The molecular mechanism of Chinese medicine Uncaria Rhynchophylla (gouteng) for inducing autophagy and protecting neurons in Parkinson's disease

Leilei Chen

Hong Kong Baptist University

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The Molecular Mechanism of Chinese Medicine *Uncaria Rhynchophylla* (Gouteng) for Inducing Autophagy and Protecting Neurons in Parkinson's Disease

CHEN Lei-Lei

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Principal Supervisor: Prof. LI Min

Hong Kong Baptist University

August 2015
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: ____________________________

Date: August 2015
ABSTRACT

Parkinson’s disease (PD) is the second most common neurodegenerative disorder characterized by the accumulation of protein aggregates (namely Lewy bodies) in dopaminergic neurons in the substantia nigra region of the brain. Alpha-synuclein (α-syn) is the major component of Lewy bodies (LBs) in PD, and impairment of the autophagy-lysosomal pathway has been linked to its accumulation. In our previous study, we identified corynoxine B (Cory B), an oxindole alkaloid isolated from Uncaria rhynchophylla (Miq.) Jacks (Gouteng in Chinese), as a Beclin-1-dependent autophagy enhancer. In this work, we continued to screen autophagy enhancers from Gouteng alkaloids, and found corynoxine (Cory), an isomer of Cory B, also induces autophagy in different neuronal cell lines and primary neurons. Meanwhile, Cory promotes the formation of autophagosomes in the fat bodies of Drosophila. By inducing autophagy, Cory promotes the clearance of wild-type and A53T α-syn in inducible PC12 cells. Interestingly, different from its enantiomer Cory B, Cory induces autophagy through the Akt/mTOR pathway as evidenced by the reduced levels of phospho-TSC2, phospho-Akt, phospho-mTOR and phospho-p70 S6 Kinase.

To identify the different pathway between Cory and Cory B, we performed phosphoproteomic study on N2a cells. With the help of iGPS (In vivo Group-based Prediction System), protein kinases which were significantly regulated by Cory or
Cory B were predicted. Based on these kinases, we drew the detailed kinase-substrates network regulated by Cory or Cory B. The structures of Cory and Cory B differ only in the stereochemistry at the spiro carbon; however, Cory has more effect on the CAMK, Trb and TSSK families, while CDK and CDKL families are more sensitive to Cory B.

Furthermore, we established a rotenone rat model of PD via injecting rotenone into the substantia nigra pars compacta (SNC) and ventral tegmental area (VTA), and evaluated the neuroprotection of Cory and Cory B on this rat model. Motor dysfunction, decreased TH level, impairment of autophagy, aggregation of α-syn and activation of microglia were all found on this PD model, which were consistent with previous reports. After the treatment of Cory or Cory B, we found that both Cory and Cory B improve motor dysfunction, increase the TH level, and inhibit microglial activation. Both Cory and Cory B decrease the puncta number of aggregated α-syn, likely due to the induction of autophagy. All these results indicate the neuroprotection of Cory and Cory B against PD.

Collectively, our findings (1) provide the original finding of Coy to be an autophagy enhancer with experimental evidences that Cory inhibited the pathway of Akt/mTOR; (2) provide cellular and animal experimental evidences for developing Cory or Cory
B as anti-PD agent, by inducing autophagy in neurons; and (3) provide candidate pathways to identify the primary molecular target of Cory or Cory B, which may turn out to be potential therapeutic targets for treating PD.

**Keywords:** Parkinson’s disease, Cory, Cory B, autophagy, phosphoproteomic, neuroprotection
ACKNOWLEDGEMENTS

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<th>Full Name</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AD</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>ALP</td>
<td>autophagy-lysosomal pathway</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AR</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>α-syn</td>
<td>alpha-synuclein</td>
</tr>
<tr>
<td>CAMK</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>Cory</td>
<td>corynoxine</td>
</tr>
<tr>
<td>Cory B</td>
<td>corynoxine B</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EtOH</td>
<td>Ethyl alcohol</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxidase</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope coded affinity tags</td>
</tr>
<tr>
<td>iGPS</td>
<td>In vivo group-based prediction system</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Immunopurification</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantitation</td>
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<tr>
<td>LBs</td>
<td>Lewy bodies</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>lethal dose, 50%</td>
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<tr>
<td>L-DOPA</td>
<td>levodopa</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LRRK2</td>
<td>leucine-repeat rich kinase 2</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption / ionization time-of-flight</td>
</tr>
<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
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<td>pS</td>
<td>phosphoserine</td>
</tr>
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<td>pT</td>
<td>phosphothreonine</td>
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<td>PTMs</td>
<td>post-translational modifications</td>
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<td>pY</td>
<td>phosphotyrosine</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Rap</td>
<td>rapamycin</td>
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<tr>
<td>RIPA</td>
<td>radio immunoprecipitation assay</td>
</tr>
<tr>
<td>SCX</td>
<td>strong cation exchange chromatography</td>
</tr>
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<td>SILAC</td>
<td>stable isotope labeling by amino acids in cell culture</td>
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<td>siRNA</td>
<td>small inference RNA</td>
</tr>
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<td>SNc</td>
<td>substantia nigra pars compacta</td>
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<tr>
<td>ssKSRs</td>
<td>site-specific kinase-substrate relations</td>
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<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>titanium dioxide</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
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<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>matrix assisted laser desorption/ionization time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>ml</td>
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</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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</tr>
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<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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CHAPTER 1

INTRODUCTION
1.1 Parkinson’s disease

Parkinson’s disease (PD) is named after James Parkinson, who firstly described the clinical symptoms of the PD patients in 1817 [1]. It is the second most common neurodegenerative disease after Alzheimer’s disease, and affects 1 ~ 2% of the population over the age of 60 [2]. Parkinson’s disease affects the movement function of patients and causes motor symptoms, including lowness of movement, tremor on a rest, rigidity and postural instability [1]. Meanwhile, non-motor symptoms are another common feature of PD. These include sensory, sleep abnormalities, autonomic dysfunction and cognitive / neurobehavioral disorders [1]. The pathological characteristics of PD is the loss of dopaminergic neurons in the substantia nigra and the accumulation of Lewy bodies (LBs), whose major component is alpha-synuclein (α-syn), in the remaining neurons [3]. Researchers have worked hard on this disease for almost two centuries, however, the molecular mechanism of neuronal degeneration is still unclear in PD, although α-syn aggregation, impairment of ubiquitin proteasome system [4], mitochondria damage and oxidative stress [5, 6] are thought to contribute to the pathogenesis of PD.

1.2 Epidemiology of PD

The prevalence of PD is estimated at 0.3% of the entire population, about 1% in persons over 60 years old and rises to 1-3% among persons over 80 years old [2, 7].
PD is an age-specific disorder, and the onset of PD is rare before 50 years old. However, the incidence is sharply increased after age of 60 [2]. Some studies reported that the prevalence of PD in men is higher than that in women [8-11], which may be due to the neuroprotection of oestrogens [12]; however, others found that there is no significant difference between men and women [13-15]. A cross-sectional survey undertaken in China reported that there are about 1.7 million PD patients over 55 years old, which are more than 40% population of PD worldwide [16]. And a survey reported that the prevalence of PD in Hong Kong Chinese people is about 0.5% over 55 years old [17].

1.3 Pathology of PD

The pathology feature of PD is dopaminergic neuronal cell loss within the substantia nigra which particularly affecting the ventral component of the pars compacta. Compared with the unaffected individuals, PD patients’ neuronal region in the brain has lost up to 70% by the time of death [3]. Lewy bodies (LBs), intracellular inclusions which are mainly composed with alpha-synuclein, are the major pathological hallmark of PD. The pathological staging of PD is based on the distribution of LBs. In the early stages, the LBs are primarily affecting the medulla oblongata/pontine tegmentum and olfactory bulb. The PD patients in the early stages behave as pre-symptomatic. During the later stages, the LBs can distribute in
substantia nigra areas of the midbrain and basal forebrain. Finally, the pathological changes can reach the neocortex [3].

1.4 Etiology of PD

There are two types of PD: inherited and sporadic. Traditionally, PD is considered a non-genetic disorder; however, about 15% of PD patients have a first-degree relative who has this disease [18]. Meanwhile, mutations of one or several specific genes contributed to at least 15% of PD patients [19]. So far, at least 15 genes mutations linked to PD are identified, including SNCA, LRRK2, PARKIN, PINK1, DJ-1 and so on [20, 21]. For the sporadic PD, it is thought to result from interactions between genetic and environmental factors, including dysfunction of mitochondrial, aggregation of disordered protein, impairment of the autophagy lysosome pathway (ALP) and ubiquitin proteasome system (UPS) and oxidative stress [22].

1.4.1 Genetics of PD

Up to now, at least 15 gene mutations were identified as the genetic factors of PD, which are detailed listed in Table 1-1. The most extensively studied PD-related genes are reviewed as below.
AD: autosomal dominant; AR: autosomal recessive;

1.4.1.1 SNCA (PARK1/PARK4)

The SNCA gene contributes to an early onset and autosomal dominant inherited Parkinsonism, which usually progressed rapidly. It was first identified in an Italian PD family with the mutation of p.Ala53Thr (A53T) [23, 24]. And then two other mutations, p.Ala30Pro (A30P) [25] and p.Glu46Lys (E46K) [26], were identified.
subsequently. These three point mutations (A30P, A53T and E46K) generate age-dependent nigrostriatal deficiency and result in familial early onset PD [27, 28]. Meanwhile, studies reported that duplication or triplication of the SNCA gene caused a familial PD [29]. The SNCA gene encodes the protein α-synuclein (α-syn) with 144 amino acids, which is natively unfolded and soluble in the cytoplasm or associated with lipid membranes [30]. Over-expression of wild type or mutant α-syn in transgenic mice or Drosophila presented progressed neuronal loss and motor dysfunction [31-34].

1.4.1.2 LRRK2 (PARK8)

The LRRK2 gene mutation (PARK8) is the most common cause of familial PD, which is linked to 5-7% PD [35]. In 2002, the LRRK2 gene mutation was first identified as a cause to autosomal dominant Parkinsonism in a Japanese family [36]. And several mutations, including R114C, R1441G, R1441H, Y1699C, G2019S, and I2020T, have been identified from then [37]. The most frequent and extensively studied mutation is G2019S, which affects about 1% patients with sporadic PD and 4% patients with inherited PD. And the risk of PD for people with G2019S mutation was 28% at 59 years old, 51% at 69 years old and 74% at 79 years old [38]. The prevalence of PD with LRRK2 mutation between different ethnicity varies widely. And the frequencies of the G2019S mutation were 0.71% of white, 0.07% of Asian
and 30.25% of Arabic PD patients [39]. However, the mechanism leading to PD by LRRK2 mutations is still not clear. Although it is suggested that LRRK2 protein, a cytosolic protein contains a kinase and GTPase domain, played an important role in neurite growth, autophagy, vesicle trafficking and cytoskeleton [40-44], the substrates and function of this protein were uncertain.

1.4.1.3 PARKIN (PARK2)/ PINK1 (PARK6)/ DJ-1 (PARK7)

PARKIN, PINK and DJ-1 lead to autosomal recessive forms of PD, with an onset range from early adolescence to sixth decade. The clinical phenotype of these three genes related PD is indistinguishable. It is reported that PARKIN leads to 15.5% of familial and 4.3% of sporadic PD. PINK1 contributes to 8.4% of familial PD and 3.7% of a combined case of sporadic and familial PD. Meanwhile, 0.8% of familial and 0.4% of sporadic PD was cause by DJ-1 [45]. All these three genes are reported to be associated with the progress of mitochondrial regulation and quality control, which caused abnormal morphology and incompetent bioenergetics [46-52]. The roles of PARKIN and PINK1 have been well characterized in this progress. When mitochondrial membrane potential was reduced, PINK1 was activated and bound to the outer membranes of impaired mitochondria. PARKIN was recruited and designated the disposal of mitochondria by autophagy via ubiquitination [53-55]. Damage of this pathway caused the accumulation of bioenergetically incompetent
mitochondria, which may give risk to degeneration of substantia nigra in PD [56]. However, the mechanism of DJ-1 on the mitochondrial pathway is uncertain now [57].

1.4.2 Deficits of mitochondrial complex I in PD

Many publications reported that environmental factors, which include pesticide exposure, head injuries, and living in the country or farming, are linked to an increased risk of PD [58, 59]. Also, living in a rural environments and drinking well water may confer an increased risk of PD, which were thought to be an indirectly exposure to pesticide [2, 60].

For many years, studies carried by human postmortem analysis thought that oxidative stress played important roles in sporadic PD [61, 62]. Meanwhile, consistent findings of deficits in mitochondrial complex I, which cause neuronal vulnerable to the excitotoxicity of glutamate, have been reported [63-67]. Maternal descendants of inheritance of PD showed increased reactive oxygen species, impaired mitochondrial complex I activity and abnormal morphology of mitochondria, indicating that deficits in mitochondrial complex I play a critical role in PD [68]. Also, a non-conservative amino acid change, caused by single-nucleotide polymorphism, within the NADH dehydrogenase 3 of mitochondrial complex I, significantly reduced the risk of PD, which indicates the role of complex I activity in sporadic PD [69].
Epidemiologic study reported that complex I inhibitors are involved in the pathologic progress of PD. MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a potential complex I inhibitors, is found to become MPP⁺ (1-methyl-4-phenyl pyridium) via active metabolite and concentrated in dopaminergic neurons, and then caused the neuron loss [70]. Also, MPTP induced inclusion bodies in old monkey [71], and α-syn aggregation in the SNc in baboons [72]. Paraquat (1, 1’-dimethyl-4,4’-5 bipyridinium), a common herbicide whose structure is similar to MPP⁺, was reported to selectively cause degeneration of dopaminergic neurons and aggregation of α-syn [73, 74]. Rotenone, another inhibitor of complex I, leads to oxidative stress and selective dopaminergic neurons death although it was not concentrated in dopaminergic neurons [75]. Meanwhile, chronic exposure to rotenone caused Lewy body like protein inclusions containing α-syn [75-77]. All these evidences that complex I inhibitors induced cell death of dopaminergic neurons, Lewy body like inclusions containing α-syn and defect in complex I system, suggest that deficits of mitochondrial complex I may be the central factor in the pathologic progress of sporadic PD (Fig.1-1) [78].
1.4.3 Impairment of ubiquitin - proteasome system (UPS) and autophagy - lysosome system (ALP) in PD

Ubiquitin - proteasome system (UPS) and autophagy - lysosome pathway (ALP) are the most important routes to keep the normal balance between the formation and degradation of cellular proteins [79]. Deficits of either UPS or ALP were found to result in the accumulation and aggregations of disorders proteins, which caused cellular toxicity and neurodegenerative diseases [80, 81].

UPS is usually responsible for the degradation of short-lived protein under normal metabolic conditions, as well as the damaged or disordered protein in nucleus, cytosol or endoplasmic reticulum. The susceptible proteins are firstly targeted by ubiquitin,
and the unfolded ubiquitinated proteins which could pass through the narrow pore of
the proteasome barrel [82]. Inhibition of UPS was reported to cause
eurodegeneration couples with the formation of Lewy body-like inclusions [83].
Dysfunction of UPS were found to be related with accumulation of disordered
proteins in PD [4, 84-87], which consistent with the genetic studies of PD causing
genes, such as SNCA, UCH-L1 and Parkin [88-90]. Also, decreased ubiquitin-ligase
enzymatic activity was found in the PD related to Parkin mutations [91, 92]. All these
findings suggested that failure of the UPS contributed to the neurodegeneration of PD
[82].

Based on the ways of substrates reach the lysosome, ALP are divided into three types:
macroautophagy (generally called autophagy), microautophagy and chaperone
mediated autophagy (CMA) (Fig.1-2) [82]. In contrast to UPS, ALP is the primary
way for the degradation of long-lived proteins and the only way to clear entire
organelles including impaired mitochondria. Large proteins such as oligomers and
aggregations that could not pass through the proteasome barrel were degraded by
autophagy [93-95]. Increased and aggregated mutation or wide-type α-syn was
implicated to be one of the causes of PD [78]. Impaired autophagy were found in the
brain of PD patients and PD animal models [96]. Meanwhile, researchers found α-syn
could be degraded by both ALP and UPS [97], and thought that impaired ALP is an
important mechanism of PD [98]. Furthermore, it is reported that wild type α-syn was
selectively translocated into lysosome and degraded by the CMA pathway, and the binding of the receptors by mutant α-syn blocked the lysosomal uptake and resulted in part of the accumulation of α-syn [98]. In a progressive mouse model of PD, α-syn aggregation was found to be caused by lysosomal malfunction [99]; and mutations in ATP13A2, which encodes a lysosomal type 5 P-type ATPase, resulted in autophagy impairment and aggregation of α-syn [100, 101]. All these evidences indicate the important role and involvement of ALP in the pathologic progress of PD.

Fig. 1-2 Autophagy-lysosome pathway (ALP) in mammalian cells [82].

1.5 Treatment of PD

Although there is no cure for Parkinson's disease, medications, surgery and multidisciplinary management can relieve the symptoms. The main aim of drug
treatment in PD is: (1) increase the level of dopamine that reaches and simulates the parts of the brain where dopamine works; (2) block the action of other chemicals that affect dopamine, such as acetylcholine.

The widely used western medicines include: (1) levodopa; (2) dopamine agonists; (3) anticholinergics; (4) MAO-B (Mono Amine Oxidase Type) inhibitors; (5) COMT (Catechol-O-Methyl Transferase) inhibitors and (6) glutamate antagonist [102, 103].

The most commonly used and most effective drug for controlling the symptom of PD is levodopa (also called L-dopa), which could be transported to the neuronal cells and converted into dopamine for the neuronal cells to use as a neurotransmitter. Levodopa is usually used combined with carbidopa or benserazide, which could increase the effectiveness and prevent or lessen the side effects of levodopa [104, 105].

Traditional Chinese medicines (TCM) have also been subjected to the treatment of PD for a long time. One of the good examples is Uncaria rhynchophylla (Miq.) Jacks., which is reported to have an effective anxiolytic agent and acts via the serotonergic nervous system [106]. Also, alkaloids extracted from Uncaria rhynchophylla (Miq.) Jacks., such as corynoxine B, rhynchophylline and isorhynchophylline, are proved to be neuroprotective with the evidences of promoting the clearance of α-syn via inducing autophagy [107, 108], reducing oxidative stress and cellular apoptosis [109],
preventing NMDA, muscarinic M1, and 5-HT2 receptors-mediated neurotoxicity during ischemia [110]. Meanwhile, many other active ingredients from Chinese herbal, such as Bak Foong Pills [111], Salvianic acid A [112], Ginkgo biloba [113], tripchlorolide [114], and Radix Polygoni Multiflori [115], are reported to have significant neuroprotection. Based on the molecular biological methods and animal models, it is more and more possible to develop the Chinese herbal medicine, especially the active components to potential therapeutic agents for PD.
CHAPTER 2

HYPOTHESIS AND OBJECTIVES
Parkinson’s disease (PD) is the second most common neurodegenerative disorder, which is hardly incurable. The available medications, surgery and multidisciplinary management for PD treatment mainly focus on relieving symptoms via increasing the level of dopamine or blocking the action of other chemicals that affect dopamine. However, these drugs such as levodopa, a most effective drug to control the symptoms of PD, usually lose efficacy and cause side effect, which result in the reduction of life quality. Therefore, it becomes an urgent need to develop new effective drugs, which directly target at the factors contributed to the PD pathogenesis.

PD is characterized by the accumulation of protein aggregates (namely Lewy bodies) in dopaminergic neurons in the substantia nigra region of the brain, whose major component is alpha-synuclein (α-syn). Genetic studies have found that duplication or tripling of the α-syn gene causes PD [29]. Point mutations (A30P, A53T and E46K) of α-syn generate age-dependent nigrostriatal deficiency and result in familial early onset PD [27, 28]. Overexpression of wild type and mutant α-syn in transgenic mice or Drosophila are correlated with neuronal dysfunction and abnormal internal organ pathology [31-34]. These findings suggest that promoting the clearance of aggregated α-syn may be an effective therapeutic strategy for treating PD.
The ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) are the most important routes for degrading aggregated/misfolded proteins, including α-syn. Recently, defects in the autophagy pathway have been observed in the brains of PD patients and of PD animal models [96]. Meanwhile, researchers found that clearance of aggregated α-syn depends more on autophagy than on the proteasome [116]. All these evidence indicate that autophagy plays an important role in PD and thereby suggests that autophagy inducers are potential drugs for treating PD.

Protein phosphorylation is one of the most important posttranslational modifications in living cells. It is involved in many regulatory functions such as cell cycle control, receptor-mediated signal transduction, differentiation, proliferation, transformation, and metabolism. Phosphorylation events are commonly probed in a targeted manner by phosphorylation-specific antibodies. In contrast, advances in proteomics technology, including quantitative labeling methods, phosphopeptide enrichment, high-accuracy mass spectrometry and associated bioinformatics now make it possible to analyze entire phosphoproteomes [117].

In our previous study, we identified an oxindole alkaloid corynoxine B (Cory B), isolated from Uncaria rhynchophylla (Miq.) Jacks (Gouteng in Chinese), as a Beclin-1-dependent autophagy enhancer. In this work, we continued to screen
autophagy enhancers from Gouteng alkaloid and study the molecular mechanism:

(1) Provide experimental evidence for developing Cory as a new autophagy enhancer from Chinese herbal medicine;

(2) Compare the network regulated by Cory or Cory B via phosphoproteomic study, and discover some novel targets involved in signaling transduction of autophagy induced by Cory and Cory B, which may turn out to be potent therapeutic targets for curing PD;

(3) Evaluate the neuroprotection of Cory and Cory B in a rotenone rat model of PD.
CHAPTER 3

Corynoxine, a natural autophagy enhancer, promotes the clearance of alpha-synuclein via Akt/mTOR pathway
3.1 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder in the world, affecting 1 ~ 2% of the population over the age of 60 [2]. It is characterized by accumulation of Lewy bodies (LBs), whose major component is alpha-synuclein (α-syn), in the substantia nigra dopaminergic neurons. Genetic studies have found that duplication or triplication of the α-syn gene causes PD [29]. Point mutations (A30P, A53T and E46K) of α-syn generate age-dependent nigrostriatal deficiency and result in familial early onset PD [27, 28]. Overexpression of wild type and mutant α-syn in transgenic mice or Drosophila are correlated with neuronal dysfunction and abnormal internal organ pathology [31-34]. These findings suggest that promoting the clearance of aggregated α-syn may be an effective therapeutic strategy for treating PD.

The ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP) are the most important routes for degrading aggregated/misfolded proteins, including α-syn. Recently, defects in the autophagy pathway have been observed in the brains of PD patients and of PD animal models [96]. Meanwhile, researchers found that clearance of aggregated α-syn depends more on autophagy than on the proteasome [116]. All these evidence indicate that autophagy plays an important role in PD and thereby suggests that autophagy inducers are potential drugs for treating PD.
In our previous study, we identified an oxindole alkaloid namely Corynoxine B (Cory B) from *Uncaria rhynchophylla* (Miq.) Jacks (Gouteng in Chinese), an herb which is routinely used in traditional Chinese medicine formulas for the treatment of symptoms relevant to PD [107] and which is also an important component of a traditional Asian herbal medicine for neurodegenerative diseases treatment called Yoku-Kan-San [118]. Cory B, as an autophagy inducer, promotes the clearance of both wild type and mutant (A30P and A53T) α-syn [107].

In this chapter, we continued to screen for new autophagy enhancers from Gouteng alkaloids and try to identify the molecular mechnism.

### 3.2 Research Materials and Methodology

#### 3.2.1 Reagents and antibodies

Corynoxine (C-13002) was purchased from Shanghai R & D Center for Standardization of Chinese Medicine. 3-MA (M9281), CQ (C6628) Rapamycin (R0395), Ara-c (C6645), DNase (D4263-5VL) and L-glutamic acid (RES5063G-A701X) were purchased from Sigma-Aldrich. SiRNA (siAtg5 and Non-target siRNA) were purchased from Cell Signaling Technology (L-064838). Goat anti-mouse (626520) and goat anti-rabbit (G21234) secondary antibodies were purchased from Invitrogen. Anti-β-actin (sc-47778) primary antibody was purchased
from Santa Cruz Biotechnology. Anti-phospho-Akt (ser473) (#9271), anti-Akt (#9272), anti-LC3 (2775), anti-phospho-mTOR (Ser2448) (#2971), anti-mTOR (#2983), anti-phospho-p70S6K (Thr389) (#9234), anti-p70S6K (#9202), anti-phospho-TSC2 (Ser939, Ser1387, Ser1462) anti-TSC2 (#8350) and anti-4E-BP1 (#9955) antibodies were purchased from Cell Signaling Technology. Anti-α-syn antibody (#610786) was purchased from BD Transduction Laboratories. Anti-cathepsin D (ab75852) antibody was purchased from Abcam. Instant Drosophila food (#173212) was purchased from Carolina Biological Supply Company.

3.2.2 Cell lines and cell culture

N2a and SH-SY5Y cells were maintained in DMEM (Invitrogen, 12800017), supplemented with 10% FBS (Invitrogen, 10099141). Inducible PC12 cells were grown in DMEM, supplemented with 10% FBS and 5% horse serum (Invitrogen, 16050122). N2a cells constitutively expressing GFP-LC3 were selected using 800 µg/ml G418 (Invitrogen, 10131027) and maintained in 200 µg/ml G418.

3.2.3 Primary neuron culture

E17 pregnant mice were injected with hydrochloride (100 mg/kg, i.p.) and embryos were transferred to a new dish with cold PBS. In the cold PBS, cortex was isolated and the meninges were gently removed under the dissecting microscope. Then tissues were transferred to a 15 ml tube containing cold PBS and washed 3 times with cold PBS,
and then added 5 ml digestion solution (2.5 ml trypsin (2.5%) plus 2.5 ml DMEM/F12 medium). The tissues were incubated at 37°C for 15-20 minutes, and then spun at 1000 rpm for 3 minutes. The digestion solution was removed. After washing the tissues with DMEM/F12 medium with 10% FBS for 3 times, we added 50 µl DNase (0.5 mg/ml) in 10 ml DMEM/F12 medium with 10% FBS, and continue to incubate the tissues at 37°C for 5 minutes. Then tissues were pipetted up and down to homogenize the cells, and spun at 1000 rpm for 3 minutes. The supernatant was discarded and the cells were suspended with neurobasal medium (Invitrogen, 21103-049) containing B27 supplement (Invitrogen, 17504-044) and glutamate (25 µM). After passing a 70-µm filter (BD, USA), cells were counted under the microscope and seeded in poly-D-lysine coated 6-well plates (2*10^6 cells/well). After 24 hours, medium was changed to neurobasal medium containing B27 and 2 mM L-glutamine. On day 3, half medium was changed to neurobasal medium containing B27, 2 mM L-glutamine and 10 µM Ara-c (Sigma, USA). Then cells were re-fed every 3 days by removing half and replacing fresh medium and maintained for at least 7 days to differentiation and maturation.

3.2.4 Drosophila culture and drug feeding

Cg-GAL4 fly lines and UAS-GFP-Atg8a fly lines were raised at 25°C on standard corn meal medium supplemented with dry yeast. Drugs were initially dissolved in
DMSO then diluted in water to desired concentrations. The drug-containing water was added to instant *Drosophila* food and mixed thoroughly. As the control, the same amount of DMSO was also mixed with instant *Drosophila* food. For the treatment, Cg-GAL4 fly lines were crossed to UAS-GFP-Atg8a fly lines at 25°C, and then transferred the 2\textsuperscript{nd} instar larvae of Cg-GAL4> UAS-GFP-Atg8a onto instant food containing different concentrations of Cory (10-100 µM), 100 µM Cory B (positive control) or 0.1% DMSO (negative control) for 12 hours at 25°C. Treated larvae were collected and fat bodies were dissected out in PBS, and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. Tissues were mounted with FluorSave™ Reagent (Merck) and imaged with a confocal microscopy.

### 3.2.5 RNA interference assay

To avoid the interaction between Cory and 3-MA or CQ, we silenced Atg5 to genetically inhibit autophagy. With the help of Lipofectamine 2000, Atg5 and Non-target siRNA (Cell Signaling Technology) were induced into N2a cells following the protocol described previously [119] with minor revision. Briefly, N2a cells were seeded into a 12-well-plate (8*10^5 cells/well). 24 hours later, 1 µg siRNA (50 µM) and 2 µl Lipofectamine 2000 were diluted with 100 µl Opti-MEMI Reduced Serum Medium separately. 5 minutes later, diluted siRNA was mixed with the diluted Lipofectamine 2000 gently and incubated for 20 minutes at the room temperature to
form complex. At last, 200 µl siRNA-Lipofectamine 2000 complex was added into each well containing cell. N2a cells were incubated at 37°C in a CO₂ incubator for 24 hours and then the cells were sub-cultured into 6-well-plate at the ratio of 1:4. 24 hours later, cells were subjected to further treatment and assay.

### 3.2.6 Transfection of plasmids into N2a cells

For transfection experiment, N2a cells were seeded in to 24-well plate at a density of 2*10^5 cells/well. 24 hours later, mRFP-GFP-LC3 plasmids were transfected into N2a cells using Lipofectamine 2000 according to the protocol described previously [119]. Briefly, medium was changed to Opti-MEMI (200 µl/well) before assay. 2 µg DNA was diluted with 100 µl Opti-MEMI and mixed gently. Then 2 µl PLUS™ Reagent was added to the diluted DNA and incubated for 5 minutes at room temperature. 5 µl Lipofectamine LTX was added into the diluted DNA and incubated for 30 minutes at room temperature. At last, 100 µl DNA-Lipofectamine LTX complex was added into each well containing cell. N2a cells were incubated at 37°C in a CO₂ incubator for 6 hours and then changed fresh medium. 48 hours after transfection, cells were subjected to treatment and assay.

### 3.2.7 Immunostaining

After treatment, N2a stable cells were fixed with 4% paraformaldehyde in PBS for 10
min at room temperature. Cells were mounted with FluoSave reagent (Calbiochem, 345789) and imaged with a confocal microscope.

### 3.2.8 Western blotting analysis

Cells were lysed with RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.35% sodium deoxycholate, 1 mM EDTA, 1% NP40, 1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml leupeptin). The boiled samples (each containing 10–20 µg of protein) were subjected to SDS-PAGE on a 10-15% acrylamide gel and transferred to PVDF membranes (GE Healthcare, RPN303F). The membranes were blocked for 1 h in TBST containing 5% non-fat milk, and then probed with the appropriate primary and secondary antibodies. The desired bands were visualized using the ECL kit (Pierce, 32106). The band density was quantified using the ImageJ program and normalized to that of the control group.

### 3.2.9 Statistical analysis

Statistical significance was assessed by using one-way ANOVA with the Newman-Keuls multiple comparison tests. Calculations were performed using ImageJ and Prism (version 5) software. Statistical significance was considered when P<0.05.
3.3 Results

3.3.1 Screen for potential autophagy enhancers from six Gouteng alkaloids

In our previous study, we identified corynoxine B as an autophagy enhancer from *Uncaria rhynchophylla* (Miq.) Jacks (Gouteng) (Fig. 3-1). Then we continued to screen for potential autophagy enhancers from other 5 Gouteng alkaloids, including corynoxine, isorhynchophylline, rhynchophylline, corynoxeine and isocorynoxeine (structures of all the 6 alkaloids were shown in Fig. 3-2). We treated N2a cells with different concentration of Gouteng alkaloids or 0.1% (v/v) DMSO for 9 hours, and found only corynoxine (Cory) and corynoxine B (Cory B) could increase the expression of LC3II, which is an autophagy specific marker (Fig. 3-3).

![Fig. 3-1 Pictures of Uncaria rhynchophylla (Miq) Jacks (Gouteng).](image)

(The pictures are provided by Dr. Chen HB, SCM, HKBU)
Fig. 3-2 Structures of 6 alkaloids extracted from Gouteng.

Fig. 3-3 Screen for potential autophagy enhancers from 6 Gouteng alkaloids. N2a cells were treated with different concentration of Gouteng alkaloids (including corynoxine, corynoxine B, isorhynchophylline, rhynchophylline, corynoxeine and isocorynoxeine), 0.2 μM rapamycin or 0.1% (v/v) DMSO for or 0.1% (v/v) DMSO for 9 hours, and then cells lysates were subjected to Western blotting analysis.
3.3.2 Cory induces autophagy in neuronal cell lines and primary neurons.

In this study, we identified Cory (PubChem CID: 10475115; the chemical structure is shown in Fig. 3-2,) as a new autophagy inducer. To determine the non-toxic dosages, we detected the relative LDH release from N2a cells treated with different concentrations of Cory for 24 hours, and then chose 25 µM for further study (Fig.3-4). Cory increased the expression of LC3II, an autophagy specific marker, in N2a, SH-SY5Y cells in a dose-dependent manner (Fig. 3-5). Also, we isolated mice cortical primary neurons from E17 ICR embryos (Fig. 3-6). After 7 days’ culture, primary neurons were treated with different concentrations of Cory. Increased LC3II was observed by western blotting (Fig. 3-7).

To confirm the enhancement of autophagy by Cory, we treated N2a cells with Cory, Cory plus 3-MA (3-methyladenine, an autophagy inhibitor) or CQ (chloroquine, a lysosomal inhibitor). Cory increased the LC3II levels, which were blocked by 3-MA and enhanced by CQ (Fig. 3-8). The results were consistent with the immunostaining assays (Fig. 3-9).

To avoid the interaction between Cory and 3-MA or CQ, we knocked down Atg5 to genetically inhibit autophagy and then tested the effects of Cory. Non-target siRNA were treated at the same time. As shown in Fig. 3-10, in Atg5 knock-down N2a cells,
Cory failed to induce autophagy.

To confirm whether Cory affect the autophagosome maturation, we employ the tfLC3 construct system which was established by Kimura et al. [120]. After transfected with mRFP-GFP-LC3 plasmid, N2a cells were treated with Cory (25 µM) with or without CQ (30 µM) for 24 hours. When cells were treated with CQ alone, the numbers of GFP and mRFP puncta are similar. However, the number of GFP puncta induced by Cory was less than that of mRFP, indicating the completion of autophagosome maturation and lysosomal degradation. And with the co-treatment of Cory and CQ, the number of GFP and mRFP puncta was also similar (Fig. 3-11). All these indicate that Cory induce autophagy in N2a cells rather inhibits lysosomal degradation.

Cathepsin D, a major lysosomal protease, is often up regulated in parallel with autophagy [121]. In this study, increased cathepsin D was also found in N2a cells (Fig. 3-12) and SH-SY5Y cells (Fig. 3-13) after treatment with Cory. However, this effect was abolished by lysosome inhibitor CQ. Meanwhile, increased LC3II induced by Cory was also enhanced in the presence of CQ (Fig. 3-13).
Fig. 3-4 The relative value of LDH after treated with Cory. N2a cells were treated with different concentrations of Cory or 0.1% (v/v) DMSO for 24 hours, and then the supernatant were collected and analyze by LDH kit. Data were presented as mean ± SEM of three independent experiments. (*p<0.05, compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).

Fig. 3-5 Cory induces autophagy in different neuronal cells. N2a (A) and
SH-SY5Y (C) cells were treated with different concentrations of Cory or 0.1% (v/v) DMSO for 6 hours or 12 hours, and then cells lysates were subjected to Western blotting analysis. Quantifications of LC3II/β-actin ratio of N2a (B) and SH-SY5Y (D) cells were shown as below. Data were presented as mean ± SEM of three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001 compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).

Fig. 3-6 Different day’s primary neurons isolated from mice cortex. E17 ICR embryonic cortical neurons were isolated and maintained in neurobasal medium 1-9 days, and observed under microscope (scale bar is shown in the picture).
Fig. 3-7 Cory induces autophagy in primary neurons. (A) Mice cortical primary neurons were isolated and maintained in neurobasal medium for 7 days, and then treated with different concentrations of Cory for 12 hours. Cells lysates were subjected to Western blotting analysis. (B) Quantifications of LC3II/β-actin ratio. Data were presented as mean ± SEM of three independent experiments. (***p<0.001 compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-8 Cory induces autophagy in N2a cells. N2a cells were pre-treated with 5 mM 3-MA or 30 µM CQ for 1 hour, and then treated with 25 µM Cory or 0.1% (v/v) DMSO for 6 hours. Cell lysates were subjected to Western blotting analysis. Quantifications of LC3II/β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (**p < 0.001 compared with Ctrl; ###p < 0.001, CQ vs. CQ+Cory; one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-9 Cory increases the formation of GFP-LC3 puncta. N2a-GFP-LC3 stable cells were pre-treated 5 mM 3-MA or 30 µM CQ for 1 hour, and then treated with 25 µM Cory or 0.1% (v/v) DMSO for 6 hours. Cells were fixed in 4% paraformaldehyde (PFA) and the formation of GFP-LC3 puncta was observed under a confocal microscope.
Fig. 3-10 Cory could not induce autophagy after silencing Atg5. N2a cells were pre-treated with siAtg5 or Non-target siRNA for 48 hours, and then treated with Cory (25 µM) for another 6 hours. Cell lysates were subjected to Western blotting analysis. Quantifications of Atg5/β-actin and LC3II /β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (***p<0.001 compared with Ctrl; ##p<0.01, NT vs. NT+Cory; one-way ANOVA with Newman-Keuls multiple test).
**Fig. 3-11 Cory doesn’t affect autophagosome maturation in N2a cells.** N2a cells were transfected with mRFP-GFP-LC3 plasmid and treated with Cory (25 µM) with or without CQ (30 µM) for 24 hours. Cells were fixed with 4% PFA and the formation of GFP/RFP-LC3 puncta was observed under a confocal microscope.
Fig. 3-12 Cory increases the expression of Cathepsin D in N2a cells. N2a cells were treated with 25 µM Cory, 30 µM CQ or 0.1% (v/v) DMSO for 12 hours. Cells lysates were subjected to Western blotting analysis. Quantifications of cathepsin D/β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (***p<0.001 compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).
Cory increases the expression of Cathepsin D and LC3II in SH-SY5Y cells. SH-SY5Y cells were treated with different concentrations of Cory, 30 µM CQ or 0.1%(v/v) DMSO for 12 hours. Cells lysates were subjected to Western blotting analysis. Quantifications of cathepsin D/β-actin and LC3II/β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001 compared with Ctrl; ***p<0.001,CQ vs. CQ+Cory; one-way ANOVA with Newman-Keuls multiple test).
3.3.3 Cory induces autophagy in *Drosophila*.

To confirm the autophagy-inducing effect of Cory *in vivo*, we firstly crossed Cg-GAL4 fly lines to UAS-GFP-Atg8a fly lines at 25°C, and got the 2\textsuperscript{nd} instar larvae of Cg-GAL4\textgreater{} UAS-GFP-Atg8a. We fed these larvae with food containing different concentrations of Cory (10-100 µM) for 12 hours at 25°C. In our previous study, Cory B was confirmed to induce autophagy in *Drosophila*; therefore we chose it as a positive control and using 0.4% DMSO as a negative control. We collected the treated larvae and prepared the fat bodies for photographing through a confocal microscope. Atg8 (Autophagy-related protein), the homolog of LC3, is an ubiquitin-like protein required for the formation of autophagosomal membranes. Compared with the control, both Cory (10-100 µM) and Cory B (100 µM) induced the puncta formation of Atg8 (Fig. 3-14), suggesting that Cory could also induce autophagy in the fat bodies of *Drosophila* larvae.
Fig. 3-14 Cory induces autophagy in Cg-GAL4>UAS-GFP-Atg8a *Drosophila* larvae fat body. Cg-GAL4 fly lines were crossed to UAS-GFP-Atg8a fly lines at 25°C, and then transferred the 2nd instar larvae of Cg-GAL4>UAS-GFP-Atg8a onto instant food containing different concentrations of Cory, 100 µM Cory B or 0.4% DMSO for 12 hours at 25°C. Collected the treated larvae and dissected out the fat body in PBS, and then fixed with 4% paraformaldehyde for 10 min at room temperature. Rinsed the tissues and mounted with FluorSave™ Reagent (Merck) and imaged with a confocal microscopy. Quantifications of autophagosome-positive spots in each group were showed in the last picture. Data were presented as mean ± SEM of three independent experiments. (***p<0.001 compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).
3.3.4 Cory promotes the degradation of wild type (WT) and mutant (A53T) α-syn via autophagy

P62/SQSTM1, an ubiquitin-binding protein, colocalizes with ubiquitinated protein aggregates in many neurodegenerative diseases. It is reported that p62 binds to LC3 and is degraded by autophagy. In this study, after the treatment of Cory (25 µM), decreased p62 was observed by western blotting, which was blocked by 3-MA (Fig. 3-15).

After treatment with Dox (doxycyline, 1 µg/ml) for 24h to induce the expression of wild type (WT) and mutant (A53T) α-syn, inducible PC12 cells [122] were treated with Cory (25 µM), 3-MA (5 mM) or CQ (20 µM) for another 48h. Western blotting results revealed significant degradation of both wild type (WT) (Fig. 3-16) and mutant (A53T) α-syn (Fig. 3-17) after the treatment with Cory. However, this effect was blocked by the autophagy inhibitor 3-MA and the lysosomal inhibitor CQ. These findings suggest that Cory promotes the clearance of wild type and mutant α-syn via autophagy.
**Fig. 3-15 Cory decreases p62 level in N2a cells.** N2a cells were treated with 25 µM Cory, 5 mM 3-MA, 0.2 µM Rapamycin or 0.1% (v/v) DMSO for 12 hours. Cell lysates were subjected to western blotting analysis. Quantifications of p62/β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001, compared with Ctrl, one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-16 Cory promotes the degradation of wild type (WT) α-syn via autophagy induction. Inducible PC12/WT-α-syn cells were treated with 1 µg/ml doxycycline (Dox) for 24 hours to induce the expression of wild type α-syn, and then treated 25 µM Cory, 20 µM CQ, 5 mM 3-MA or 0.1% (v/v) DMSO for another 48 hours. Cell lysates were subjected to western blotting analysis. Quantifications of α-syn (WT)/β-actin ratio were described as followed. Data were presented as mean ± SEM of three independent experiments. (###p < 0.001, Dox vs. Ctrl; ***p < 0.001, Dox+Cory vs. Dox; $p<0.05, $p<0.05, Dox+3-MA vs. Dox+3-MA+Cory; one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-17 Cory promotes the degradation of mutant (A53T) α-syn via autophagy induction. Inducible PC12/A53T-α-syn cells were treated with 1 µg/ml doxycycline (Dox) for 24 hours to induce the expression of wild type or mutant α-syn, and then treated 25 µM Cory, 20 µM CQ, 5 mM 3-MA or 0.1% (v/v) DMSO for another 48 hours. Cell lysates were subjected to western blotting analysis. Quantifications of α-syn (A53T)/β-actin ratio were separately described on the right hand. Data were presented as mean ± SEM of three independent experiments. (###p<0.001, Dox vs. Ctrl; **p<0.01, Dox+Cory vs. Dox; $p<0.05$, Dox+3-MA vs. Dox+3-MA+Cory; one-way ANOVA with Newman-Keuls multiple test).
3.3.5 Cory induces autophagy through Akt/mTOR pathway

mTOR, the mammalian target of rapamycin, is a classical autophagy regulator [123]. In order to determine by which pathway Cory induces autophagy, we firstly detected the phosphorylation of mTOR at Ser2481 and Ser2448. We found decreased phospho-mTOR (Ser2448) (Fig. 3-19C, D), which is a key participant in autophagy. Decreased p-TSC2 (Ser939) (Fig. 3-18), phospho-Akt (Ser473) and phospho-p70S6K (Thr389) were also found (Fig. 3-19A, B, E and F). Since these proteins act as either upstream or downstream of mTOR, our finding indicates that the Akt/mTOR pathway is involved in Cory-induced autophagy. However, Cory has no effect on 4E-BP 1 (Fig. 3-20), which is another downstream protein of mTOR.
**Fig. 3-18 Decreased p-TSC2 (Ser939) induced by Cory.** N2a cells were treated with 25 μM Cory or 0.1% (v/v) DMSO for 6 hours, and then cells lysates were subjected to analyze phospho and total TSC2 by western blotting. Quantifications of p-TSC2/t-TSC2 ratios were separately described. Data were presented as mean ± SEM of three independent experiments. (***p<0.001 compared with ctrl; one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-19 Cory induces autophagy through Akt/mTOR pathway. N2a cells were treated with 25 µM Cory or 0.1% (v/v) DMSO for different time, and then cells lysates were subjected to analyze phospho and total Akt (A), mTOR (C), p70 S6 Kinase (E) by western blotting. Quantifications of p-Akt/t-Akt (B) p-mTOR/t-mTOR (D) and p-p70S6K/t-p70S6K (F) ratios were separately described on the right hand. Data were presented as mean ± SEM of three independent experiments. (**p < 0.01, ***p < 0.001 compared with ctrl; one-way ANOVA with Newman-Keuls multiple test).
Fig. 3-20 Cory has no effect on 4E-BP1. N2a cells were treated with 25 µM Cory or 0.1% (v/v) DMSO for 6 hours, and then cells lysates were subjected to analyze phospho and total TSC2 by western blotting.
3.4 Discussion

Autophagy is a process by which a cell itself degrades disordered proteins or dysfunctional organelles to maintain basal homeostasis. Defective autophagy is thought to account for neurodegenerative diseases, including Parkinson’s disease, Huntington’s disease and Alzheimer’s disease [96, 124-127]. Recently, more and more publications reported that autophagy enhancers, such as rapamycin, could promote the clearance of abnormal protein and prevent neuron death/loss in neurodegenerative diseases [128-133]. Thus, inducing autophagy seems like an appropriate method if not ideal therapeutic approach.

However, autophagy induction is more difficult in neuronal cells than in non-neuronal cells[134]. In our previous study, we identified Cory B from *Uncaria rhynchophylia* (Miq.) Jacks (Gouteng in Chinese), a Chinese herb, which is reported to have neuroprotective properties [135] and which is routinely used in traditional Chinese medicine formulas for the treatment of symptoms relevant to PD. Furthermore, it appears to be a good autophagy inducer in both neuronal cell lines and primary neurons [107]. Meanwhile, in previous clinical trials, herbal preparation containing Gouteng has been shown to relieve PD symptoms [136]. So, we continued to screen for autophagy inducers from five isomers of Cory B, namely Cory, corynoxeine, isocorynoxeine, rynchophylline and isorynchophylline (Fig. 3-2), which are all
extracted from Gouteng, and found only Cory and Cory B could induce autophagy in N2a cells (Fig. 3-3). Although there is no report about the safety and toxicity of Cory, before all experiment, we performed LDH assay (Fig. 3-4) and got the safe dose for further study. Our study showed that Cory could also induce autophagy in different neuronal cell lines, including N2a and SH-SY5Y, and primary neurons (Fig. 3-5----3-13). Also, Cory doesn’t affect the autophagy maturation (Fig. 3-11).

Autophagy was firstly identified in yeast as a response to starvation, and many components of the autophagy pathway have been identified through genetics and biochemical studies. However, some essential components of the yeast pathway are missing in higher organisms. Thus, the autophagy pathway in more complex systems demands further study. Because of its easy culture, short generation time and rapid mutation, Drosophila is well-suited for genetic analysis. With respect to autophagy, it represents an intermediate between yeast and mammals. As the major nutrient storage organ, the fat body of larvae is particularly sensitive and readily responds to autophagy induced by starvation [137]. The Cg-GAL4>UAS-GFP-Atg8a fly lines are obtained by crossing Cg-GAL4 fly lines with UAS-GFP-Atg8a fly lines. As the homolog of LC3, Atg8 aggregates in puncta of the fat body of the 2nd instar after the treatment of Cory, as well as Cory B (Fig. 3-14). Our data suggested that Cory could induce autophagy in vivo.
Alpha-syn is a small soluble protein expressed primarily at presynaptic terminals in the central nervous system. Strong evidence has demonstrated that aggregation of α-syn contributes to dopaminergic cell loss and PD [138, 139]. Promoting the clearance of α-syn is thought to be a therapeutic method for treating PD [140]. Our data showed that Cory significantly degraded both wild type and mutant α-syn and its effect were abolished by autophagy inhibitor and lysosomal inhibitor (Fig. 3-15---3-17). These results indicate that Cory promotes the clearance of wild type and mutant α-syn through autophagy.

Cory and Cory B, which two substituents in the D-ring have a cis-relationship, and the compounds differ only in the stereochemistry at the spiro carbon. In our previous study, we proved that Cory B induces autophagy in a Beclin-1 dependent manner. Although Cory is the isomer of Cory B, we identified Cory induced autophagy via the Akt/mTOR pathway (Fig. 3-18---Fig. 3-19). As decreased phospho-mTOR occurred about 5 hours later than phospho-p70S6K (Fig. 3-18), which is the downstream of mTOR, the pathway involved in the Cory-induced autophagy may be multi-regulated.

Rapamycin, a lipophilic macrolide, was the first mTOR inhibitor discovered; thereafter, rapamycin analogs (rapalogs), including sirolimus, temsirolimus, everolimus and deforolimus, were named as the first generation of mTOR inhibitors.
The second generation is known as ATP-competitive mTOR kinase inhibitors, such as quinostatin, furoquinoline, PI-103, NVP-BEZ235, and GSK2126458 and so on [141-145]. Meanwhile, small molecule enhancers of rapamycin (SMER), such as SMER10 and SMER 28, which are quinazoline derivatives, were found to induce autophagy and promote the clearance of disordered protein [146]. However, there is no report about oxindole derivatives as mTOR inhibitors. Hence, our study about Cory, an oxindole alkaloid, constitutes an original finding. In summary, our study provides experimental evidence for developing Cory as a new autophagy enhancer from Chinese herbal medicine, which may have potential application in the prevention or treatment of PD.
CHAPTER 4

Phosphoproteomic study of corynoxine and corynoxine B

on neuronal cell culture
4.1 Introduction

The pathological characteristic of Parkinson’s disease is the abnormal accumulation of alpha-synuclein, which forms inclusions called Lewy bodies [3] in neurons, as well as cell death of dopaminergic neurons in substantia nigra. Mutations in alpha-synuclein, a major protein in Lewy bodies, are the remote causes of the early-onset PD. Thus to enhance the clearance of accumulated α-syn via autophagy is a potent strategy for curing PD.

Lots of studies indicate that nutrient related signaling pathways control the activity of autophagy in mammals. These pathways include Beclin 1/Vps34 lipid kinase complex [147], AMPK [148] insulin, amino acid and mTOR kinase complex [149-151]. Among those components involved in the regulation of autophagy, mTOR is a key component that coordinately regulates autophagy in response to cellular physiological conditions and environmental stress [123]. However, autophagy in neurons is highly specialized and uniquely regulated with the molecular mechanism which is quite different from the classical one [152]. It is proposed that starvation, the most commonly used autophagy induction method, cannot induce large-scale autophagy in neurons due to the ability of neurons to utilize multiple energy sources [153]. To attain a good understanding of neuronal autophagy at molecular and cellular levels is of all-important for the purpose to reveal the real character of autophagy in
neurodegenerative diseases including PD.

Protein phosphorylation is one of the most important post-translational modifications (PTMs) in living cells. It is involved in many regulatory functions such as cell cycle control, receptor-mediated signal transduction, differentiation, proliferation, transformation, and metabolism. Phosphorylation events are commonly probed in a targeted manner by phosphorylation-specific antibodies. In contrast, advances in phosphoproteomic techniques, including quantitative labeling methods, phosphopeptide enrichment, high-accuracy mass spectrometry and associated bioinformatics now make it possible to analyze entire phosphoproteomes [117]. Many techniques based on stable isotope labeling for protein quantization by MS such as isotope-coded affinity tags (ICAT), metabolic labeling by $^{15}$N-incorporation, stable isotope labeling by amino acids in culture (SILAC), enzymatic $^{18}$O-labeling and the recently introduced chemical labeling by tandem mass tags (known as iTRAQ$^{TM}$). SILAC and iTRAQ are currently the most frequently used techniques in quantitative MS-based phosphoproteomics. Many different strategies for up-front selective enrichment of phosphopeptides before MS analysis have been reported. The most successful to date have been the affinity- and antibody-based methods including Immobilized Metal Affinity Chromatography (IMAC) [154], Titanium Dioxide (TiO$_2$) Enrichment [155], Strong Cation Exchange Chromatography (SCX) [156], Hydrophilic Interaction Chromatography (HILIC) [157] and Immunopurification (IP)
with immobilized anti-phosphotyrosine antibodies [158]. Mass spectrometers measure the mass-to-charge ratio of an ion by manipulating ions in electric and/or magnetic fields or by measuring their time-of-flight (TOF). Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) is one of the most widely used MS techniques. In the process of MALDI, the analyte is mixed with an excess of ultraviolet-absorbing matrix, which is normally a low-molecular-weight aromatic acid. On irradiation with a focused laser beam of the appropriate wavelength, the excess matrix molecules sublime and transfer the embedded non-volatile analyte molecules into the gas phase. After numerous ion–molecule collisions in the plume of ions and molecules, singly protonated analyte ions are formed, which are accelerated by electric potentials into a TOF mass analyzer, by which can detect the analyte ions. Analyte ions with different m/z have the same energy but different velocities upon acceleration in the ion source.

In chapter 3 and our previous study, we identify Cory and Cory B as autophagy enhancers, which could promote the clearance of α-syn via autophagy. However, the mechanisms involved in these progresses are not totally clear. In this study, we performed phosphoproteomic study to compare the phosphorylation regulatory network and try to identify the protein kinases which may play an important role in Cory and Cory B induced autophagy.
4.2 Material and Methods

4.2.1 Reagents

H$_2$O was purchased from Thermo; ACN (Acetonitrile) and EtOH (ethyl alcohol) was purchased from Fisher Chemical; FA (formic acid) was purchased from Fluka; DMEM medium was purchased from Pierce; DMEM-L and FBS were purchased from Gibico; SILAC$^{TM}$ Protein Identification and Quantitation Media Kit was purchased from Thermo; Sequencing Grade Modified Trypsin was purchased from Promega; TFA (trifluoroacetic acid), IAA (iodoacetamide) and DTT (dithiothreitol) were purchased from Sigma; 2-D Quant kit was purchased from GE Healthcare; IMAC beads was purchased from Dalian Institute of Chemical Physics.

4.2.2 Stable isotope labeling and compounds treatment of N2a cells

The cells were grown to 80% confluence in high glucose (4.5 g/liter) Dulbecco’s modified Eagle’s medium (with glutamine and sodium pyruvate) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 95% air and 5% CO$_2$. The cells were labeled with “heavy” ($^{13}$C$_6^{15}$N$_2$-Lysine, $^{13}$C$_6^{15}$N$_4$-Arginine), “medium” ($^{13}$C$_4^{14}$N$_2$-Lysine, $^{13}$C$_6^{14}$N$_4$-Arginine) or “light” ($^{12}$C$_6^{14}$N$_2$-Lysine, $^{12}$C$_6^{14}$N$_4$-Arginine) using a SILAC Protein Quantitation Kit (Pierce, Thermo) according to manufacturer’s instructions. Briefly, the cell line was grown in DMEM supplemented with 10% FBS...
and the “heavy”, “medium” or “light” forms of L-lysine for more than six generations before treatment, to achieve more than 96% labeling efficiency. After that, the cells were further expanded in SILAC media to desired cell number (~5*10^8) into 15*150 cm^2 flasks.

The “light” labeled cells were then treated with 15 µM Cory for 3 hours and the “medium” labeled cells were treated with 15 µM Cory B for 3 hours. The “heavy” labeled cells were treated with same amount of 0.1% DMSO for 3 hours. After treatment, the cells were harvested and washed twice with ice-cold PBS. After snap freezing in liquid nitrogen, cell pellets were stored in -80 °C freezer for future use.

4.2.3 Isolation of peptide and determination of labeling efficiency

N2a cells were grown in light or heavy medium for 3 and 6 days. The labeling efficiency was determined according to a previous published protocol [159] with small modifications. The harvested cell pellet was treated with 100 µl of 0.1 % trifluoroacetic acid (TFA) for 5 minutes at 4°C. Then, the cell pellet was incubated in a sonication bath for 5 minutes and centrifuged for 10 minutes at 14000 rpm at 4°C. The supernatant was concentrated by speed vac and then purified by µ-C_{18} ZipTips (Millipore, Billerica, MA). The elute was preceded to Nano-LC/Ion Trap MS (Ultimate 3000, Diones) (amaZon ETD, BRUKER) mass spectrometry as described
below. In the MASCOT search engine, “none” enzyme was selected and N-acetyl (protein) was chosen as variable modification.

4.2.4 Total protein extraction for phosphoproteomic profiling Crude Protein Extraction

The harvested “heavy”, “medium” and “light” labeled cells were lysed with lysis buffer (100 mM Tris-Cl, 2mM EDTA, 1.0 % Triton X-100, pH 7.2) supplemented with Phosphatase Inhibitor Cocktail and Protease Inhibitor Cocktail on ice using a high intensity ultrasonic processor (Scientz) for 30 minutes, respectively. The supernatants were saved after centrifuge at 20,000g for 10 minutes at 4 °C. The protein concentration was determined with 2-D Quant kit according to the manufacturer’s instructions.

4.2.5 Protein digestion in solution

For digestion, the protein solution was reduced with 10 mM DTT for 1 hour at 56 °C and alkylated with 20 mM IAA for 45 minutes at room temperature, which were protected from light. Finally, trypsin gold was added at the ratio of 1:50 (trypsin-to-protein) overnight and 1:100 (trypsin-to-protein) for another 4 hours.

4.2.6 HPLC Fractionation
The sample was then fractionated into fractions by high pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 µm particles, 4.6 mm ID, and 250 mm length). Briefly, peptides were first separated with a gradient of 2% to 60% acetonitrile in 10 mM ammonium bicarbonate pH 10 over 80 minutes into 80 fractions. Then, the peptides were combined into 12 fractions and dried by vacuum centrifuging.

4.2.7 IMAC Enrichment

For phosphopeptides enrichment, fractionated peptide mixtures were first incubated with IMAC microspheres suspension by vibration. The IMAC microspheres with enriched phosphopeptides were collected by centrifugation, and the supernatant was removed. To remove nonspecifically adsorbed peptides, the IMAC microspheres were washed with 50% ACN/6% TFA and 30% ACN/0.1% TFA, sequentially. To elute the enriched phosphopeptides from the IMAC microspheres, elution buffer containing 10% NH₄OH was added and the enriched phosphopeptides were eluted with vibration. The supernatant containing phosphopeptides was collected and lyophilized for LC-MS/MS analysis.

4.2.8 LC-MS/MS Analysis

Peptides were dissolved in solvent A (0.1% FA in 2% ACN), directly loaded onto a
reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific) with a linear gradient of 5–20% solvent B (0.1% FA in 98% ACN) for 50 min, 20–35% solvent B for 10 min, and 35–80% solvent B for 10 min at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system. The resulting peptides were analyzed by Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific).

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using 28% NCE; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 10 MS/MS scans was applied for the top 10 precursor ions above a threshold ion count of 2E4 in the MS survey scan with 5.0s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800.

4.2.9 Database search
The resulting MS/MS data was processed using MaxQuant with integrated Andromeda search engine (v.1.4.1.2). Tandem mass spectra were searched against Swiss Prot_mouse database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages, 4 modifications per peptide and 5 charges. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carb amidomethylation on Cys was specified as fixed modification and oxidation on Met, Phosphorylation on Ser, Thr, Tyr and acetylation on protein N-terminal were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 1%. Minimum peptide length was set at 7. All the other parameters in MaxQuant were set to default values.
4.3 Results

4.3.1 Basic information of phosphorylation sites identified with LC/MS

In this study, proteome-wild profiling of phosphorylation regulated by Cory or Cory B was carried out on N2a cells with IMAC enrichment of phosphopeptides followed by MS (mass spectrometry) identification. Totally 5555 phosphorylation sites were profiled from the phosphopeptides enriched by IMAC, and the number of phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) were 4861 (2), 666 and 28 respectively (Fig. 4-1). Although most phosphopeptides were down-regulated, the number of up/down regulated phosphopeptides between Cory and Cory B is similar (Fig. 4-2). Meanwhile, the ratio distribution of phosphopeptides induced by Cory or Cory B and the motif distribution involved in these progresses were shown in the heat maps (Fig. 4-3, Fig 4-4).
Fig. 4-1 Distribution of three types of phosphorylation sites, including phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY).

Fig. 4-2 Up- or down-regulated phosphorylation sites induced by Cory and Cory B, respectively.
Fig. 4-3 The heat map of ratio distribution of phosphorylation sites induced by Cory and Cory B.
Fig. 4-4 The heat map of phosphorylation sites motif distribution calculated by Motif-X.
4.3.2 Enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for phosphoproteins.

To understand the biological function of cellular phosphorylation induced by Cory and Cory B, we annotated these phosphorylated proteins by the enrichment analysis in the Gene Ontology (GO) function term and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Among the GO annotations, most of the significantly enriched biological processed are chromosome organization and chromatin organization (Fig. 4-5). Most of the significantly enriched molecular function are RNA binding and nucleotide binding (Fig. 4-6). And most of the significantly enriched cellular components are intracellular non-membrane-bounded organelle and non-membrane-bounded organelle (Fig. 4-7), indicating their involvement in the pathways of autophagy induced by Cory and Cory B.

Furthermore, to better understand the pathways regulated by Cory and Cory B, we performed the enrichment analysis of the KEGG pathways. Most interestingly, both the neurotrophin signaling pathway [160-162] and MAPK signaling pathway[163, 164], which were reported to be related to neurodegenerative disease and autophagy, are significantly regulated by Cory and Cory B (Fig. 4-8).
Fig. 4-5 Biological process of GO annotation (top15) for phosphoproteins that are regulated by Cory or Cory B.
Fig. 4-6 Molecular function of GO annotation regulated by Cory or Cory B.
Fig. 4-7 Cellular component of GO annotation regulated by Cory or Cory B.
Fig. 4-8 The most over-represented KEGG pathways regulated by Cory or Cory B.
4.3.3 The kinase activity analysis based on the information of phosphorylated sites.

We employed the software of iGPS [165] to predict the site-specific kinase-substrate relations (ssKSRs) for all the identified phosphorylated proteins in the dataset, and quantitative analysis every phosphorylation sites. To evaluate the kinase activity, defined $KA_T(K)$ as the value of kinase K in the “treatment” group, and $KSW_T(i|K)$ represented as the transformed number of the kinase K’s phosphorylation sites (Eq. 1).

At the same time, defined the $KA_C(K)$ and $KSW_C(i|K)$ in the “control” group (Eq. 2).

And $ratio(i)$ represented as the value of H/L ratio for each phosphorylation site (H: treatment group; L: control group).

\[ KA_T(K) = \sum_{i \in N_T} KSW_T(i|K) \cdot KSW_T(i) = \begin{cases} \left\lfloor \frac{ratio(i)}{1}, \quad ratio(i) \geq 1 \right. \\ 1, \quad ratio(i) < 1 \end{cases} \]  
(Eq. 1)

\[ KA_C(K) = \sum_{i \in N_C} KSW_C(i|K) \cdot KSW_C(i) = \begin{cases} \left\lfloor \frac{1}{ratio(i)} \right\rfloor, \quad ratio(i) \geq 1 \\ 1, \quad ratio(i) < 1 \end{cases} \]  
(Eq. 2)
And the significant difference of the kinase activity between “treatment” and “control” group were analysis by Yates' chi-squared ($\chi^2$) test.

**Table 4-1 Yates' chi-squared ($\chi^2$) test**

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Control</th>
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<tbody>
<tr>
<td>Up regulated sites of kinase $K$</td>
<td>$a$</td>
<td>$b$</td>
</tr>
<tr>
<td>Up regulated sites of other kinases</td>
<td>$c$</td>
<td>$d$</td>
</tr>
<tr>
<td></td>
<td>$N_l$</td>
<td>$N_w$</td>
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</table>

Then we calculated the $E$-ratio followed Eq. 3. When $E$-ratio $>1$, the kinase activity was upregulated in the “treatment” group, and when $E$-ratio $<1$, the kinase activity was upregulated in the “control” group.

$$E - ratio = \frac{a}{b} \frac{N_l}{N_y} \frac{N_i}{N} \quad (\text{Eq. 3})$$

The value of $\chi^2$ was calculated by the Eq. 4. And the $p$-value was calculated with the CHIDIST ($\chi^2$, degrees freedom) in the Excel (freedom=1).

$$\chi^2_{Yates} = \frac{N(\max(0, | \frac{ad - bc}{N_y} - N / 2)))^2}{N_iN_yN_jN_n} \quad (\text{Eq. 4})$$
According to calculation, we got a list of up/down regulated kinase by Cory or Cory B. And the most significantly regulated kinases were shown in the Fig. 4-9----Fig. 4-12. In the group of Cory, 4 members of CAMK family (CAMK 2a, CAMK 2g, CAMK 1a, and CAMK 1d), 3 members of Trb family (Trb1, Trb2, and Trb 3) and 5 members of TSSK family (TSSK1, TSSK2, TSSK3, TSSK4 and TSSK5) are all significantly down regulated (Fig. 4-10). On the other hand, with the treatment of Cory B, 5 members of CDKL family (CDKL1, CDKL2, CDKL3, CDKL4, and CDKL5) are significantly up regulated (Fig. 4-11). Meanwhile, western blotting results confirmed the prediction of iGPS that down-regulated p70S6K, a downstream of mTOR[166], was only found in the group of Cory (Fig. 4-13), indicating different kinase families were involved in the pathway reduced by Cory or Cory B. At the same time, many members of CDK and PLK families are both up-regulated after the treatment of Cory and Cory B (Fig. 4-9, Fig 4-11), suggesting some pathways are shared between these two compounds. Based on the significantly regulated kinases and substrates, we drew the kinase–substrate phosphorylation network regulated by Cory (Fig. 4-14) or Cory B (Fig. 4-15), which showed the detailed information of Cory and Cory B.
Fig. 4-9 The protein kinases up-regulated by Cory.
Fig. 4-10 The protein kinases down-regulated by Cory.
Fig. 4-11 The protein kinases up-regulated by Cory B.
Fig. 4-12 The protein kinases down-regulated by Cory B.
Fig. 4-13 Cory, not Cory B, down-regulates the activity of p70S6K. N2a cells were treated with 25 µM of Cory, Cory B or 0.1% (v/v) DMSO for 6 hours, and the cells lysates were subjected to western blotting analysis. Quantifications of p-p70S6K/t-p70S6K ratio were shown as mean ± SEM of three independent experiments. (***p < 0.001 compared with Ctrl; one-way ANOVA with Newman-Keuls multiple test).
Fig. 4-14 The kinase–substrate phosphorylation network regulated by Cory.
Fig. 4-15 The kinase–substrate phosphorylation network regulated by Cory B.
4.3.4 Differences between Cory and Cory B

To identify the difference between the pathway regulated by Cory or Cory B, we compared the topology structure of Cory and Cory B, including numbers of node, interaction, kinase, substrate and hub gene, which was detailed shown in Table 4-1. Most items between these two compounds are similar, although the numbers of Cory seem to be a little more than that of Cory B. However, the number of interaction of Cory (4604) was much more than that of Cory B (2896). Then with the help of MOCDE v1.4, we got the most close-interacted sub-network of Cory or Cory B (Fig. 4-16, Fig. 4-17). From the Fig. 4-16, we found the substrates of both TSSK and Trb families were significantly enriched in sub-network of Cory. However, the phosphorylated substrates of CDKL and CDK families were over-represented in the sub-network of Cory B (Fig. 4-17), indicating that different kinases involved in the autophagy that regulated by Cory or Cory B.
Table 4-2 Comparison between Cory and Cory B based on the topology structure.

<table>
<thead>
<tr>
<th>Items</th>
<th>Cory</th>
<th>Cory B</th>
<th>Common</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node</td>
<td>623</td>
<td>573</td>
<td>508</td>
</tr>
<tr>
<td>Interaction</td>
<td>4604</td>
<td>2896</td>
<td>2277</td>
</tr>
<tr>
<td>Kinase</td>
<td>79</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Substrate</td>
<td>551</td>
<td>532</td>
<td>479</td>
</tr>
<tr>
<td>Hub gene (10%)</td>
<td>62</td>
<td>57</td>
<td>23</td>
</tr>
</tbody>
</table>

![Fig. 4-16 The most close-interacted sub-network of Cory.](image-url)
Fig. 4-17 The most close-interacted sub-network of Cory B.
4.4 Discussion

Proteomics is a large scale study of proteins, especially their functions and involved pathway [167]. In the past, quantitative proteomics was performed by 2D (two dimensional) gel electrophoresis. Recently, mass spectrometric methods based on SILAC (stable isotope labeling by amino acid in cell culture) have given more promise to study the stimulation and pathway in a cell culture system [168]. In this study, we used stable isotope ($^{13}$C and $^{15}$N) to label the lysine and arginine and got 3 different groups of N2a cells with light, middle or heavy molecular weight. As the 3 groups of cells after treatment mixed, and the following steps were then performed together, it avoided some system errors and made the results credible.

Totally 5555(2) phosphorylation sites were identified in this study (Fig. 4-1----Fig. 4-4). And with the GO enrichment analysis, RNA binding and chromosome/chromatin organization stood out (Fig. 4-5----Fig. 4-7), indicating that mitogen may be active by Cory or Cory B. Consistent with these results, in the enrichment analysis of KEGG pathway, MAPK (mitogen activated protein kinase) signaling pathway was significantly affected, as well as neurotrophin signaling pathway and insulin signaling pathway (Fig. 4-8). Autophagy is usually regulated via MAPK pathway [169] and MAPK was found to be involved in the regulation of amyloid precursor protein (APP) by rasagiline, which is a anti-Parkinson drug [170]. Also, it is reported that insulin signaling pathway triggered the clearance of huntingtin
aggregates via autophagy [171] and defective autophagy caused insulin resistance [172]. Meanwhile, neurotrophic effects resulting from the insulin/insulin receptor system was considered as important in the etiology of PD [162, 173]. All these suggested the potential effect of Cory or Cory B in PD via regulating autophagy.

The software of iGPS has a good performance on prediction of in vivo site-specific kinase-substrate relation from the phosphoproteomic data [165]. From its prediction, we found that CAMK, Trb and TSSK families were significantly up regulated after treated with Cory (Fig. 4-9,10). Researchers found that synaptic and motor deficits in PD were linked to the abnormal of Ca$^{2+}$-calmodulin-dependent protein kinase II (CAMK II) [174]. Trb3, a novel inducible gene under ER stress, was found to directly interact with and inhibit AKT, which is a partner of autophagy [175], and silent the Trb3 showed significant neuroprotection to primary neurons against Aβ in AD [176]. According to previous reports, down regulation of CAMK and Trb families by Cory indicated the potential neuroprotection. However, in the Cory B group, CDKL family was more affected (Fig. 4-11, 12).

PLK2, a member of PLK family, was found to promote the clearance of α-syn in PD. CDK5, a members of CDK family, was thought be required for proper development of the mammalian central nervous system [177], and inhibition of CDK5 by flavopiridol attenuates the loss of dopaminergic neurons caused by MPTP [178]. Both
of the two families were up regulated in the group of Cory and Cory B, however, CDK family was richer in the most close-interacted sub-networks of Cory B, and the ranking of PLK family members was topper in Cory group than that of Cory B (Fig. 4-9,11,15,16), indicating their different response to Cory or Cory B.

Collectively, in this phosphoproteomic study, MAPK, neurotrophin and insulin signaling pathways were enriched after the treatment of Cory and Cory B. Although the structure of these two compounds are quite similar, Cory has more effect on the CAMK, Trb and TSSK families, however, CDK and CDKL families are more sensitive to Cory B.
CHAPTER 5

Neuroprotection study of corynoxine and corynoxine B

on a rotenone rat model of Parkinson’s disease
5.1 Introduction

PD is the second most common neurodegenerative disorder after AD, which is characterized by tremor, rigidity, brady kinesia and postural instability [179]. And its pathologic features include dopaminergic neurons loss in the SNc and α-syn aggregation in neurons [180]. Since James Parkinson gave the first detailed description of this disease in 1817 [181], PD has been studied for almost two centuries; however, there are still no effective drugs to cure this disease.

Autophagy-lysosome pathway (ALP) is one of the major ways to clear disordered, especially long-lived protein, such as α-syn [182, 183]. And impaired autophagy is thought be related to the pathologic development both in patients and animal models of PD [96, 184]. Therefore, autophagy enhancers are suggested to be a new strategy for PD treatment. In our previous, we identified two alkaloids from Chinese herb Gouteng, corynoxine (Cory) [185] and corynoxine B (Cory B) [107], as the new autophagy enhancers, which could promote the clearance of α-syn in a cell model of PD. However, the microenvironment between in vitro and in vivo cells is quite different, and the value of cells models is limit. Therefore, it becomes necessary to evaluate the neuroprotection of Cory and Cory B on animal models of PD before clinical trial.

As impairment of mitochondrial complex I was found to be a major factor that
contributed to neurodegeneration [186], inhibitors of mitochondrial complex I, such as rotenone[187], are widely used to induce Parkinson-like symptoms in animals. In this study, we stereotaxical injected rotenone into SNc and VTA (ventral tegmental area), two important regions for the pathogenesis of PD [188-190], to establish a rat model of PD and provide more experimental evidences to develop Cory and Cory B to be the potential drugs for PD treatment.

5.2 Materials and methods

5.2.1 Regent

Apomorphine (Y0001465) and Rotenone (R8875) were purchased from Sigma; Cory and Cory B were purchased from Aktin Chemicals Inc (Chengdu, China); Anti-Tyrosine Hydroxylase (TH) antibody (AB152) was purchased from Merck Millipore; Anti-Iba-1 (019-19741) was purchased from Wako Pure Chemical Industries; Anti-β-actin sc-47778) was purchased from Santa Cruz Biotechnology; Anti-LC3 (2775), anti-p62 (5114) and anti-α-syn (2628) were purchased from cell signaling technology; Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 594 conjugate (R37117), Goat anti-mouse (626520) and goat anti-rabbit (G21234) secondary antibodies were purchased from Invitrogen; VECTASTAIN Elite ABC kit (PK-6101) was purchased from VECTOR LAB.

5.2.2 Animals
ICR mouse (10, 25~30g) and SD rat (82,300±10g) were purchased from animal lab of Hong Kong Chinese University.

5.2.3 Treatment

Srague-Dwaley rats were pre-treated with different concentration of Cory and Cory B for 1 week. After injected with rotenone, the rats were continued to be treated with compounds for another 4 weeks.

5.2.4 Acute toxicity study of Cory and Cory B

Eight week-old 10 female Srague-Dwaley rats weighing 200±10g were used in the study. The rats were placed separately in laboratory animal houses at 20-25°C with 50–60% humidity and 12 h light/dark cycle with the lights off at 7 PM. The rats were fed with standard diet from Lab Diet, allow to access distilled water ad libitum, and acclimated to laboratory conditions for 7 days.

Test Up and Down procedure was followed the OECD guideline[191]. Acute Oral Toxicity (Guideline 425) Statistical Program (AOT425StatPgm) developed by US Environmental protection agency was used. Firstly, dissolved the Cory B in DMSO, and then diluted with normal saline to desired concentration. And Cory was dissolved in normal saline directly. The final injection volume for each rat was 1.0 ml. Before
dosing, each rat was fasted overnight. The first dosage was set as 80 mg/kg, which was followed the LD$_{50}$ report of rynchophylline [192], which is an isomer of Cory and Cory B. And the assumed dose progression factor of 1.2 was use in the study. Dosing continues depending on the 48 hours outcomes after dosing. And the test stops when one of the following stopping criteria first is met: (a) 3 consecutive animal survive at the upper bound; (b) 5 reversals occur in any 6 consecutive animal tested; (c) at least 4 animal have followed the first reversal and the specified likelihood-ratios exceed the critical value.

5.2.5 TH (Tyrosine Hydroxylase) staining

The protocol is followed the VECTASTAIN Elite ABC kit. Working solution was prepared before staining. Frozen brain sections (SNc: 30µm, Striatum: 25 µm) were prepared with Cryostat Series (7721.160 GB, SHANDON). Sections were rinsed with PBS for 3 times (3 minutes/time). After incubating with 3% H$_2$O$_2$ for 10 minutes, sections were rinsed with PBS for 3 times (3 minutes/time). Then sections were blocked with normal serum for 30 minutes, and rinsed with PBS for 3 times (3 minutes/time). Sections were incubate with primary antibody (1:500) overnight at 4 °C. Then after rinsing with PBS for 3 times (3 minutes/time), sections were incubated with Elite second antibody for 30 minutes at room temperature. After rinsing with PBS for 3 times (3 minutes/time), sections were incubated with Elite ABC reagent for
another 30 minutes at room temperature. Sections were rinse with PBS for 3 times (3 minutes/time), and incubated with DAB reagent for 2 minutes. At last, the slides were mounted and images were taken with confocal.

5.2.6 Fluoresce immunostaining

Working solution was prepare the before staining. Frozen brain sections (SNc: 30µm, Striatum: 25 µm) were prepared with Cryostat Series (7721.160 GB, SHANDON). Sections were rinse with PBS for 3 times (3 minutes/time). After incubating with 3% H₂O₂ for 10 minutes, sections were rinsed with PBS for 3 times (3 minutes/time). Sections were blocked with normal serum for 30 minutes, and rinsed with PBS for 3 times (3 minutes/time). Sections were incubated with primary antibody overnight at 4 °C. Then sections were rinsed with PBS for 3 times (3 minutes/time), and incubated them with Alexa Fluor 594 second antibody for 30 minutes at room temperature. At last, sections were rinsed with PBS for 3 times (3 minutes/time), slides were mounted and images were taken with confocal.

5.2.7 Western blotting analysis

The protocol is followed with 3.2.8.

5.2.8 Statistical analysis

Statistical analysis is followed with 3.2.9
5.3 Results

5.3.1 Acute toxicity study of Cory and Cory B

To confirm the safe dosage of Cory and Cory B on rat, we performed acute toxicity study. It was reported that the LD_{50} (i.v.) of rhynchophylline, the isomer of Cory and Cory B, is 105 mg/kg in mice [192]. So the limit test with dose of 2000 mg/kg was skipped and performed the main test directly. Ten female Sprague-Dwaley rats (Eight week-old, five rats for each compound) weighing 200±10g were used in the study. According the experiment date of Cory (Table 5-1) and Cory B (Table 5-2), the LD_{50} of Cory and Cory B were estimated at 96.89 and 80 mg/kg respectively (Table 5-3).

**Table 5-1: Experiment data of Cory acute toxicity**

<table>
<thead>
<tr>
<th>Test sequence</th>
<th>Animal ID</th>
<th>Dose (mg/kg)</th>
<th>Short-term Result</th>
<th>Long-term Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>80</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>105</td>
<td>X</td>
<td>X</td>
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<td>80</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>105</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>80</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

(X=Died, O=Survived, Short-term=48 hours, Long-term=14 days)
Table 5-2: Experiment data of Cory B acute toxicity

<table>
<thead>
<tr>
<th>Test sequence</th>
<th>Animal ID</th>
<th>Dose (mg/kg)</th>
<th>Short-term Result</th>
<th>Long-term Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>80</td>
<td>O</td>
<td>O</td>
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<td>2</td>
<td>2</td>
<td>105</td>
<td>X</td>
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<tr>
<td>5</td>
<td>5</td>
<td>80</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

(X=Died, O=Survived, Short-term=48 hours, Long-term=14 days)

Table 5-3: Estimated LD$_{50}$ of Cory and Cory B

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LD$_{50}$(mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cory</td>
<td>96.89</td>
</tr>
<tr>
<td>Cory B</td>
<td>80</td>
</tr>
</tbody>
</table>
5.3.2 Cory B induces autophagy in normal mouse

Before establishing the rotenone rat model, we detected the autophagy induction on mouse by Cory B. ICR (Institute of Cancer Research) mouse were given Cory B (20 mg/kg) for 21 days by oral administration. After infusion, frontal cortex and liver were separated for western blotting assay. From Fig. 5-1, we found Cory B could significantly increase the LC3II level, indicating that 20 mg/kg of Cory B induced autophagy in mouse. As the body surface area of mouse is twice of that of rat, we set 10, 5, 2.5 mg/kg as high (H), middle (M) and low (L) dosage for the following experiments.
Fig. 5-1 Cory B induces autophagy in ICR mouse. ICR mouse were given Cory B (20 mg/kg) for 21 days by administration. After infusion, frontal cortex and liver were subjected to western blotting assay. Data were presented as mean ± SEM of three independent experiments. (**p<0.001 compared with control; one-way ANOVA with Newman-Keuls multiple test).

5.3.3 Establishing the rotenone rat model of PD

Eight week-old 72 male Srague-Dwaley rats weighing 300±10g were divided into 9 groups. The detailed groups are as followed: sham; model; sinemet; Cory-L (2.5 mg/kg); Cory-M (5 mg/kg); Cory-H (10 mg/kg); Cory B-L (2.5 mg/kg); Cory B-M (5 mg/kg); Cory B-H (10 mg/kg).
Rotenone (3 µg/µl) was dissolved in DMSO and kept from light before using. According to Bao’s rat cerebral stereotaxic atlas, injected 1 µl of rotenone into the right side substantia nigra compacta (SNc) (AP: -5.3 mm; ML: 2.0 mm; DV: -8.0 mm) and ventral tegmental area (VTA) (AP: -5.3 mm; ML: 0.9 mm; DV: -8.0 mm) respectively (Fig. 5-2). The sham group received the same amount of DMSO.

**Fig. 5-2 Establishing the rotenone rat model of PD.**

**5.3.4 Cory and Cory B improve the motor dysfunction**

At the end of 2nd and 4th week after model built, rotation was induced by apomorphine, which is a non-selective dopamine agonist and activates both D1-like and D2-like receptors[193]. Each rat was received 2.5 mg/kg apomorphine (i.p.) and placed in the rotometer. 35 minutes of video was taken for each rat. After 5 minutes of adaptation, rotations to the left and right were counted respectively and the net positive rotations of 30 minutes were calculated. At the end of 2nd week, all the dosage of Cory and Cory B decreased the rotations induced by apomorphine (Fig. 5-3). With the
developed toxin caused by rotenone, only the low and middle dosage of Cory and the high dosage of Cory B could decrease the number of rotation (Fig. 5-4). However, the high dosage of Cory has no significant effect.

![Graph showing Apomorphine-induced rotation/30 min](image)

**Fig. 5-3 Cory and Cory B improve the motor function after 2 weeks’ post-injection.** Rotation test induced by apomorphine (2.5mg/kg) was performed at the end 2\textsuperscript{nd} week after rotenone rat model establishment. Rotations to the left and right were counted respectively and the net positive rotations of 30 minutes were totaled. Data were presented as mean ± SEM of three independent experiments. (**p < 0.01, ***p < 0.001 compared with Model; n=5; one-way ANOVA with Newman-Keuls multiple test).
Fig. 5-4 Cory and Cory B improve the motor function after 4 weeks’ post-injection. Rotation test induced by apomorphine (2.5mg/kg) was performed at the end 4th week after rotenone rat model establishment. Rotations to the left and right were counted respectively and the net positive rotations of 30 minutes were totaled. Data were presented as mean ± SEM of three independent experiments. (*p < 0.05, **p < 0.01 compared with Model; n=5; one-way ANOVA with Newman-Keuls multiple test).

5.3.5 Cory and Cory B increase TH level.

Tyrosine hydroxylase (TH) is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) [194]. It is a rate-limiting enzyme, which controls the first step of dopamine biosynthesis. Compared with the group of sham, decreased expression of TH was observed in the
right side of SNc in the group of model (Fig. 5-5). And both Cory and Cory B significantly increased the TH level (Fig. 5-5). Also, from the immunostaining results, decreased TH-positive neurons were found in the right side of SNc, VTA and striatum in the group of model (Fig. 5-6, Fig. 5-7). However, with the treatment of Cory and Cory B, TH-positive neurons’ loss was prevented (Fig. 5-6, Fig. 5-7).

**Fig. 5-5 Cory and Cory B increase the level of TH.** The right side of SNc was isolated from the brain tissue. After protein extraction, samples were subjected to western blotting assay. Data were presented as mean ± SEM of three independent experiments. (*p<0.05, **p<0.001 compared with model; n=5; one-way ANOVA with Newman-Keuls multiple test).
Fig. 5-6 Cory and Cory B prevent TH-positive neurons loss in the right side of SNC and VTA.
Fig. 5-7 Cory and Cory B prevent TH-positive neurons loss in the right side of striatum.
5.3.6 Cory and Cory B induce autophagy and decrease α-syn aggregation in the rotenone rat model of PD

Increased α-syn aggregation is one of the characters of PD, which is reported to be linked with the impaired autophagy. Right side of SNC, which is injected with rotenone, was isolated from brain tissue and the autophagy markers, LC3 and p62, were detected by western blotting. Decreased LC3II and increased p62 were found in the group of model (Fig. 5-8). Also, α-syn aggregations were significantly increased in the group of model (Fig. 5-9, Fig. 5-10). Compared with the group of model, Cory and Cory B increased the expression of LC3II and decreased the expression of p62, indicated autophagy induction (Fig. 5-8). Meanwhile, the aggregated α-syn was significantly decreased (Fig. 5-9, Fig. 5-10).
Fig. 5-8 Cory and Cory B induce autophagy in the rotenone rat model of PD. The right side of SNc was isolated from the brain tissue. After protein extraction, samples were subjected to western blotting assay. Data were presented as mean ± SEM of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 compared with model; n=5; one-way ANOVA with Newman-Keuls multiple test).
Fig. 5-9 Cory and Cory B decrease the aggregated α-syn in the right side of SNc.
Fig. 5-10 Cory and Cory B decrease the aggregated α-syn in the left side of SNc.
Fig. 5-11 Non-significant α-syn aggregations are found in striatum.
5.3.7 Cory and Cory B decrease active microglia cells

In the rotenone rat model of PD, microglia cells are always active. And in this study, we selected brain sections of SNc, and performed immunostaining with the antibody of Iba-1, which is a marker of microglia cell. In the group of model, microglia cells were significantly increased both in the right and left side. And the number of right side is more than that of left (Fig. 5-12, Fig. 5-13). Compared with the group of model, the actived microglia cells were significantly decreased in the group of Cory and Cory B (Fig. 5-12, Fig. 5-13). However, the low dosage of Cory has no significant effect on the left side of SNc (Fig. 5-13).
Fig. 5-12 Cory and Cory B decrease the number of actived microglia cells in the right side of SNc.
Fig. 5-13 Cory and Cory B decrease the number of activated microglia cells in the left side of SNc.
5.4 Discussion

In 2000, rotenone was the first time to be reported to reproduce features of PD by chronic systemic exposure, which is similar to MPTP [186], and animal models reduced by rotenone have been investigated since then. Because of the lipophilic nature, it is easier for rotenone than MPTP to cross the blood brain barrier, as well as the cell membrane. Also, rotenone up regulated α-syn expression and aggregation both in cell [195] and rat models [190, 196], which is one of the major pathologic features of PD. Consider of the peripheral toxicity and general health problem rather than a specific motor deficit characteristic of PD symptoms, which is caused by systemic administration of rotenone [197], in this study, we employed a rat model of PD which was stereotaxical injected rotenone into SNc and VTA [190], to evaluate the neuroprotection of Cory and Cory B.

Firstly, we performed acute toxicity study, which is followed the Acute Oral Toxicity (Guideline 425) Statistical Program (AOT425StatPgm) developed by US Environmental protection agency, and got the LD$_{50}$ of Cory and Cory B on rats. Because of similar chemical structure, the values of these two compounds are close. And the toxicity of Cory, whose LD$_{50}$ is 96.89 mg/kg, seems a bit lower than that of Cory B (LD$_{50}$=80mg/kg) (Table 1-3). According to the acute toxicity study and the results of the preliminary experiment on mouse, we set the dosage for the following
Apomorphine is a dopamine receptor agonist, and apomorphine-induced rotation in rat model of PD is usually used to estimate the motor impairment [190, 198, 199]. At the early stage (2nd week) of this study, both Cory and Cory B decreased the number of rotation induced by apomorphine, indicating a protection on motor dysfunction (Fig. 5-3). However, at the end of 4th week, only high dosage of Cory B significantly against the rotation, which may be due to the development of rotenone toxicity (Fig. 5-4). An interesting result is that, high dosage of Cory has no effect, which may be due to the toxicity of Cory, but the low and middle dosage could significant decrease the apomorphine induced rotation (Fig.5-4), indicating that the effective dosage of Cory may be lower than Cory B. Tyrosine hydroxylase (TH) is the enzyme responsible for catalyzing the conversion of the amino acidL-tyrosine toL-3, 4-dihydroxyphenylalanine (L-DOPA) [194]. It is a rate-limiting enzyme which controls the biosynthesis of catecholamine (dopamine, noradrenaline and adrenaline), the central and sympathetic neurotransmitters and adrenomedullary hormones [194, 200]. Decreased TH level was found in the dopamine neurons in Parkinson's disease [201-204]. In this study, the expression of TH was significantly decreased in the group of model. However, both Cory and Cory B increased the level of TH in a dose-dependent manner (Fig.5-5). Meanwhile, in the lesion side of model, the TH-positive neurons were lost in SNC, VTA and striatum (Fig.5-6, Fig.5-7). However,
both Cory and Cory B prevented this progress, indicating the protection of neurons loss.

In our previous study, we provided evidences that both Cory and Cory B induced autophagy in neuronal cells and *Drosophila*. And in this study, decreased p62, a substrate of autophagy, and increased LC3II indicated that both Cory and Cory B induced autophagy in the rotenone rat model of PD (Fig.5-8). Meanwhile, depressed autophagy was found in the group of model and sinemet group (Fig.5-8). Consistent with previous study [190, 195, 196], increased α-syn aggregation were found in the lesion side of SNc in the group of model (Fig.5-9). Although the number of aggregation in the left side of SNc is less than that of the lesion side, it is really more than that of sham group (Fig.5-10). However, maybe because of the autophagy induction, which is thought to be an effective route to clear aggregated α-syn[97, 205], the numbers of aggregation are significantly decreased both in Cory and Cory B groups (Fig.5-9, Fig.5-10), indicating a promotion of α-syn clearance of these two compounds via autophagy.

Recently, inflammation is found to be a critical factor response to the progress of neurodegeneration [206, 207]. And microglia, the resident innate immune cells in the central nervous system, is found to be actived and produce toxic factors (IL-1, TNF-α,
NO, PGE2, superoxide) to neuron as a response to environmental toxins, such as rotenone [206, 208, 209]. In this study, significantly increased microglia was found in the lesion side of SNC in the group of model (Fig.5-12). And a slightly increased microglia was also found in the left side of SNC in the group of model (Fig.5-13). Both Cory and Cory B decreased the number of actived microglia in a dosage dependent manner, and Cory B seemed to more effective (Fig.5-12, Fig.5-13), indicating an anti-inflammation effect of these two compounds.

Collectively, in this study, Cory and Cory B showed a neuroprotection on a rotenone rat model of PD via against motor dysfunction, increasing TH level, decreased α-syn aggregation via autophagy and anti-inflammation, which provides experimental evidences to develop Cory and Cory B to potential drugs for the treatment of PD.
CHAPTER 6

DISCUSSION AND CONCLUSIONS
6.1 Identify oxindole derivatives as mTOR inhibitor

Rapamycin, a lipophilic macrolide, was the first one to be identified as, which thereby induced autophagy. After that, researchers named the rapamycin analogs as the first generation of mTOR inhibitors [210]. The second generation is known as ATP-competitive mTOR kinase inhibitors, which target the mTOR kinase domain [211]. At the same time, some quinazoline derivatives, named as SMER (small molecule enhancers of rapamycin), were found to induce autophagy [132]. However, there is no similar report about oxindole derivatives. In this study, we identified Cory, an oxindole alkaloid, as an autophagy enhancer, which induced autophagy in different neuronal cell lines and primary neurons, as well as in mice and Drosophila. Cory inhibited the pathway of Akt/mTOR, which provided the original finding of oxindole derivatives to be an mTOR inhibitor.

6.2 Alpha-synuclein clearance via autophagy to against PD

Although the mechanisms of inducing neurodegeneration remains not understand completely, the accumulation of α-syn was thought to be an important cause to PD [212]. Autophagy lysosome pathway is the major route to degrade long-lived and disordered proteins involved in neurodegenerative diseases [93, 213]. And deficits of autophagy contribute to the neurotoxicity caused by disordered protein, including α-syn, Aβ and huntingtin [213, 214]. Thus, promoting the clearance of α-syn by
autophagy enhancer is suggested to be a therapeutic strategy for PD treatment [214]. In this study, we indentified Cory as a new autophagy enhancer, which promotes the clearance of over-expressed wild type and mutant α-syn in a cell system. Also, in a rotenone rat model of PD, maybe due to autophagy induction by Cory and Cory B, decreased the puncta number of α-syn was found in the lessened SNc. All these findings provide experimental evidences to develop Cory or Cory B to be potential therapeutic drugs for PD treatment.

6.3 Identify different signaling pathway via phosphoproteomic analysis

Although the structures of Cory and Cory B are similar, which two substituents in the D-ring have a cis-relationship, and the compounds differ only in the stereochemistry at the spiro carbon, the manners they induced autophagy are quite different. In our previous study, we found Cory B induced autophagy in a Beclin-1 dependent manner, and did not affect the mTOR pathway [107]. However, in this study, Cory was identified as an mTOR inhibitor [185].

Recently, LC/MS methods based on SILAC make it effective to study the stimulation and pathway via quantitative proteomic [168]. With the help of iGPS, software predicts the in vivo site-specific relation [165], we got a list of kinases regulated by Cory or Cory B, and drew the detailed kinase-substrate network for each compound.
After comparison, we found Cory has more effect on the CAMK, Trb and TSSK families, however, CDK and CDKL families are more sensitive to Cory B. All these findings provide a direction to identify the primary target of Cory or Cory B, and a method to study different pathways between similar compounds.

6.4 Limitation and future work

In this work we identify that Cory induces autophagy through Akt/mTOR pathway, however, the primary target of Cory needs to be further identified. Also, decreased p-mTOR happened after 12 hours’ treatment of Cory (Fig. 3-19), indicate that other pathways upstream of mTOR may be regulated by Cory. Therefore, in the future research work, based on the results of phosphoproteomics, which provides a direction to identify the primary targets of Cory or Cory B, we will identify the key kinases and related substrates responsible for Cory or Cory B induced autophagy.

Meanwhile, in the animal work, we have confirmed the neuroprotective effects of Cory and Cory B, however, the mechanisms of these two compounds need to be identified in rotenone cell model.
REFERENCE


20. Houlden, H. and A.B. Singleton, *The genetics and neuropathology of Parkinson's


37. Floris, G., et al., Genetic analysis for five LRRK2 mutations in a Sardinian parkinsonian population: Importance of G2019S and R1441C mutations in sporadic...


46. Gandhi, S., et al., PINK1-associated Parkinson's disease is caused by neuronal...


64. Schapira, A.H.V., Mitochondria in the aetiology and pathogenesis of Parkinson's


73. Thiruchelvam, M., et al., The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: Implications for


100. Ramirez, A., et al., *Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase*. Nature Genetics,


108. Song, J.X., et al., *HMGB1 is involved in autophagy inhibition caused by SNCA/alpha-synuclein overexpression: A process modulated by the natural


Yuan, H., et al., LPS-induced autophagy is mediated by oxidative signaling in cardiomyocytes and is associated with cytoprotection. Am J Physiol Heart Circ.


138. Lo Bianco, C., et al., alpha-Synucleinopathy and selective dopaminergic


146. Sarkar, S., et al., Small molecules enhance autophagy and reduce toxicity in


163. Kim, E.K. and E.J. Choi, *Pathological roles of MAPK signaling pathways in*


195. Sala, G., et al., Rotenone upregulates alpha-synuclein and myocyte enhancer


204. Breese, G.R. and T.D. Traylor, Developmental characteristics of brain


212. Duda, J.E., V.M. Lee, and J.Q. Trojanowski, *Neuropathology of synuclein*


PUBLICATIONS LIST

Original Research Articles in Refereed International Journals:

(*Correspondence author)

(1) Chen L. L., Song J.X., Lu J.H., Yuan Z.W., Liu L.F., Durairajan S.S.K., Li M.*


(Jia-Wei-Liu-Jun-Zi Tang) for Non-motor Symptoms in Parkinson’s Disease”,

**Mov. Disord.** 2014, 29: (Suppl.1): P571. 〔IF 2014: 5.68〕


Abstracts Presented at International Conferences:


Akt/mTOR pathway and promotes the clearance of alpha-synuclein”. Poster presentation at Keystone Symposia on Autophagy: Fundamentals to Disease (E2), Hyatt Regency Austin, Austin, Texas, USA, 23-28 May 2014.


neurons independent of mTOR inhibition”, 7th International Symposium on Autophagy (7thISA), Huangshan, China, 19-23 March 2015. (Wins the Best Poster Prize)


5-9 May 2013.


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