 - 85% similarity. Penicillin derivatives exhibited between 67 - 85% similarity, pain relievers exhibited between 47 – 83% similarity, and antifungal drugs exhibited between 40 – 84% similarity.

a. Clustering diagram of penicillin derivatives
Amoxicillin, and penicillin exhibited 90% similarity; ampicillin exhibited ~85% similarity with amoxicillin/penicillin; methicillin exhibited ~80% similarity with amoxicillin/penicillin/ampicillin; and dicloxacillin demonstrated only a ~67% similarity with other penicillin derivatives.

b. Clustering diagram of common pain relievers
Ibuprofen and ketoprofen exhibited highest similarity (~82%), fenoprofen and naproxen sodium exhibited ~80% similarity; aspirin exhibited ~78% similarity with fenoprofen and naproxen sodium and ~70% similarity with ibuprofen and ketoprofen. Etodolac exhibited only 47% similarity with other pain relievers.
c. Clustering diagram of common antifungal drugs
Itraconazole and ketoconazole exhibited ~86% similarity; clotrimazole exhibited ~63% similarity with itraconazole/ketoconazole; fluconazole and terbinafine exhibited ~50 – 51% similarity to itraconazole, ketoconazole, and clotrimazole; and ciclopirox exhibited only ~40% similarity with all other antifungals.

Figure 3.4 Clustering diagrams of similar drugs
As clustering analysis may not provide conclusive evidence of similar function or behavior of compounds, we performed molecular docking studies of the potentially PD-related compounds and tubulin inhibitors for further evaluation.

3.2 Molecular docking calculations: Potentially PD-related compounds & tubulin inhibitors.
We performed docking calculations of the potentially PD-related compounds with tubulin using AutoDock Vina. Docking calculations were performed with molecules in rigid conformation (all rotatable bonds non-rotatable) and relaxed conformation (all rotatable bonds rotatable). All molecules were docked on all three potential binding sites and five binding poses were generated for each conformation. The binding range (lowest and highest binding affinity of the five poses) is summarized in Table 3.2.
Table 3.2  Binding affinities for potentially PD-related compounds and known tubulin inhibitors.

<table>
<thead>
<tr>
<th>Compounds Evaluated</th>
<th>Colchicine Site</th>
<th>Vinblastine Site</th>
<th>Sbolidotin Site</th>
<th>Taxol Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
</tr>
<tr>
<td>Aspartame</td>
<td>Low: -8.7 High: -7.6</td>
<td>Low: -6.4 High: -6.3</td>
<td>Low: -5.7 High: -5.7</td>
<td>Low: -8.4 High: -7.9</td>
</tr>
<tr>
<td>MPP+</td>
<td>Low: -6.8 High: -6.3</td>
<td>Low: -5.9 High: -5.5</td>
<td>Low: -5.6 High: -5.3</td>
<td>Low: -6.2 High: -5.4</td>
</tr>
<tr>
<td>MPTP</td>
<td>Low: -6.7 High: -6.6</td>
<td>Low: -5.7 High: -5.6</td>
<td>Low: -5.6 High: -5.4</td>
<td>Low: -5.9 High: -5.7</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Low: -6.2 High: -5.8</td>
<td>NA</td>
<td>Low: -5.7 High: -5.3</td>
<td>NA</td>
</tr>
<tr>
<td>Toluene</td>
<td>Low: -4.4 High: -4.4</td>
<td>NA</td>
<td>Low: -4.2 High: -4.2</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tubulin Inhibitors</th>
<th>Colchicine Site</th>
<th>Vinblastine Site</th>
<th>Sbolidotin Site</th>
<th>Taxol Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Low: -12.0 High: -9.0</td>
<td>Low: -9.4 High: -9.0</td>
<td>Low: -6.9 High: -6.8</td>
<td>Low: -8.5 High: -8.3</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>High (+11.5)</td>
<td>Low: -5.4 High: NA</td>
<td>Low: -10.7 High: -8.1</td>
<td>Low: -4.9 High: -4.0</td>
</tr>
<tr>
<td>Sbolidotin</td>
<td>High (+9.2)</td>
<td>Low: -7.5 High: -6.8</td>
<td>Low: -6.8 High: -6.7</td>
<td>Low: -8.0 High: -7.6</td>
</tr>
<tr>
<td>Taxol</td>
<td>NA High (+44.6)</td>
<td>Low: -4.5 High: -3.1</td>
<td>Low: -9.1 High: -8.1</td>
<td>Low: -3.0 High: -0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tubulin Inhibitors</th>
<th>Colchicine Site</th>
<th>Vinblastine Site</th>
<th>Sbolidotin Site</th>
<th>Taxol Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
<td>All bonds non-rotatable</td>
<td>All bonds rotatable</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Low: -12.0 High: -9.0</td>
<td>Low: -9.4 High: -9.0</td>
<td>Low: -6.9 High: -6.8</td>
<td>Low: -8.5 High: -8.3</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>High (+11.5)</td>
<td>Low: -5.4 High: NA</td>
<td>Low: -10.7 High: -8.1</td>
<td>Low: -4.9 High: -4.0</td>
</tr>
<tr>
<td>Sbolidotin</td>
<td>High (+9.2)</td>
<td>Low: -7.5 High: -6.8</td>
<td>Low: -6.8 High: -6.7</td>
<td>Low: -8.0 High: -7.6</td>
</tr>
<tr>
<td>Taxol</td>
<td>NA High (+44.6)</td>
<td>Low: -4.5 High: -3.1</td>
<td>Low: -9.1 High: -8.1</td>
<td>Low: -3.0 High: -0.7</td>
</tr>
</tbody>
</table>

Only 2 configurations were generated for colchicine, and only 1 configuration for all other inhibitors with all bonds non-rotatable (other than for taxol on the sbolidotin site), as indicated in table.

In evaluating docking results, the lowest (most negative) binding affinities were considered to be the most likely binding configurations, as discussed within AutoDock Vina Tutorial [35] and as demonstrated within our docking results for the tubulin inhibitors compared with their known crystal structures from their PDB configurations, and within our “Controls” section (docking results compared to known crystallographic structures).

All potentially PD-related compounds that we evaluated exhibited the lowest calculated binding affinities on the colchicine site of tubulin. This is consistent with our hypothesis that PD-related compounds would bind to a “depolymerization site” on
tubulin. We had expected that aspartame would demonstrate its lowest calculated affinity value on the soblidotin site, given its chemical similarity to soblidotin; though calculated binding affinity for aspartame on the colchicine site was 0.3 kcal/mole lower than calculated affinity for aspartame on the soblidotin site (-8.7 kcal/mole and -8.4 kcal/mole respectively). We would recommend further binding studies to determine the specific binding site, and to evaluate the potential of aspartame to bind to either site.

Binding affinities were lowest for rotenone and aspartame, followed by MPP+, MPTP, saccharin, and toluene. Results show that MPP+ demonstrated only a slightly lower binding affinity than MPTP. Lowest affinity values were generally found with all bonds nonrotatable. Vinblastine, soblidotin, and taxol did not exhibit binding on the colchicine site in their configurations with all bonds nonrotatable but did exhibit binding with all bonds rotatable, likely due to the larger size of these molecules exhibiting difficulty fitting within the colchicine binding site.

As expected, all tubulin inhibitors had the lowest binding affinities in their respective binding sites. Figure 3.5 illustrates our determined binding configurations of all 4 tubulin inhibitors with all bonds nonrotatable and with all bonds rotatable compared with their PDB configurations.
a. Docked configuration of tubulin inhibitors compared with their PDB configurations with all bonds nonrotatable.

b. Docked configuration of tubulin inhibitors compared with their PDB configurations with all bonds rotatable.

**Figure 3.5 Docking configurations of tubulin inhibitors vs. PDB configurations.**
Figure 3.5a shows the tubulin inhibitors in their docking configurations with all rotatable bonds nonrotatable compared to the PDB configuration. Figure 3.5b shows the tubulin inhibitors in their docking configurations with all rotatable bonds rotatable compared with the PDB configuration. Binding affinities for each of the 5 configurations are shown as follows: red (lowest affinity), orange (2nd lowest affinity), yellow (3rd lowest affinity), green (4th lowest affinity), blue (5th lowest affinity). PDB configurations are shown in magenta.
As all of the 6 potentially PD-related compounds that we evaluated exhibited the lowest binding affinity on the colchicine site of tubulin, we compared our obtained docking configurations with that of the PDB configuration of colchicine (Figure 3.6). Docking results are shown separately for configurations with all bonds nonrotatable and with all bonds rotatable, and are compared to the PDB configuration of colchicine.

**Figure 3.6 Docking positions of potentially PD-related molecules compared to that of colchicine.** Figure 3.6a shows all molecules in their determined docking configurations with all rotatable bonds nonrotatable compared with the PDB configuration of colchicine. Figure 3.6b shows all molecules in their determined docking configurations with all rotatable bonds rotatable compared to the PDB configuration of colchicine. Binding affinities for each of the 5 configurations illustrated are illustrated as follows: red (lowest affinity), orange (2nd lowest affinity), yellow (3rd lowest affinity), green (4th lowest affinity), blue (5th lowest affinity). PDB configuration of colchicine is shown in magenta.
Figure 3.7 illustrates the configurations of the potentially PD-related compounds and colchicine on the colchicine site of tubulin showing hydrophobicity surfaces. Poses shown are the lowest calculated binding affinity configurations. All compounds are shown within the colchicine pocket on tubulin. The pocket lies in a hydrophobic cleft on beta-tubulin [36] (shown in Figure 3.7b). The remainder of the colchicine site is a nonpolar hydrophilic area on alpha-tubulin (shown in Figure 3.7c). Note that the shape of the pocket, most visible in Figure 3.7b, colchicine on beta tubulin, conforms to the molecular structure of colchicine. As molecular dynamics were beyond the scope of our current study, we utilized the configuration of alpha-beta tubulin in its conformation when bound with colchicine to provide representational view of the possible docking locations of the PD-related compounds that we evaluated. We can observe (Figure 3.7b) that all potentially PD-related compounds appear to dock within highly hydrophobic areas of beta-tubulin, even though they do not completely conform to the pocket (as expected).
a. Illustration of alpha-beta tubulin “broken apart” to illustrate binding location of colchicine.

b. Colchicine and the 6 potentially PD-related compounds on β-tubulin.

c. Colchicine and the 6 potentially PD-related compounds on α-tubulin.

**Figure 3.7 Docking results of molecules on α- and β-tubulin.**
Views of molecules in their docking conformations. Figure 3.7a illustrates the configuration of alpha-beta tubulin and shows the two molecules (alpha and beta tubulin) “broken apart” and rotated slightly to illustrate colchicine docked within the molecules (circled). Figures 3.7 b and c show all potentially PD-related molecules on enlarged views in the colchicine binding area of alpha and beta tubulin. Hydrophobic surfaces are shown in orange, hydrophilic surfaces in blue, neutral surfaces in white. Colchicine is shown in magenta, rotenone in purple, aspartame in yellow, MPP+ in red, MPTP in green, saccharin in light orange, and toluene in light blue. All molecules are shown with hetero-atoms designated. Hydrophobic surface area is approximately 10Å x 10Å.
We calculated physicochemical interactions between the 6 potentially PD-related compounds and the 4 tubulin inhibitors with tubulin. Table 3.3 lists the hydrogen bonds identified for the potentially PD-related molecules and colchicine as docked in their lowest binding affinity configurations, and Appendix A.1 lists VdW contacts identified. As noted in Table 3.3, 3 hydrogen bonds were found for colchicine, 2 hydrogen bonds were found for both rotenone and saccharin, and 1 for aspartame. No hydrogen bonds were found for MPP+, MPTP or toluene. Hydrogen bonds were not expected for MPP+ or toluene, as both compounds have no hydrogen donors or acceptors. CYS241β was found to act as a hydrogen bond donor for colchicine, aspartame, and saccharin. The majority of hydrogen bonds identified between the potentially PD-related compounds and tubulin were on beta-tubulin; only 2 of the 8 hydrogen bonds identified were with alpha-tubulin.

It is worth noting that though the molecular weight of saccharin is low (183.18 g/mol), the number of hydrogen bonds determined is the same as the number determined for rotenone (MW 394.42 g/mol). This could indicate strength in the interaction between saccharin and tubulin as hydrogen bonds are the usual modes by which drugs bind to receptors [37].
Table 3.3 Hydrogen bonds identified for potentially PD-related molecules and colchicine.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine (CN)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Rotenone (ROT)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Aspartame (ASPTM)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>MPP+ (MPP+)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>MPTP (MPTP)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Saccharin (SAC)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Toluene (TOL)</td>
<td>Cys241βSG</td>
<td>CNS1</td>
<td>Ser178αOG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*Don. = Donor, Acc. = Acceptor, α = backbone, β, G, D = sidechains.*

Ligand-interaction diagrams are shown in Figure 3.8. All molecules evaluated (colchicine and the potentially PD-related molecules) exhibited Van der Waals interactions with all or some of the following amino acid residues: Ala250, Ala316, Asn258, Cys241, Ile378, Leu242, Leu248, Leu255, Lys254, and Lys352 on beta-tubulin; and Ser178, Thr179, and Val181 on alpha-tubulin. Forty-seven VdW contacts were found for colchicine, 58 for rotenone, 31 for aspartame, 20 for MPP+ and MPTP, 25 for saccharin, and 16 for toluene (Ref Appendix A1). Residues common to all molecules evaluated were Ala250 and Leu255 on beta-tubulin. All but MPTP had VdW contacts with Val238 on beta tubulin, and all but MPP+ and MPTP had VdW contacts with Leu242 on beta-tubulin.

The majority of VdW contacts found were with beta-tubulin; minimal contacts were found with alpha-tubulin. Only 4 of 47 VdW contacts occurred with alpha-tubulin for colchicine, only 4 of 58 for rotenone, 2 of 31 for aspartame, 1 of 20 for MPTP, and none for saccharin, toluene, and MPP+. As illustrated within Figure 3.8, the majority of interactions between the potentially PD-related compounds that we evaluated and tubulin and between colchicine and tubulin are with one of the three functional groups discussed.
within Section 3.1 “Chemical Similarity Evaluation”: alkyl, amine, and aromatic groups, in additional to interaction with oxygen atoms.
Aspartame

MPP+

MPTP
Figure 3.8 Ligand interaction diagrams
Table 3.4 summarizes the results of our binding study of potentially PD-related molecules and colchicine, illustrating lowest calculated binding affinities, number of hydrogen bonds, and number of VdW contacts for each compound evaluated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (kcal/mole)*</th>
<th>Number of hydrogen bonds</th>
<th>Number of VdW contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>colchicine</td>
<td>-12.0</td>
<td>3</td>
<td>47</td>
</tr>
<tr>
<td>rotenone</td>
<td>-10.7</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td>aspartame</td>
<td>-8.7</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>MPP+</td>
<td>-6.8</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>MPTP</td>
<td>-6.7</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>saccharin</td>
<td>-6.1</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>toluene</td>
<td>-4.4</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

*Binding affinities from Table 3.2, number of hydrogen bonds from Table 3.3, Number of VdW contacts from Appendix A1. Colchicine is shown bold italics for comparison purposes.

Our docking results suggest that the binding affinity of rotenone on the colchicine site of tubulin is close to the binding affinity of colchicine. Though previous binding studies have also identified that rotenone binds to the colchicine site of tubulin [2], we could not find binding values for comparison. Our results also show a significant binding affinity for aspartame on the colchicine site. We also did not find comparative binding values for aspartame on tubulin, as to our knowledge, binding of aspartame to tubulin has not been evaluated in previously. Though affinity values for the remaining potentially PD-related compounds that we evaluated are less significant, we suggest that these smaller compounds (MTPT/MPP+, saccharin, and toluene) may exhibit non-specific binding to tubulin, effectively serving to inhibit microtubule function through attachment to numerous sites.

To illustrate the potential for non-specific binding with tubulin and the smaller potentially PD-related molecules evaluated (< 185 g/mol), we compared affinity values
generated for all five poses for the potentially PD-related compounds on each of the 4
tubulin inhibitors (Table 3.5). As illustrated in Table 3.5, in general, binding affinities
are lower for higher molecular weight compounds, due to the larger number of bonds
available (refer to Table 3.2). The binding affinities for the 4 tubulin inhibitors
(colchicine, soblidotin, vinblastine, taxol, listed in order of increasing molecular weight)
on their known binding sites were -12.0, -13.8, -16.3, and -15.4 kcal/mole, respectively).
The molecular weights of these compounds are, respectively, 399, 701, 810, and 853
g/mol, respectively. Vinblastine exhibited a slightly larger binding affinity than taxol,
though taxol has a slightly higher molecular weight.

Additionally, binding affinity values for the compounds with known binding sites
on that specific site (e.g. colchicine and rotenone on the colchicine site) exhibit a wider
range of values than those compounds that appear to exhibit non-specific binding. The
two generated poses for colchicine on the colchicine site exhibit a range of 3 kcal/mole,
and the values generated for rotenone on the colchicine site exhibit a range of 0.8
kcal/mole. In contrast, values generated for MPTP, MPP+, and toluene on the colchicine
site all have a range of 0.4 kcal/mole, and in many cases, two or more of the five poses
have the same binding affinity value.
Table 3.5. Review of calculated binding affinity ranges: Potentially PD-related compounds and colchicine on all 4 tubulin inhibitor binding sites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding affinity values (kcal/mole)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colchicine site</td>
</tr>
<tr>
<td>colchicine</td>
<td>-12.0, -9.0</td>
</tr>
<tr>
<td>aspartame</td>
<td>-8.7, -8.6, -8.4, -8.4</td>
</tr>
<tr>
<td>MPP+</td>
<td>-6.8, -6.8, -6.5, -6.4, -6.4</td>
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<tr>
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<tr>
<td>saccharin</td>
<td>-6.2, -6.1, -6.0, -5.8, -5.8</td>
</tr>
<tr>
<td>toluene</td>
<td>-4.4, -4.4, -4.4, -4.3, -4.3</td>
</tr>
</tbody>
</table>

*Values with all bonds nonrotatable

Now that we have discussed binding of tubulin inhibitors to tubulin and have calculated the binding affinity of potentially PD-related compounds on tubulin, we shall next evaluate physichochemical interactions of tubulin dimers to further understand the action of tubulin inhibitors.

### 3.3 Alpha-beta tubulin interactions

As numerous studies have evaluated the interactions between alpha and beta tubulin [38] [39] [40], we will not elaborate greatly on this topic, but will provide enough information to understand the relationship between alpha-beta protein-protein interactions and the method of action of tubulin inhibitors.

We calculated the physicochemical interactions between alpha and beta tubulin molecules: 6 hydrogen bonds and 128 VdW contacts were identified. Hydrogen bonds
identified were between residues Glu97, Asp98, Thr179, and Val260 of alpha-tubulin and Arg164, Lys254, Val260, and Lys352 of beta-tubulin. VdW contacts identified were between residues Glu71, Glu97, Asp98, Ala100, Asn101, Arg105, Pro175, Ser178, Ala180, Val181, Glu220, Arg221, Thr279, Lys394, Leu397, Met398, Lys401, Ala403, Phe404, His406, and Trp407 of alpha-tubulin and residues Arg164, Arg249, Asp251, Arg253, Lys254, Val257, Arg258, Val260, Pro261, Phe262, Pro263, Met325, Glu345, Trp346, Ile347, Pro348, Asn349, Lys352, and Ala438 of beta-tubulin. (Reference Appendix A2.)

As discussed within Section 3.3 “Physicochemical Interactions”, residues involved in the binding of tubulin inhibitors to tubulin were found to be Ala 250β, Ala 316β, Asn 258β, Cys 241β, Ile 378β, Leu 242β, Leu 248β, 2 Leu55β, Lys 254β, Lys352β, Ser 178α, Thr 179α, and Val 181α. Thus, 4 of the 40 residues that were found to contribute to the binding of alpha to beta tubulin (Ser 179 and Val 181 on alpha-tubulin and Lys 254 and Lys 252 on beta-tubulin) were found to also contribute to binding of tubulin inhibitors to tubulin.

We also evaluated the electrostatic potential of alpha and beta tubulin molecules and dimer to dimer interaction. Figure 3.9 is a depiction of the coulombic surfaces of 2 interacting tubulin dimers.
As illustrated in Figure 3.9, both alpha and beta tubulin molecules are strongly electro-negative, beta-tubulin slightly more so than alpha tubulin (AESOP analysis identified a charge of -16e for alpha-tubulin, and -18e for beta-tubulin). We noted that alpha-beta dimers attach at small electropositive and electronegative areas of each molecule (note yellow arrows above).

Analysis of physicochemical interactions between alpha1 and beta1 (initial tubulin dimer) revealed 6 hydrogen bonds and 128VdW contacts, whereas an analysis of interactions between dimers (beta1-alpha2) revealed 0 hydrogen bonds and only 65 VdW contacts. It is evident that interaction between alpha and beta tubulin molecules within dimers is much stronger than between dimers. This “weak” interaction between dimers is critical to the dynamic nature of microtubules [41]. (Recall Figure 1.2, formation of microtubules, which illustrates that tubulin dimers of alpha- and beta-tubulin molecules attach and detach to microtubules during cellular function.)
Within the next section, we evaluated control molecules to obtain representative binding affinity values for known ligands on their standard receptors, as well as to understand the potential for non-PD related compounds to bind with tubulin.

3.4 Controls

To investigate the potential binding of non PD-related compounds to tubulin, as well as to gauge typical binding values for compounds on their standard receptors, we evaluated 6 non PD-related molecules (common drugs or biological substances), and compared their potential for binding to their specific receptors with their potential for binding with tubulin. Compounds evaluated include a pain reliever (ibuprofen), a PARP inhibitor (quinazolinedione sulfonamide), a fat produced by the body (cholesterol), a drug used to regulate epilepsy (topomax), an anti-depressant (doxepin), and a biological compound essential for vision, skin, teeth, and bone health (retinol). We chose these compounds as they are either frequently used or commonly present within the human body, and their crystal structures bound to their human (or closely-related eukaryote) receptors were readily available in the Protein Data Bank. Figure 3.10 illustrates the chemical structure of these molecules. Again, the three groups we discussed previously (methyl, alkyl, and amine groups) are present.
Figure 3.10 Chemical structure of controls. Chemical structure of the 6 control molecules that we evaluated, illustrating similar functional groups.

The determined docking configurations of our control compounds on their standard receptors compared to their determined docking configurations on the colchicine site of tubulin are shown in Table 3.6. The colchicine site was used for consistency with our previous results. All molecules were docked with all potentially rotatable bonds non-rotatable and five poses were generated. The binding range of the five poses (highest and lowest affinities) is shown and the full set of binding affinity values is provided in italics. Our results showed that all compounds exhibited a larger binding affinity on their standard receptors than on tubulin (shown in bold). This results helps to provide understanding to the theory that high concentrations of compounds can lead to binding to non-standard receptors if all standard receptors are occupied (lowest affinity sites become occupied, compounds have the opportunity to bind to other areas). [42]
Table 3.6 Binding values of molecules to colchicine site of tubulin and to their standard receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
<th>PDB ID/Description</th>
<th>Receptor organism</th>
<th>Standard receptor</th>
<th>Colchicine site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibuprofen</td>
<td>Pain reliever</td>
<td>1EQG (COX-1 with ibuprofen)</td>
<td>Ovis aries</td>
<td>Low: -9.4</td>
<td>Low: -7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High: -6.5</td>
<td>High: -6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(-9.4, -8.6, -6.7, -6.5*)</td>
<td></td>
</tr>
<tr>
<td>quinazolinedione</td>
<td>Poly (ADP-ribose) polymerase</td>
<td>3R7X (Quinazolinedione sulfonamide with GluR2)</td>
<td>Human</td>
<td>Low: -9.8</td>
<td>Low: -8.9</td>
</tr>
<tr>
<td>sulfonamide</td>
<td>inhibitor</td>
<td></td>
<td></td>
<td>High: -7.8</td>
<td>High: -8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(-9.8, -8.5, -7.8, -7.7, -7.6)</td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>Fat produced by liver, crucial</td>
<td>3GKI (Niemann-Pick C1 protein with cholesterol)</td>
<td>Human</td>
<td>Low: -15.0</td>
<td>Low: -11.3</td>
</tr>
<tr>
<td></td>
<td>for body functions</td>
<td></td>
<td></td>
<td>High: NA*</td>
<td>High: -8.6</td>
</tr>
<tr>
<td>topomax</td>
<td>Drug used to treat epilepsy</td>
<td>3LXE (Human carbonic anhydrase with topomax)</td>
<td>Human</td>
<td>Low: -9.4</td>
<td>Low: -7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High: -7.4</td>
<td>High: -6.3</td>
</tr>
<tr>
<td>doxepin</td>
<td>Used to treat depression</td>
<td>3RZE (Human histamine receptor with doxepin)</td>
<td>Human</td>
<td>Low: -12.0</td>
<td>Low: -9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High: NA*</td>
<td>High: -8.1</td>
</tr>
<tr>
<td>retinol</td>
<td>Form of vitamin A</td>
<td>1BRP (RBP with retinol)</td>
<td>Human</td>
<td>Low: -12.7</td>
<td>Low: -9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High: NA*</td>
<td>High: -8.7</td>
</tr>
</tbody>
</table>

*Only 1 pose generated for cholesterol, doxepin, and retinol and only 4 poses generated for ibuprofen. Affinity values of all binding poses generated shown in italics.

Per our docking calculations, the binding affinity values of known ligands on their standard receptors range from -15.0 to -9.2 kcal/mole. This is within the range of the calculated affinity of rotenone on the colchicine site (-10.7 kcal/mole), and just slightly larger than the calculated affinity of aspartame on the colchicine site (-8.7 kcal/mole).

The five poses generated for each of the control molecules are shown in Figure 3.11, compared to their PDB configurations. Again, the pose with the lowest affinity value most closely matched the PDB configuration of the known crystallographic structure of each compound. The lowest binding affinity configurations generated in our docking results have consistently yielded poses that closely match the known configurations of each molecule (i.e. tubulin inhibitors and control molecules with known crystallographic structures). This provides a high level of confidence in our results.
Figure 3.11 Docking configurations of control molecules compared with their PD configurations
Docking poses generated for control molecules compared with their PD configurations. Binding affinities for each of the 5 configurations illustrated are illustrated as follows: red (lowest affinity), orange (2nd lowest affinity), yellow (3rd lowest affinity), green (4th lowest affinity), blue (5th lowest affinity). PDB configurations are shown in magenta. The lowest affinity poses for ibuprofen and cholesterol overlap their PDB configuration poses (i.e. the red pose, lowest affinity, is barely visible for these 2 compounds).

Having presented our similarity analysis and docking results of potentially PD-related compounds with tubulin, we next discuss further considerations including genes and risk factors involved in PD, entry of the potentially PD-related compounds into the bloodstream/potential to cross the BBB, and we present our hypothesis which ties all PD-related factors (compounds, genes, cellular injuries) to a common trigger.
Chapter 4 – Additional Information

4.1 Genes implicated in PD

Among the genes known to be related to early-onset PD are: LRRK2, PARK2, PARK7, PINK1 and SNCA [5]. Though the specific function of these genes is not fully understood, the following is a summary of their suspected roles (Table 4.1).

Table 4.1 Suspected function of genes involved in PD

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Suspected function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
<td>Cytoskeleton integrity</td>
<td>[3]</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkinson protein 2, E3 ubiquitin protein ligase (parkin)</td>
<td>Provides instructions for making protein parkin. Parkin helps to degrade unneeded proteins, is involved in maintenance of mitochondria, and is known to stabilize microtubules.</td>
<td>[3], [43]</td>
</tr>
<tr>
<td>PARK7</td>
<td>Parkinson protein 7</td>
<td>Provides instructions for making protein DJ1. DJ1 is important for protection against oxidative stress, chaperone in protein folding.</td>
<td>[3]</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN induced putative kinase 1</td>
<td>Provides instructions for making protein PTEN. PTEN. Help protect mitochondria from malfunctioning during periods of cellular stress.</td>
<td>[3]</td>
</tr>
<tr>
<td>SNCA</td>
<td>synuclein, alpha (non A4 component of amyloid precursor)</td>
<td>Provides instructions for making protein alpha-synuclein, which regulates dopamine production and is also believed to be involved in cytoskeleton function.</td>
<td>[3], [44]</td>
</tr>
</tbody>
</table>

As we have illustrated (bold in table above), all of the genes listed above are involved in either cytoskeletal/microtubule function and/or protection against oxidative stress, the main triggers suggested to be involved in PD. Additionally, PARK2 and PINK1 are involved in mitochondrial function, also implicated in PD. Evidence is emerging that microtubules may be involved in proper mitochondrial function [45], potentially explaining the connection between microtubule inhibition and mitochondrial dysfunction.
We had anticipated performing binding studies of parkin to alpha-beta tubulin. As parkin is believed to act as a microtubule stabilizer, we would predict that parkin would bind near the taxol binding site of tubulin [46]. However, a PDB structure of parkin was unavailable for docking simulations.

### 4.2 Further considerations

In order for the compounds that we evaluated to interact with microtubules within neurons, they must be capable of entering the bloodstream intact and, further, of penetrating the blood brain barrier (BBB). We shall consider the potential for these to occur, beginning with entry into the bloodstream.

#### 4.2.1 Entry of chemicals into the bloodstream

Two of the compounds that we evaluated, rotenone and toluene, can potentially enter the bloodstream via the respiratory tract through inhalation of compound vapors [47, 48]. MPTP is a byproduct of the synthetic drug “meperidine”. MPP+ is a metabolite of MPTP, which can enter the bloodstream after injection of meperidine [49]. The artificial sweeteners saccharin and aspartame would need to pass through the intestinal wall and/or exhibit absorption through oral mucosa to enter the bloodstream. Cohen-Addad et al [50] illustrated that ingested saccharin enters the bloodstream in measurements of human mother-infant pairs, and Matthews et al illustrated that ingested saccharin enters the bloodstream in rats [51]. We can thus conclude the saccharin enters the bloodstream once ingested. Regarding the last compound, aspartame, it is reported that, upon ingestion, aspartame does not enter the bloodstream intact but is broken down in the gut into phenylalanine, aspartic acid and methanol [52]. However, to our
knowledge no studies of the buccal and/or sublingual penetration of aspartame have been published, nor studies on intestinal absorption in individuals with potentially compromised intestinal penetration, such as persons with diabetes and other disorders [53]. We recommend evaluation of these potential routes of entry and illustrate the potential for sublingual penetration of aspartame in the ensuing pages.

4.2.2 Penetration of BBB

Regarding the potential for the compounds that we evaluated to penetrate the BBB, we shall consider the following criteria currently utilized in drug design in target molecule selection [54], a more stringent version of Lipinski’s Rule of Five: BBB penetration is considered to be likely if: i) Molecular weight ≤400, ii) Log p ≤ 5, iii) hydrogen bond donors ≤ 3, and iv) hydrogen bond acceptors ≤ 7. Referring to Table 3.1, we see that all of the 6 molecules that we evaluated meet these criteria. It should be noted that all but two of the compounds evaluated (saccharin and aspartame) are currently considered to be harmful to the human body, though saccharin has been banned in the country of Canada due to potential toxicity studies [9]. The method of action of each known toxin has not been specifically identified, though evidence is emerging for at least two of these toxins (rotenone and MPTP/MPP+) that microtubule disruption is involved [55, 56].

4.2.3 Evaluation of sublingual penetration of aspartame

Given the considerable chemical similarity we identified between aspartame and soblidotin and the significant calculated binding affinity for aspartame on the colchicine site of tubulin, we evaluated further the potential of aspartame to act as a tubulin
inhibitor, and therefore discuss a proposed mechanism of entry of aspartame into the bloodstream. We hypothesized that, given its low molecular weight and structural similarity to known sublingual drugs, aspartame may potentially enter the bloodstream intact via the sublingual route. We compared aspartame to eight sublingual drugs: epinephrine, estradiol, isoproterenol, isoxsuprine, oxytocin, thiocolchicoside, triazolam, and zolpidem through both chemical clustering analysis as well as through evaluation of properties known to contribute to sublingual penetration.

Figure 4.1 illustrates the chemical structure of these 8 sublingual drugs and aspartame. As illustrated, all chemicals share common functional groups (alkyl and aromatic groups), suggesting the potential for similar chemical behavior.

![Chemical Structure of Drugs](image)

**Figure 4.1. Chemical structure of 8 sublingual drugs compared to aspartame.** Chemical structure of the 8 sublingual drugs evaluated compared to aspartame illustrating similar functional groups.

We also performed a clustering analysis of aspartame and these 8 sublingual drugs (Figure 4.2). As illustrated, aspartame exhibited ~68% similarity to oxytocin, and ~60% similarity to isoxsuprine, isoproterenol, and epinephrine. Note that isoproterenol
and epinephrine differ only by an additional methyl group in isoproterenol, though they exhibited only a ~80% similarity in our clustering analysis.

\[ \text{Figure 4.2 Clustering diagram of sublingual drugs and aspartame.} \]

Clustering diagram of the 8 sublingual drugs evaluated and aspartame. Aspartame ~68% similarity to oxytocin, and ~60% similarity to isoxsuprine, isoproterenol, and epinephrine.

We evaluated what are considered to be the two most pertinent properties for sublingual penetration (logP and molecular weight) [57] of these eight drugs and compared these properties with those of aspartame. Values are shown in Table 4.2.

It has been suggested that drugs that exhibit optimum sublingual penetration generally have low molecular weight (<600 g/mol) and a logP in the range of 1.6 - 3.3 [57]. However, as illustrated in Table 4.2, the range of logP values for known sublingual drugs is much wider (the chemicals that we evaluated have logP values ranging from -2.6 to 4). The logP of aspartame is -2.7, just slightly below the logP of oxytocin. We suggest that logP may not be the only indication of sublingual penetration, and provide a brief discussion of logP to illustrate.

Lipophilicity of a compound (the tendency of the compound to partition between lipophilic organic phase and polar aqueous phase), is measured by the logarithm of partition coefficient P (logP) between these two phases. Compounds with higher
lipophilicity generally have higher permeation across biological membranes (up to a certain limit). Drugs with a logP greater than 3.3 are considered to be so oil-soluble that it is difficult for sufficiently high levels of drug to be soluble in the aqueous salivary fluids. Drugs less lipophilic than those with a logP of 1 would not be absorbed to any great extent and would thus require large doses by this route [58].

We have discussed that large doses of compounds would likely be required to trigger damage that might cause PD. As large doses of compounds would be needed for sublingual absorbance of compounds with logP less than 1 (aspartame has a logP of -2.7), we suggest that the same underlying cause that we proposed may be present in toxin-induced damage (large dosages of compounds) might be present in sublingual penetration of certain compounds, such as aspartame. I.e., if large doses of foods or beverages containing aspartame are ingested, this may lead to some of the aspartame present in these foods and beverages being absorbed sublingually.
Table 4.2 Chemical properties of chemicals administered sublingually compared to aspartame

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Treatment/Use</th>
<th>ChemSpider ID</th>
<th>PubChem ID</th>
<th>LogP</th>
<th>MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>epinephrine [59]</td>
<td>cardiac arrest</td>
<td>5611</td>
<td>5816</td>
<td>-1.4</td>
<td>183.20</td>
</tr>
<tr>
<td>estradiol [60]</td>
<td>tubulin inhibitor</td>
<td>5554</td>
<td>5757</td>
<td>4</td>
<td>272.38</td>
</tr>
<tr>
<td>isoproterenol [61]</td>
<td>bradycardia, asthma</td>
<td>3647</td>
<td>3779</td>
<td>-0.6</td>
<td>211.26</td>
</tr>
<tr>
<td>isosuprine [62]</td>
<td>vasodilator</td>
<td>3651</td>
<td>3783</td>
<td>2.8</td>
<td>301.38</td>
</tr>
<tr>
<td>oxytocin [63]</td>
<td>induce labor</td>
<td>388434</td>
<td>439302</td>
<td>-2.6</td>
<td>1007.19</td>
</tr>
<tr>
<td>thiocolchicoside [64]</td>
<td>muscle relaxant</td>
<td>65053</td>
<td>72067</td>
<td>-0.4</td>
<td>563.61</td>
</tr>
<tr>
<td>triazolam [65]</td>
<td>sleep disorder</td>
<td>5355</td>
<td>5556</td>
<td>2.4</td>
<td>343.21</td>
</tr>
<tr>
<td>zolpidem [66]</td>
<td>sleep disorders</td>
<td>5530</td>
<td>5732</td>
<td>2.5</td>
<td>307.39</td>
</tr>
<tr>
<td>Aspartame</td>
<td>sweetener</td>
<td>118630</td>
<td>134601</td>
<td>-2.7</td>
<td>294.30</td>
</tr>
</tbody>
</table>

References in “Chemical Name” column refer to sublingual penetration studies.

Table 4.2 illustrates that the molecular weight and logP of aspartame is within a range comparable to known sublingual drugs. We have also discussed that aspartame and known sublingual drugs share similar functional groups, and have discussed the possibility of large doses of aspartame to potentially lead to sublingual penetration. We thus recommend further evaluation of sublingual penetration of aspartame.

Within the next section we discuss the risk factors involved in PD and relate them to the proposed PD cascade that we have evaluated.

4.2.4 The 4 risk factors in PD

Four main factors have been implicated in PD. These include: 1) genetics [67], 2) toxins [68], 3) head trauma [67], 4) age [68]. We have discussed two of these 4 main factors (toxins and genetics), and shall now provide an overview of the remaining two factors: head trauma and age.

To our knowledge, no studies have related the biochemical rationale for the relationship between head trauma and PD; the relationship has only been linked statistically. (Head trauma has been associated with a higher risk of PD [69].) We propose that the same trigger illustrated in Figure 1.1, excess cytosolic dopamine...
followed by ROS production, is common in both head-trauma related PD and toxin-induced PD. The following is our rationale: Studies evaluating traumatic brain injury (TBI) note that large amounts of dopamine are expelled into the cytosol of neurons during head trauma [70]. We have discussed that when high levels of dopamine are expelled into the cytosol, ROS can form, leading to α-syn aggregation (Figure 1.1) and PD pathology. Thus, we suggest that influx of dopamine into cellular cytosol and the ensuing production of ROS is common to both TBI-induced and toxin-induced PD.

We also propose that the same initial trigger (ROS) exists in the final risk factor in PD (aging). It is reported that approximately 85% of known cases are diagnosed in people over 60 years [69]. We suggest the following in relation to this fact: Oxidation of proteins by ROS can generate a range of reactive products such as protein hydroperoxides that can generate additional radicals particularly upon interaction with transition metal ions. Most oxidized proteins that are functionally inactive are rapidly removed; however, some can gradually accumulate with time and thereby contribute to the damage associated with aging as well as various diseases. [71]. ROS form as natural byproducts of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [71]. However, high levels of ROS, which can accumulate during cellular stresses, can be extremely damaging to cells. We propose that high levels of ROS and the ensuing build-up of oxidized proteins, whether caused by head trauma, natural aging, or through toxin binding to tubulin, (3 of the 4 risk factors in PD) can be a trigger for PD pathology.
We have discussed in Section 4.1 how the remaining risk factor, genetic mutations, can be related to the inability of cells to protect against oxidative stress and microtubule dysfunction. Thus, we have tied all 4 PD risk factors to tubulin inhibition/microtubule depolymerization and/or ROS, as we illustrate in Figure 4.3.

**Figure 4.3 Proposed interaction of 4 PD-related factors to microtubule inhibition & ROS**
Our proposed relationship between the main causal factors in PD (genetics, toxins, head trauma, and aging) and microtubule disruption and/or ROS.

Having discussed the relationship between the main risk factors in PD to microtubule disruption and/or ROS production, we shall now evaluate the proposed method of propagation of PD.

**4.3 Proposed Method of Propagation of PD**

Only recently (within the past 3-4 years) have studies been conducted evaluating the potential mechanism of the propagation of PD, with the majority of studies suggesting that PD is propagated through the transfer of mis-folded α-syn from cell to cell. It appears that aggregated α-syn species are especially prone to uptake and have the
potential to “seed” fibrillization of endogenous α-syn, in a similar manner as prion disorders [72].

Though inter-cellular transfer of α-syn has not been proven at this time, aggregation of α-syn has been evidenced. To evaluate the binding potential of α-syn proteins to each other, we conducted further docking studies. We utilized residues 1 to 100 of α-syn for the ligand and the full protein, residues 1-140, as the receptor (see Figure 4.4 for a representation of the structure of α-syn). We removed a small portion of the “tail” of the protein in the ligand model as the docking program we utilized is intended for ligand-protein docking rather than protein-protein docking [35]. Our results are thus cursory, and we recommend further α-syn protein-protein docking studies. However, our study may suggest potential binding values and interacting residues in α-syn binding.

Figure 4.4 Ribbon structure of α-syn protein
Arrows indicate residues 100-140, removed in the α-syn “ligand” used for our docking study.

Our docking results of the α-syn “ligand” on the α-syn protein generated 1 pose with binding affinity of -14.5 kcal/mole. This value is between the calculated affinity of taxol and soblidotin on their own binding sites (see Table 3.2). Figure 4.5 illustrates the
results of our study. The full $\alpha$-syn protein is shown in purple, the $\alpha$-syn “ligand” in orange. No hydrogen bonds were found between the $\alpha$-syn ligand and receptor, though 103 VdW contacts were identified (see Appendix A-3).

![Figure 4.5 Predicted docking position of $\alpha$-syn fibrils.](image)

Predicted docking position of $\alpha$-syn ligand on $\alpha$-syn protein. $\alpha$-syn protein is shown in purple and the $\alpha$-syn “ligand” is shown in yellow.

Residues 60-90 have been identified as the potential interacting residues in $\alpha$-syn binding [73]. However, our docking results suggest that a wider range of residues may be involved. Residues 4, 7, 15, 22, 26, 29, 32, 33, 52, 56, 59, 63, 67, 70, 71, 74, 87, 92, 94 and 107 of the $\alpha$-syn full protein (receptor) were found to interact, as were residues 3, 10, 16, 20, 27, 31, 34, 38, 41, 42, 46, 50, 54, 58, 61, 65, 66, 80, and 99 of the $\alpha$-syn “ligand”.

Having evaluated potential causes and mechanisms of PD, we next look at commonalities among neural disorders and propose a relationship between potentially carcinogenic substances and PD-related compounds.
4.4 Commonalities among neurodegenerative disorders, carcinogenic substances, and PD-related compounds

It has been noted that neurodegenerative disorders share common mechanisms of propagation. Neural diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS), and prion diseases all involve protein aggregation and inclusion formation in areas of the brain that degenerate. Protein aggregates generally consist of fibers of mis-folded protein with beta-sheet conformation (amyloids) [74]. Abnormal aggregation of α-syn, huntingtin, superoxide dismutase (SOD), and alpha-beta peptide proteins are present in PD, HD, ALS, and AD, respectively.

In the majority of neural disorders, proteins impacted are generally in a natively unfolded state and become mis-folded and aggregated through the course of the disease process. Varying factors are considered to be involved in the aggregation process, though a major factor is known to be oxidative stress [75]. We have discussed that oxidative stress is considered to be an initial trigger and major factor in PD.

We also propose that there is also a relationship between carcinogenic substances and substances related to neural disorders (particularly PD). As discussed, compounds that have been associated with PD include solvents such as toluene, heavy metals, MPTP/MPP+, pesticides such as rotenone, and, by speculation, the artificial sweeteners saccharin and aspartame. These compounds have also been implicated in potential carcinogenic activity or as generators of ROS (Table 4.3 provides references).
Table 4.3 Summary of potential toxicity of PD-related compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Implicated in</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>Potential carcinogenic activity and oxidative stress</td>
<td>[76]</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Potential carcinogenic activity and oxidative stress</td>
<td>[77]</td>
</tr>
<tr>
<td>MPTP/MPP+</td>
<td>Oxidative stress</td>
<td>[78], [79]</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Potential carcinogenic and anti-carcinogenic activity</td>
<td>[80], [81]</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Potential carcinogenic activity (banned in Canada)</td>
<td>[82]</td>
</tr>
<tr>
<td>Aspartame</td>
<td>Potential carcinogenic activity</td>
<td>[83]</td>
</tr>
</tbody>
</table>

There is a seeming paradox in the function and action of tubulin inhibitors: i.e., rotenone has been found to potentially exhibit both carcinogenic and anti-carcinogenic activity as it can inhibit the growth of cancer cells but also may contribute to certain cancers. This phenomenon is explained by Schumacker [84] as follows: “Evidence suggests that transformed cells use ROS signals to drive proliferation and other events required for tumor progression. This confers a state of increased basal oxidative stress, making them vulnerable to chemotherapeutic agents that further augment ROS generation or that weaken antioxidant defenses of the cell. In this respect, it appears that tumor cells may die by the same systems they require.” Thus, the action of tubulin inhibitors may involve a complex balance between concentration levels, stage of disease, and other factors.

We have identified a repeating theme common to both neurodegenerative disorders and cancer -- overproduction of ROS, and recommend further evaluation of this observation to understand potential common treatments and/or prevention techniques.

We shall now evaluate potential therapies relating to the two potential causes of PD that we have discussed: ROS accumulation and α-syn aggregation.
4.5 Potential PD Therapies

As we begin to understand probable causes and potential pathways of propagation of PD, we can start to evaluate potential therapies accordingly. We evaluated prospective therapies for two potential factors in PD: α-syn aggregation and ROS. We begin with a discussion of therapies for elimination of ROS: anti-oxidants.

4.5.1 Potential therapies for ROS

We have mentioned throughout this paper the potential involvement of ROS in PD. Thus, it is worth providing a discussion of the functions of ROS in biological systems. As discussed, ROS are chemically reactive molecules that form as a natural byproducts of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [71]. However, high levels of ROS can accumulate during cellular stresses and can be damaging to cells. Reactive oxygen species include hydrogen peroxide (H₂O₂), hypochlorous acid (HClO), and free radicals such as the hydroxyl radical (-OH) and the superoxide anion (O₂-). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation or by oxidizing DNA or proteins. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms [85].

We have also discussed that the brain (particularly areas harboring dopamine neurons) is vulnerable to oxidative injury, due to high metabolic rate and elevated levels of polyunsaturated lipids, the target of lipid peroxidation [86]. Consequently, antioxidants are commonly used as medications to treat various forms of brain injury. Sodium thiopental and propofol are used to treat prevent tissue damage caused when
blood supply returns to the tissue after periods of ischemia, and also to treat traumatic brain injury [87] [88]. These compounds (sodium thiopental and propofol) appear to alleviate oxidative stress in neurons, thus preventing neurological damage.

Anti-oxidants are also being investigated as possible treatments for neuro-degenerative diseases [89] [90]. Anti-oxidants known to exhibit successful blood brain barrier penetration include vitamins E, C, and A, and coenzyme Q10. As discussed by Gilgun-Sherki et al [75], studies have not yet shown conclusive evidence of the potential of these anti-oxidants to prevent or treat PD, though there is some evidence to suggest they may be successful in high doses and/or when administered in combination with each other.

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). Water-soluble antioxidants tend to react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants tend to protect cell membranes from lipid peroxidation [91]. Figure 4.6 shows the chemical structure of all anti-oxidants we have discussed. Vitamin C is water-soluble, while vitamins A, E, coenzyme Q10, sodium thiopental, and propofol are lipid-soluble [92] [93] [94] [95]. As can be seen in Figure 4.6, similar chemical groups (alkyl and methyl groups) are found in vitamins E, A, coenzyme Q10, and propofol, sodium thiopental contains methyl groups, though vitamin C contains neither of these two groups, which can indicate the difference in solubility.
Figure 4.6 Chemical structure of potential anti-oxidants for treatment of PD

Though still in the early stages of evaluation, antioxidants may prove to be a successful preventive treatment for PD, and/or for use in the early stages of the disease. As of yet, antioxidants evaluated in PD include mainly the vitamins we have discussed, to our knowledge no studies have evaluated propofol and sodium thiopental as a PD treatment. As propofol and sodium thiopental have proven to be successful in the treatment of stroke and brain injuries, and given the relationship we have illustrated between brain injury and PD, we recommend that these compounds be evaluated for potential PD treatments as well.

We now shall evaluate potential treatments for the second major factor in PD, α-syn aggregation, and shall evaluate through molecular docking calculations potential α-syn aggregation inhibitors.
4.5.2 Potential therapies for α-syn aggregation

It is generally accepted that aggregation of α-syn is involved in PD (whether or not propagation of α-syn from cell to cell has been proven), thus therapies preventing α-syn protein to protein binding may be promising. Curcumin [96] and geldanamycin [97] are two compounds that have shown the potential to prevent α-syn clustering.

Figure 4.7 shows the chemical structure of curcumin and geldanamycin. Both compounds share two of the functional groups that we have discussed function in binding (alkyl and methyl groups).

![Curcumin and Geldanamycin](image)

**Figure 4.7 Chemical structures of curcumin and geldanamycin.**

We conducted molecular docking studies of curcumin and geldanamycin on α-syn to understand if these compounds may potentially bind to residues that are active in α-syn protein to protein binding (aggregation). Five poses were generated for each compound with all bonds non-rotatable. Calculated binding affinity values for curcumin were: -6.6, -6.1, -5.3, -5.2, -5.1 kcal/mole and for geldanamycin were: -7.1, -6.5, -6.0, -6.0, and -5.6 kcal/mole. These values are between the range of values found for MPTP/MPP+ and saccharin on the colchicine site of tubulin (refer to Table 3.2).
Poses generated for curcumin docked on α-syn are shown in Figure 4.8a, and for geldanamycin in Figure 4.8b. The α-syn “ligand” is shown in Figure 4.8 for reference. Poses generated for curcumin and geldanamycin had similar binding behavior, binding near residues 15-50 of α-syn.

**a. Docking poses of curcumin on α-syn.**
Calculated docking positions of curcumin on α-syn.

**b. Docking poses of geldanamycin on α-syn.**
Calculated docking positions of geldanamycin on α-syn.

**Figure 4.8 Docking positions of curcumin and geldanamycin on α-syn**
Calculated docking positions of curcumin and geldanamycin on α-syn. α-syn protein is shown in purple, α-syn “ligand” in yellow. Poses generated for curcumin and geldanamycin are shown as follows from lowest to highest binding affinity: red, orange, yellow, green, and blue.

Physicochemical interactions for the 2 lowest binding affinities for each compound were analyzed. The lowest affinity binding pose of curcumin was found to
have 1 hydrogen bond with α-syn (O4 of curcumin and Glu35 of α-syn), and 43 VdW contacts with α-syn (see Appendix A-3). 2 of the VdW contacts were with Lys32 of α-syn, which was also found to have 14 contacts from the α-syn protein to the α-syn “ligand” in our α-syn binding study. The lowest binding pose of geldanamycin was found to have 1 hydrogen bond (Val 40 of α-syn and O7 of geldanamycin), and 48 VdW contacts with α-syn. The second lowest affinity binding pose of curcumin was found to have no hydrogen bonds and 43 VdW contacts with α-syn. The second lowest affinity pose of geldanamycin was found to have 1 hydrogen bond (between Lys96 of α-syn and O3 of geldanamycin) and 40 VdW contacts with α-syn. 8 of the VdW contacts were with Phe94 of α-syn, which was also found to have 9 contacts from the α-syn protein to the α-syn “ligand” in our α-syn binding study. (Reference Appendix A-3 for a summary of physicochemical interactions.)

We propose that curcumin and geldanamycin could exhibit non-specific binding to α-syn, as exhibited by their close range of binding values generated between poses. These compounds could act as antagonists to prevent α-syn aggregation through binding with residues involved in α-syn protein to protein binding.
Chapter 5 – Conclusions and recommendations

We have utilized a multi-faceted approach to elucidate a probable cause and potential treatments of a complex disorder (PD). We have compared the function and chemical structure of compounds suspected of triggering PD pathology to tubulin inhibitors, identifying not only chemical similarity but also demonstrating through molecular docking simulations the tendency for potentially PD-related compounds to bind to the colchicine site of tubulin (a known depolymerization site). We identified considerable chemical similarity between rotenone and colchicine and between aspartame and soblidotin, and our docking results show that the calculated binding affinities for rotenone and aspartame are on the order of known ligands on their standard receptors.

We related known causal factors in PD, including cellular insults, genes, and PD-related compounds, to a suggested PD cascade involving tubulin inhibition and ROS production. We have demonstrated computationally a possible aggregation mechanism of α-syn proteins to one another and of have calculated the binding sites of potential aggregation inhibitors on α-syn. We have thus provided a collective body of evidence to help substantiate the hypothesis that PD may be brought on by tubulin inhibition leading to excessive ROS production, triggering a PD cascade.

Given our findings, we recommend further evaluation of the artificial sweeteners saccharin and aspartame, and we also suggest that sublingual penetration of aspartame be studied in a clinical setting. Lastly, we propose that guidelines for release of new drugs and food additives should include similarity evaluation of the proposed compounds to known tubulin inhibitors and carcinogens.
Author’s Note

I began my research several years ago not quite knowing where it would take me and unsure exactly where to begin. Potential causes of PD were not well elucidated even 4 to 5 years ago, and literature searches fell short of providing any consequential courses of study. I came across an eloquent article by Sidhu et al [10], and I fervently studied their illustrative diagram of a proposed PD cascade (Figure 1.1). Their reference to cytoskeletal insult as a potential trigger led me to further literature searches on the cytoskeleton and thus microtubules and their function. I began to research compounds that were potentially related to PD, and compounds that were utilized to mimic PD pathology. At the time, I found only rotenone. Articles unrelated to PD identified that rotenone was indeed involved in microtubule dysfunction. Once I made the connection between microtubule dysfunction and tubulin inhibitors, my research began to take shape. In the past year or two, studies are beginning to suggest that microtubule dysfunction is involved in PD, and I am strongly convicted that this hypothesis is valid.

My hope is that my research will shed further light on the cause and potential therapies for PD, and may lead to reconsideration of food additives that may possess similarities to tubulin inhibitors. I hope that my approach might be used as an example for future courses of research: utilizing the “big-picture” by collecting all current knowledge and suspect causes of ailments, looking for clues from other diseases and other areas of research possibly unrelated to the specific disorder but related to the suspect causes, thereby elucidating prospective courses of study that could lead to identification of seemingly elusive cures for devastating disorders.
### APPENDIX A.

Table A1. Van der Waals contacts for colchicine and the 6 potentially PD-related compounds evaluated.

<table>
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<tr>
<th>COLCHICINE</th>
<th>BOTONEONE</th>
<th>ASPARTAME</th>
<th>MFP+</th>
<th>MPTP</th>
<th>SACCHARIN</th>
<th>TOLENE</th>
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<td>A = alpha tubulin</td>
<td></td>
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<td></td>
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<tr>
<td>B = beta tubulin</td>
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<td><strong>ALPHA 1</strong></td>
<td><strong>BETA 1</strong></td>
<td><strong>ALPHA 1 (cont.)</strong></td>
<td><strong>BETA 1 (cont.)</strong></td>
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**TOTAL** 63
**TABLE A.3 VAN DER WAALS CONTACTS: α-SYN DOCKING STUDY**

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<th>CURC1</th>
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<th>CURC2</th>
<th>ALPHA-SYN</th>
<th>GELD1</th>
<th>ALPHA-SYN</th>
<th>GELD2</th>
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<th>ALPHAPROTEIN</th>
<th>ALPHALIGAND</th>
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**Note:** The table lists various contacts between different amino acids, indicating van der Waals interactions in the context of an α-SYN docking study.


