Acetaminophen Overdose as a Potential Risk Factor for Parkinson's Disease

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ARTICLE

Acetaminophen Overdose as a Potential Risk Factor for Parkinson's Disease

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Four complementary approaches were used to investigate acetaminophen overdose as a risk factor for Parkinson's disease (PD). Circulating microRNAs (miRNAs) serum profiles from acetaminophen-overdosed patients were compared with patients with terminal PD, revealing four shared miRNAs. Similarities were found among molecular structures of dopamine (DA), acetaminophen, and two known PD inducers indicating affinity for dopaminergic transport. Potential interactions between acetaminophen and the human DA transporter were confirmed by molecular docking modeling and binding free energy calculations. Thus, it is plausible that acetaminophen is taken up by the dopaminergic transport system into the *substantia nigra* (SN). A ChEMBL query identified proteins that are similarly targeted by DA and acetaminophen. Here, we highlight CYP3A4, present in the SN, a predominant metabolizer of acetaminophen into its toxic metabolite N-acetyl-p-benzoquinone imine and shown to be regulated in PD. Overall, based on our results, we hypothesize that overdosing of acetaminophen is a potential risk factor for parkinsonism.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Parkinson's disease (PD) is a neurodegenerative disorder of mostly unknown etiology. Acetaminophen is a widely used analgesic/antipyretic agent. Although acetaminophen is safe when used at therapeutic doses, acetaminophen poisonings are frequent. Recently, we have demonstrated that the serum of acetaminophen-overdose patients features some acetaminophen-induced microRNAs seeming to originate from the brain, indicating a potential brain perturbation.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ Considering that acetaminophen is widely used and can pass the human blood-brain barrier, we set out to investigate whether exposure to toxic levels of acetaminophen increases risk for development of PD.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ The present work suggests that it is plausible that acetaminophen is taken up by the dopaminergic transport system into the *substantia nigra* (SN). We emphasize CYP3A4, which is present in the SN, a predominant metabolizer of acetaminophen into its toxic metabolite *N*-acetyl-p-benzoquinone imine and shown to be regulated in PD.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ These results suggest that acetaminophen overdose may predispose for the development of PD.

Parkinson's disease (PD) affects about 0.5% of the population aged 60–69, 1% aged 70–79, and up to 2% aged above 80 years of age.[1] Familial mutations are responsible for ~ 10% of PD cases, leaving 90% sporadic cases for which causes are poorly understood. The toxicant 1-methyl-4-phenylpyridinium (MPP^+) is taken up by dopamine (DA) transporters (DATs)[2] and known to induce parkinsonism, which implies that exogenous factors may indeed induce PD. In addition, environmental toxicants, including pesticides (e.g., Paraquat), pollutants (metals and organochlorides), as well as dietary contaminants and drugs, such as methamphetamine (METH) have been suggested to cause sporadic PD.[3,4]

Acetaminophen is a widely used analgesic/antipyretic agent. Although acetaminophen is relatively safe when used at therapeutic doses, acetaminophen poisonings are quite frequent. In fact, acetaminophen is responsible for >70,000 visits to the hospital and is responsible for around 300–400 deaths/year in the United States alone.[5] Acetaminophen toxicity is also a major issue in acetaminophen-opioid products, which led the US Food and Drug Administration (FDA) to limit the dose of acetaminophen in these combinations to 325 mg/dose.[5] Hepatocellular injury due to formation of reactive metabolites is considered as a hallmark of acetaminophen toxicity. In rodent studies, acetaminophen has been shown to also cause damage to the brain, although it is not entirely clear

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whether brain injury was a consequence of the liver failure and subsequent oxidative stress or was caused by direct action of acetaminophen and its toxic metabolites in the brain.[6]

Therapeutic doses of acetaminophen were found not to influence PD risk.[7–9] However, recently we have demonstrated that the serum of acetaminophen-overdose patients features a distinct profile of organ-typical microRNAs (miRNAs) mainly reflecting liver injury.[10] Interestingly, some acetaminophen overdose-induced miRNAs seemed to originate from the brain, which may indicate a potential brain perturbation. Considering that acetaminophen at high dose was also found to cause brain perturbations in animal studies, and acetaminophen poisonings occur quite frequently, we set out to investigate whether exposure to toxic levels of acetaminophen increases risk for neurodegeneration and development of PD.

Because evaluating the potential role of acetaminophen poisoning in PD development in humans is technically and ethically extremely challenging, we designed a novel experimental approach by combining a noninvasive biomarker-based analysis with several *in silico* methods. First, we compared serum miRNA profiles in acetaminophen-overdose patients to miRNA profiles reported in the serum of patients with PD. Next, the molecular structure of acetaminophen was investigated for structural similarities with DA and known PD-inducing toxicants, which may indicate similar modes of interactions. Furthermore, molecular docking modeling and binding free energy calculations were performed *in silico* to inspect potential common binding modes for the DAT for DA and acetaminophen. In addition, binding affinities are calculated for those docked poses that present optimal binding interactions between acetaminophen with DAT. Finally, protein targets of acetaminophen and DA were compared and evaluated through *in silico* approaches thereby focusing on potential metabolic activation of acetaminophen into N-acetyl-p-benzoquinone imine (NAPQI), its major toxic metabolite.

RESULTS

Serum miRNA in patients with PD and subjects with acetaminophen overdose

To determine whether acetaminophen overdose might increase PD risk, the levels of circulating miRNAs in serum from both patients with PD[11–13] and subjects who were hospitalized with acetaminophen overdose[10,14] were compared (**Table 1**). Serum levels of three circulating miRNAs, hsa-miR-192, hsa-miR-24, and hsa-miR-30c, showed the same directional change (increase or decrease when compared with healthy subjects) in patients with PD and in subjects with acetaminophen overdose. This demonstrates a 14% overlap with known PD-related miRNAs.

According to miRTarBase, these three miRNAs target 2,679 gene transcripts. These target genes were compared

Table 1 Circulating miRNAs associated with PD and differentially expressed after acetaminophen overdose detected in human blood

miRNA	PD Di	FC	*P* value	Acetaminophen overdose Di	FC	*P* value	Reference for PD
miR-1253	↓	N.A.	N.A.	x	—	—	Khoo *et al.*[12]
miR-1826	↑	N.A.	N.A.	x	—	—	Discovery set: 32 PD patients, 32 healthy individuals
miR-192	↑	N.A.	N.A.	↑	2.26	4.58E-10	Replication set: 42 PD patients, 30 healthy individuals
miR-200a	↓	N.A.	N.A.	x	—	—	Validation set: 30 PD patients, 8 healthy individuals
miR-222	↑	N.A.	N.A.	x	—	—	
miR-450b-3p	↓	N.A.	N.A.	x	—	—	
miR-455-3p	↓	N.A.	N.A.	x	—	—	
miR-485-5p	↓	N.A.	N.A.	x	—	—	
miR-488	↑	N.A.	N.A.	x	—	—	
miR-505	↑	N.A.	N.A.	x	—	—	
miR-506	↑	N.A.	N.A.	x	—	—	
miR-518c	↓	N.A.	N.A.	x	—	—	
miR-626	↑	N.A.	N.A.	x	—	—	
miR-1274	↑	4.34	0.12	x	—	—	Vallelunga *et al.*[13]
miR-148	↓	–1.53	0.039	x	—	—	Discovery set: 6 PD patients, 6 healthy individuals
miR-24	↑	2.94	0,03	↑	3.15	3.11E-21	Validation set: 25 PD, 25 healthy individuals
miR-30c	↓	–1.53	0.036	↓	–1.34	3.61E-05	
miR-34	↑	1.76	0.07	x	—	—	
miR-19a	↓	–1.2	0.11	x	—	—	Botta-Orfila *et al.*[11]
miR-19b	↓	–1.45	0.0024	x	—	—	Discovery set: 10 PD patients, 10 familial PD, 10 healthy individuals
miR-29c	↓	–1.78	1.53E-5	x	—	—	Validation set 1: 20 PD patients, 20 familial PD, 20 healthy individuals
							Validation set 2: 65 PD patients, 65 healthy individuals

miRNAs changing in the same direction for PD and acetaminophen are marked in bold.
↓, decreased expression; ↑, increased expression; Di, direction of expression; FC, fold change; miRNA, microRNA; N.A., not available; PD, Parkinson's disease; x, not detected.

with data from a meta-analysis of gene expression studies in the *substantia nigra* (SN) of deceased patients with PD.[15] These three miRNAs target a total of 90 gene transcripts that seemed differentially expressed in SN from patients with PD. These 90 genes are involved in DA metabolism, vesicle management, apoptosis, autophagy, protein degradation, cell cycle, and mitochondrial functioning—all hallmarks of PD according to the PD map.[16] Both hsa-miR-192 and hsa-miR-24 serum levels were increased in patients with PD and upon acetaminophen overdose, and, as expected, 72% of their target genes are downregulated in SN of patients with PD. Hsa-miR-30c serum levels were decreased in patients with PD and in subjects with acetaminophen overdose, whereas 18% of its targets are upregulated in SN of patients with PD (**Table S1**).

Structural similarities among DA, known PD inducers, and acetaminophen

The structural similarities among DA, acetaminophen, MPP^+, Paraquat, and METH are reported in **Figure 1**. The Tanimoto coefficient, an evaluation method for assessing structural similarities between chemicals, is 0.39 between DA and MPP^+, 0.36 between DA and Paraquat, 0.58 between DA and METH, and 0.59 between DA and acetaminophen (**Figure 1**). These values show that DA is structurally more similar to acetaminophen than it is to MPP^+, being the best defined PD-related toxicant.

Molecular docking of acetaminophen to DATs

The plausibility that acetaminophen is taken up into dopaminergic cells in the SN was evaluated by molecular docking modeling, a method recently suggested for evaluating drug/transporter interactions.[17] The docking protocol was first validated by redocking of DA into drosophila DAT, because this is the only available crystal structure of DAT, and by comparing the resulting docked pose with a reported high-resolution structure for the complex formed between DA and DAT. The superposed docking pose and experimental complex structure were very similar, as indicated by a root mean squared deviation of 0.232 Å. This illustrates that the docking protocol is valid and can be used to determine a likely interaction between acetaminophen and DAT. A potential binding pose for the complex of acetaminophen with DAT was obtained after the docking process, which was then structurally analyzed (**Figure 2**). The hydroxyl residue on the phenol ring of acetaminophen likely interacts with the aspartate residue in position 121 (D121) of the DAT, similar to how one of the hydroxyl groups of DA interacts with its receptor. Additionally, the binding free energy calculation suggests that acetaminophen binds to

	DA	APAP	MPP^+	Paraquat	METH
DA	1	0.59	0.39	0.36	0.58
APAP	0.59	1	0.45	0.44	0.47
MPP^+	0.39	0.45	1	0.93	0.54
Paraquat	0.36	0.44	0.93	1	0.50
METH	0.58	0.47	0.54	0.50	1

Figure 1 Similarities between molecular structures. Similarities between molecular structures of dopamine (DA), acetaminophen (APAP), 1-methyl-4-phenylpyridinium MPP^+, Paraquat, and methamphetamine (METH) were evaluated using the Tanimoto coefficient. (https://pubchem.ncbi.nlm.nih.gov/score_matrix/score_matrix.cgi)

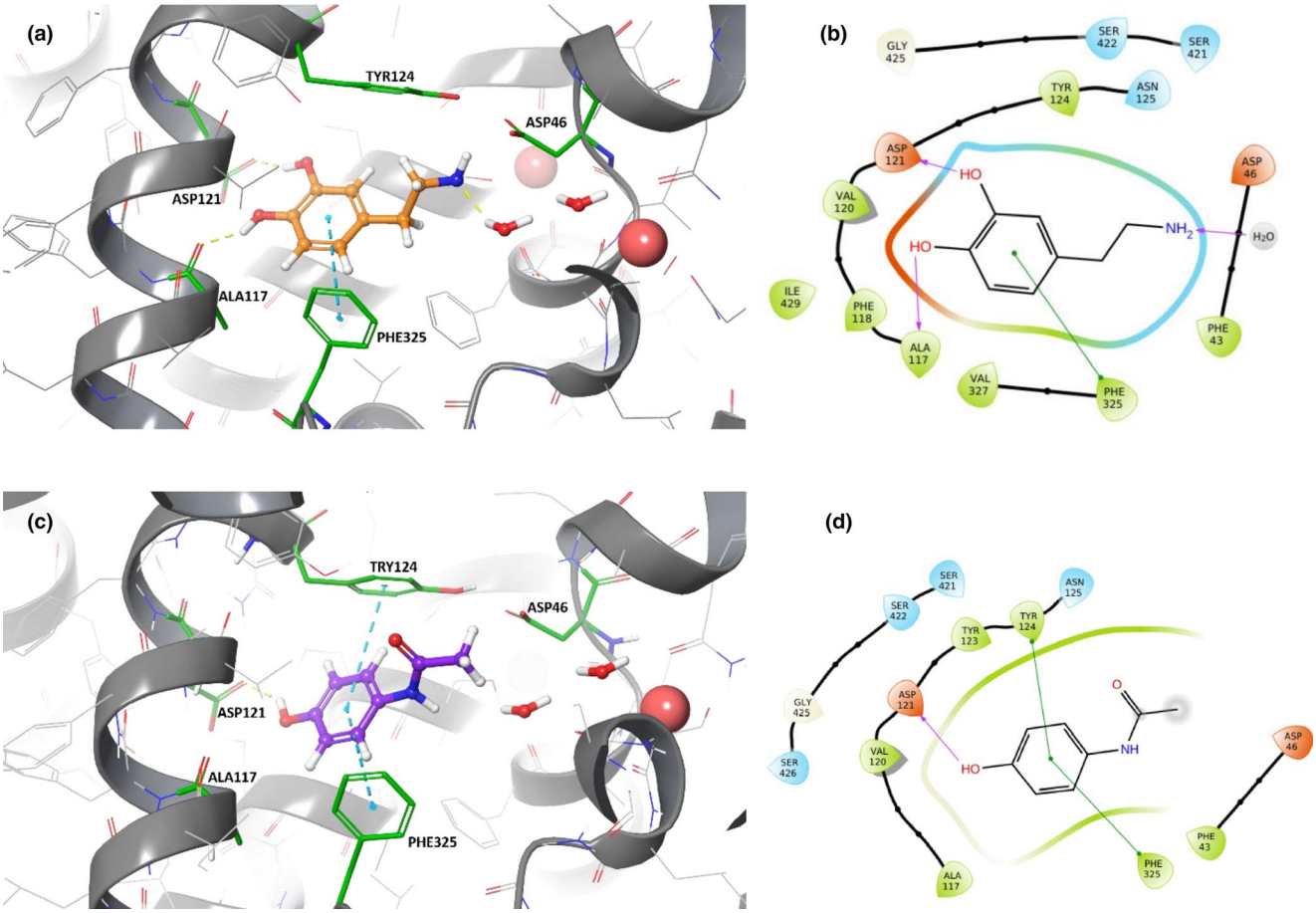

Figure 2 Molecular docking modeling of dopamine (DA) (orange) and acetaminophen (purple) to DA transporter (DAT; represented by dark grey structure, key residues are colored by green) protein using Schrödinger Glide software. Binding pose of (**a**) DA and (**c**) acetaminophen to DAT; 2D interaction diagram of (**b**) DA and (**d**) acetaminophen with DAT. DA binds to DAT by formation of H-bonds at ASP121, ALA117 and water molecule sites, and pi-pi stacking with PHE325; acetaminophen binds to DAT by formation of H-bonds at ASP121 site, and pi-pi stacking at PHE325, TYR124 sites. Analysis of the interaction patterns reveals that ASP121 and ALA117 serve as H-bonds acceptors and water serves as H-bond donor.

DAT with almost the same affinity as DA (DA: –18.07 kcal/mole; acetaminophen: –16.29 kcal/mole), but significantly less strong as compared with the strong DA inhibitor cocaine (–37.22 kcal/mole; **Table 2**).

Transferability from drosophila to human

Sequence alignments (**Figure S1**) showed that drosophila DAT and human DAT possess a very conserved binding pocket (80% sequence identity), except for D121G and A117S mutations. However, DA seemed to bind to the drosophila DAT structure enriched with D121G and A117S mutations, with comparable binding free energy as it binds to the drosophila wild type (DA-drosophila DAT: –18.07 kcal/mole, DA-mutated drosophila DAT: –16.00 kcal/mole). Per-residue decomposition calculations revealed that the energy contribution of A121 (–4.24 kcal/mole) drops to –1.33 kcal/mole when it is mutated to G. In contrast, A117 mutated to S increased interaction contribution from –0.75 kcal/mole to –1.47 kcal/mole.

Table 2 Binding free energies of DA, acetaminophen, and cocaine with DAT, calculated by the MM/PBSA method

System	Binding free energy (kcal/mole)
Dopamine_DAT	–18.07 ± 2.56
Acetaminophen_DAT	–16.29 ± 2.39
Cocaine_DAT	–37.22 ± 4.52

DA, dopamine; DAT, dopamine transporter; MM/PBSA, Molecular Mechanics Poisson-Boltzmann Surface Area.

Protein targets of DA and acetaminophen

Human protein targets for DA (82) and acetaminophen (137) were retrieved from ChEMBL. Forty-five protein targets are in common between the two molecules (**Figure 3**). These targets include adrenergic, serotonin, and DA receptors; norepinephrine, serotonin, and DA transporters; various cytochrome P450 and UDP-glucuronosyltransferase isoforms; and microtubule-associated protein tau (**Table 3**). All of these targets are associated with neurotransmitter signaling or detoxification of potentially toxic xenobiotics and endogenous compounds.[18] Furthermore, these are

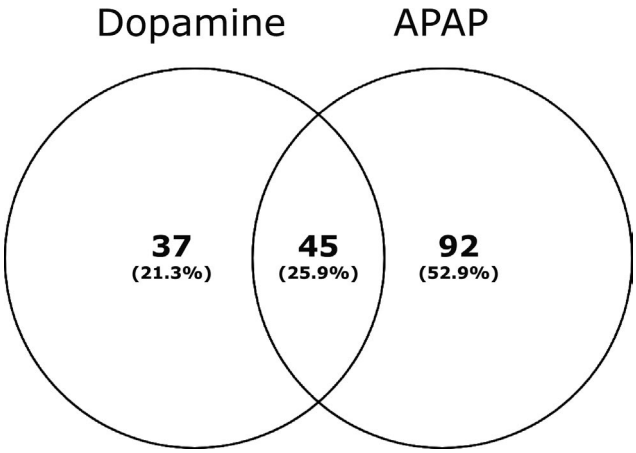

Figure 3 Common human protein targets for dopamine (DA) and acetaminophen (APAP) based on a ChEMBL human protein target search. Forty-five protein targets appear in common between DA and APAP.

intimately linked to the central nervous system in general and to the dopaminergic system specifically. After comparison with the SN-PD dataset, it was revealed that nine of the genes coding for these proteins are also differentially expressed in the SN of deceased patients with PD (ALDH1A1, CA14, CYP2C9, CYP3A4, DRD2, GLS, HTR2A, MAPK1, and MAPT). Among these nine genes, CYP2C9 and CYP3A4, which code for acetaminophen-metabolizing enzymes, are upregulated in SN-PD; all others are decreased in abundance.

DISCUSSION

There is increasing evidence that implicates exposure to chemicals, such as MPP^+, Paraquat, and METH, in the development of PD.[19,20] These chemicals are known to enter dopaminergic neurons (DNs) via DAT.[21,22] Therefore, we evaluated the potential of chemical-DAT interaction using *in silico* modeling approaches. We showed that DA and acetaminophen are molecules of comparable size and conformation, as confirmed by the Tanimoto coefficient score (**Figure 1**), whereas MPP+, Paraquat, and METH are structurally more different from DA than acetaminophen. Therefore, it is possible that acetaminophen fits into the recognition site of DAT. This has been confirmed in the known 3D structure of DAT.[23] Binding of DA to DAT is facilitated by the catechol group of DA (i.e., the benzene ring binding two hydroxyl groups).[23] The catechol group is highly similar to the phenol group of acetaminophen, except for one missing hydroxyl group. Whereas D121 of DAT forms two hydrogen bonds with DA, one respectively with each hydroxyl group, it is likely to bind acetaminophen by means of one hydrogen bond with its single hydroxyl group.

Because structural similarities between molecules are no guarantee that these molecules will, in fact, interact with the same proteins, molecular docking modeling was carried out to further evaluate the likelihood of acetaminophen interaction with DAT. Docking results suggest that it is indeed possible that acetaminophen binds to DAT in a comparable manner as DA, as was estimated from docking poses and their corresponding docking score. Further support comes from molecular dynamic simulations performed with the acetaminophen-DAT and DA-DAT complexes; this showed that the acetaminophen-DAT interaction is stable with a similar conformation as observed for DA (**Figure 2**). Binding affinity was predicted by calculating binding free energy, which yielded energy levels that would allow interaction, yet not so strong as to be able to completely displace DA. In contrast, a similar approach showed that cocaine displays an associated binding free energy greatly superior to DA, in line with the observation that cocaine is known to be a strong inhibitor of DAT,[24] thereby confirming that acetaminophen is not an inhibitor of DAT but instead may be easily transported into SN cells. Therefore, we conclude that acetaminophen may interact with DAT by means of binding at

Table 3 Forty-five human protein targets in common between DA and acetaminophen according to ChEMBL[39]

45 human protein targets in common between DA and acetaminophen		
Aldehyde dehydrogenase 1A1	Carbonic anhydrase XIV	Norepinephrine transporter
Alpha-2a adrenergic receptor	Chromobox protein homolog 1	Serotonin 2a (5-HT2a) receptor
Beta-1 adrenergic receptor	**Cytochrome P450 1A2**	Serotonin 2c (5-HT2c) receptor
Beta-2 adrenergic receptor	**Cytochrome P450 2C19**	Serotonin transporter
Bile salt export pump	**Cytochrome P450 2C9**	Solute carrier family 22 member 1
Carbonic anhydrase I	**Cytochrome P450 2D6**	Solute carrier organic anion transporter family member 1B1
Carbonic anhydrase II	**Cytochrome P450 3A4**	Solute carrier organic anion transporter family member 1B3
Carbonic anhydrase III	**Dopamine D1 receptor**	Sulfotransferase 1A1
Carbonic anhydrase IV	**Dopamine D2 receptor**	Tyrosine-protein kinase FYN
Carbonic anhydrase IX	**Dopamine D3 receptor**	**UDP-glucuronosyltransferase 1–1**
Carbonic anhydrase VA	**Dopamine D4 receptor**	**UDP-glucuronosyltransferase 1–10**
Carbonic anhydrase VB	**Dopamine transporter**	**UDP-glucuronosyltransferase 1–7**
Carbonic anhydrase VI	Glutaminase kidney isoform, mitochondrial	**UDP-glucuronosyltransferase 1A4**
Carbonic anhydrase VII	MAP kinase ERK2	**UDP-glucuronosyltransferase 2B15**
Carbonic anhydrase XII	Microtubule-associated protein tau	**UDP-glucuronosyltransferase 2B7**

Proteins of the dopaminergic system and proteins involved in the catabolism of potentially toxic xenobiotics and endogenous compounds are marked in bold.
DA, dopamine.

the same position as DA and, thus, may indeed be taken up into dopaminergic SN neurons.

Due to the lack of human or even mammal DAT crystal structures, the only available structure from drosophila was used. Even though there are two mutations in the binding pocket of human DAT compared with drosophila, the overall interaction with DA is likely to be conserved in human DAT, as verified by artificially mutating the binding pocket of drosophila into the human sequence *in silico*. We suggest that the molecular docking results and binding free energies obtained using the experimentally verified drosophila DAT crystal structure are, therefore, transferable to human DAT.

The link of acetaminophen and DA is further evaluated by the comparison of protein targets from ChEMBL for both molecules. The analysis returned 45 protein targets in common between the two molecules (**Figure 3**). These include DA receptors, DAT, and isoforms of cytochrome P450, including CYP3A4, CYP2C9, CYP1A2, and CYP2D6, which are associated with oxidative acetaminophen metabolism, including the formation of the major toxic metabolite NAPQI.[25] This further indicates that acetaminophen at overdose may be taken up into and oxidized into NAPQI within the DNs of the SN, thus causing cytotoxicity and thereby contributing to the onset of PD. The link with PD seems to be confirmed by the fact that CYP3A4 and CYP2C9 levels were found to be relatively increased in the SN of patients with PD.[15] Through combining various *in silico* approaches we therefore hypothesize that acetaminophen at overdose enters dopaminergic neurons via DAT, leading to SN injury upon metabolization into NAPQI. This is underlined by the observation that acetaminophen generates reactive oxygen species and decreases glutathione levels in the neuroblastoma cell line SH-SY5Y.[6] Additionally, in mixed primary cultures of rat astrocytes and oligodendrocytes, acetaminophen induced decreased cell proliferation and a dose-dependent cell death.[6]

The analysis of serum miRNA profiles in overdosed patients again suggests a possible link between PD and toxic exposure to acetaminophen. The miRNAs hsa-miR-192, hsa-miR-24, and hsa-miR-30c all vary in the same direction in PD and in acetaminophen-overdose patients. Although it is acknowledged that some of these miRNAs are also known to be released by other target organs injured by acetaminophen overdose, in particular miR-192 in rat liver,[26] and miRNA-30c after kidney damage,[27] the total set of three miRNAs target 90 genes that have been shown differentially expressed in SN from deceased patients with PD. Overall, this suggests that these miRNAs, released into the circulation after acetaminophen overdose, may originate from acetaminophen-affected dopaminergic neurons in the SN where they are involved in PD-related gene regulation. Other brain-related miRNAs detected in blood of acetaminophen overdosed patients, but not in patients with PD, were the frontal orbital gyrus-enriched miRNAs miR-125 b-3p and hsa-miR-125b-5p, but this region of the brain is only affected in a very late stage in a minority of patients with PD.

Our combination of *in silico* and biological approaches suggests a plausible acetaminophen interaction with the PD-relevant dopaminergic cells, although further confirmation from *in vitro* experiments is needed. We further hypothesize that excess intake of acetaminophen might lead to accumulation of the cytotoxic metabolite NAPQI in SN DNs that may contribute to increased risk of the development of PD. To test this hypothesis, cohorts of patients having overdosed on acetaminophen should be followed up to investigate whether shortly after recovering, parkinsonism has actually developed.

METHODS

Serum miRNA in patients with PD and subjects with acetaminophen overdose

Circulating miRNAs characteristically present in the serum of acetaminophen-overdose patients compared with normal controls were gathered from a publication by Krauskopf *et al*.[10] The miRNA data by Krauskopf *et al*.[10] were generated using Next Generation Sequencing technology, enabling reliable quantification of in particular low-abundance miRNAs. Circulating miRNAs were found to be stable even under conditions as harsh as boiling, extreme pH, long-time storage at room temperature, and multiple freeze-thaw cycles.[28,29] Details regarding sample collection, sample preparation, sequencing, and data processing can be found in Krauskopf *et al*.,[10,14] and see **Table S2** for patient characteristics and levels of liver damage markers. Circulating miRNAs characteristically present in the serum of patients with PD were gathered from Khoo *et al*.[12] (miRNA quantification by microarray), Botta-Orfila *et al*.[11] (miRNA quantification by quantitative real-time polymerase chain reaction), and Vallelunga *et al*.[13] (miRNA quantification by low-density array).

The overlap of circulating miRNAs between acetaminophen overdose and PD was determined by uploading the two separate lists of characteristic miRNAs to the online Venn diagram generator Venny.[30]

Target genes regulated by the overlapping miRNAs were found by querying the online miRNA database miRTarBase.[31]

To compare the genes regulated by the miRNAs of interest to genes differentially expressed in the SN of deceased patients with PD, gene expression data were downloaded from the PD map.[16] The data used for constructing this PD map originate from a meta-analysis collecting the results from several gene expression analyses on the SN of deceased patients with PD vs. deceased healthy persons.[15] The two gene lists (miRNA targeted genes and PD-related genes) were further uploaded to Venny, and the list of overlapping genes was retrieved.

Structural similarities

To quantify the structural similarities of DA, acetaminophen, MPP^+, Paraquat, and METH, each molecule was queried in PubChem,[32] uploaded into the PubChem Score Matrix Service, and the Score Type was set to 2D Similarity (https://pubchem.ncbi.nlm.nih.gov/score_matrix/score_matrix-help.html). This tool uses the Tanimoto index to evaluate structural similarities between molecules.

Molecular docking of acetaminophen to DATs

The crystal structure of drosophila DAT, the only crystal structure available for DAT, in complex with DA at a resolution of 2.89 Å (PDB-ID 4XP1) was downloaded from the Protein Data Bank,[33] only the protein structure and structural waters directly involved in ligand binding were used as the receptor. The docking program Schrödinger Glide[34] (released in 2017) was used; the grid was defined by a rectangular box of 10 Å in the x, y, and z directions centered on the ligand, which covered all the key residues in the cavity. All ligands were prepared by the Schrödinger LigPrep Wizard before docking by applying Glide SP (standard precision) where Glide was set to write out at most 10 poses per ligand and post-docking minimization was performed to let ligand fit well with full flexibility. The original Protein Data Bank (PDB)-entry was then taken, and the ligand was removed, to create an apo structure for the transporter. Dopamine was next redocked to the DAT to optimize the docking protocol, which was subsequently used to dock all other ligands analyzed in this study. The best redocked pose was ranked on top according to the docking score and was superposed with the experimental structure complex.[23] The associated root mean squared deviation was calculated between them by PYMOL[35] to estimate docking accuracy.

Binding free energy calculation

The best docking poses of all ligands with DAT, as judged by their corresponding docking scores, were taken as starting structures for molecular dynamic simulations.[36] Ligand partial atomic charges were calculated using the AM1-BCC method[37] with the ANTECHAMBER program of Amber 14.[38] Topology and coordinate files were generated in tleap for each DAT-ligand system, Amber FF14SB force field[38,39] was used to prepare proteins, and Amber GAFF2[38] was carried out for ligand preparations. To complete every complex system, it was first solvated with a transferable intermolecular potential with 3 points (TIP3P) water box[40,41] with a radius of 10.0 Å and neutralized by adding Cl^- or Na^+ ions. Finally, a 50 ns molecular dynamic simulation was run after minimization and equilibration steps for every complex system. After molecular dynamic simulations, the binding free energy was calculated by applying the Molecular Mechanics Poisson-Boltzmann Surface Area method.[42] Free energy decomposition was performed to calculate the energy contribution per residue for all residues in the system.

Validation from drosophila to human

To verify the validity of extrapolating the results obtained from the drosophila DAT crystal structure to the human DAT, multiple sequence alignments were performed. Sequences related to the binding pocket of DAT were identified by choosing residues within 4.5 Å of the DA ligand. Of all 10 residues within that distance of the ligand, only D121 and A117 are not conserved in human DAT. These amino acids are structurally close and are replaced by G and S, respectively, in the human isoform. To evaluate the effect of the mutations, D121G and A117S were inserted *in silico* into the drosophila crystal structure to mimic the human sequence. The DA docking pose was modeled and binding free energies were calculated as described above.

Protein targets of DA and acetaminophen

To identify protein targets for DA and acetaminophen, the molecules were queried in ChEMBL,[43] after which the protein targets were directly downloaded from the "compound target summary" within the compound report card of each molecule. The protein lists were then uploaded to Venny, and the list of overlapping proteins was retrieved. The overlapping proteins were furthermore compared with the SN-PD dataset by use of Venny.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Table S1.
Table S2.
Figure S1.

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