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Synthesis and Characterization of Brain Penetrant Prodrug of Neuroprotective D-264: Potential Therapeutic Application in the Treatment of Parkinson's Disease

Fahd Dholkawala[#1],Chandrashekhar Voshavar[##1], Aloke K. Dutta[1]*

1. Wayne State University, Department of Pharmaceutical Sciences, Detroit, MI 48202

Correspondence Address:

Aloke K. Dutta, Ph.D.

Department of Pharmaceutical Sciences

Eugene Applebaum College of Pharmacy & Health Sciences, Rm# 3128

Detroit, MI 48202

Tel: 1-313-577-1064, Fax: 1-313-577-2033, e-mail: adutta@wayne.edu

[#] **FD and CV contributed equally to this manuscript**

[#] Present address: Advinus therapeutics Ltd., Quantum towers, RGP Phase I, Hinjwadi, Pune 411057, Maharashtra, India.

[##] Present address: Bioorganic Chemistry Laboratory, NCU, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bengaluru 560064, Karnataka, India.

Abstract

Parkinson's disease (PD) is one of the major debilitating neurodegenerative disorders affecting millions of people worldwide. Progressive loss of dopamine neurons resulting in development of motor dysfunction and other related non-motor symptoms is the hallmark of PD. Previously, we have reported on the neuroprotective property of a potent D3 preferring agonist D-264. In our goal to increase the bioavailability of D-264 in the brain, we have synthesized a modified cysteine based prodrug of D-264 and evaluated it's potential in crossing the blood brain barrier. Herein, we report the synthesis of a novel modified cysteine conjugated prodrug of potent neuroprotective D3 preferring agonist D-264 and systematic evaluation of the hydrolysis pattern of the prodrug to yield D-264 at different time intervals in rat plasma and brain homogenates using HPLC analysis. Furthermore, we have also performed *in vivo* experiments with the prodrug to evaluate its enhanced brain penetration ability.

Keywords: Parkinson's disease, Multifunctional Dopamine agonist, D-264, Modified cysteine, Blood-brain barrier, Prodrug

1. Introduction

Parkinson's disease (PD) is a complex progressive neurodegenerative disorder characterized by uncontrollable tremors, rigidity, slowness of movement, bradykinesia and postural imbalances[1]. PD also has symptoms of depression as well as dementia. It stands as the second most prevalent neurodegenerative disease after Alzheimer's disease and it is estimated that PD affects 1-2% of the people older than 65 years of age[2]. The PD produces broad spectrum symptoms with motor and non-motor features and their severity varies according to the stage of the disease. Although PD pathogenesis is not well understood, it is multi-factorial in nature where oxidative stress, protein aggregation, mitochondrial dysfunction, genetic and environmental factors are strongly implicated in the progression of PD[2-5]. The pathological hallmark of PD is loss of dopaminergic neurons in the substantia nigra pars compacta (SNPc) region of the brain. Other major pathological features of PD include presence of α-synuclein rich cytoplasmic filamentous protein aggregates called lewy bodies in dead or dying neurons of the SNPc along with mitochondrial dysfunction due to oxidative stress, increased amounts of iron, etc. [6]. Most of the pathological features of PD appear when about 75% of the nigral dopaminergic neurons are degenerated[7]. Progressive degeneration of dopaminergic neurons leads to profound striatal dopamine deficiency. Thus, dopamine deficiency in the SN region leads to motor symptoms and worsen as dopamine depletes further[8, 9]. Since, the pathogenesis of PD is multi-factorial with a great degree of complexity, research towards development of agents focusing on a single aspect of pathogenetic factors may not be effective for the PD treatment. In our on-going drug discovery studies, previously we have developed a library of D2/D3 agonist molecules in which D-264 was found to be one of the potent D3 preferring agonists[10]. D-264 exhibited potent neuroprotection in two animal models[11]. Also, this D3 receptor-preferring agonist D-264 exhibited neurotrophin-like property which elevated BDNF and

GDNF levels in MPTP- and Lactacystin-treated mice[11]. Further, D-264 showed *in vitro* neuroprotection in MN9D cells against MPP^+ induced toxicity and showed *in vivo* efficacy in reversing hypolocomotion in reserpinized rats[12]. Although D-264 exhibited potent D3 preferring agonist properties as well as very good neuroprotection in MPTP & Lactacystin PD animal models, this drug seems to be less bioavailable in the brain if dissolved in saline, due to its sluggish brain penetration. However, it seems bioavailability in the brain increases if the D-264 is dissolved in 5% beta-hydroxypropylcyclodextrin solution[12]. There are multiple ways of enhancing the bioavailability of certain drugs to make them more efficacious. In this regard, prodrug approach seems to be an appropriate method and a clinically viable technology for enhancing the efficacy without compromising on the pharmacological activity of the parent drug. One of the best and most reliable strategy for delivering active drugs with low BBB crossing ability is to design an effective prodrug that can potentially cross the BBB to release the parent drug after hydrolysis[13, 14]. Carrier mediated transport (CMT) systems are key in the transportation of various nutrients, hormones into the central nervous system. With the advent of technology in understanding membrane transport mechanisms and identification of various receptors involved in the transportation particularly in the blood-brain barrier system provided a great impact on the development of carrier mediated transportation of drugs targeting the CNS. As the CMT systems are known for their high stereo specificity for the substrates, drugs targeting CNS/brain are not readily transported by CMT system. There are number of CMT systems such as LAT1, GLUT1 and MCT1, CAT1, NCT1 and SVCT2 each having their specific substrates to be transported across the BBB to the CNS[15, 16]. Further, it is also reported that uptake of L-cysteine by brain synaptosomes occurs mostly via X^-_{AG} family of glutamate receptors (Excitatory Amino Acid Transporter-EAAT) with low-affinity, sodium dependent ion channel type mechanism[17]. In this

regard, prodrug approach was developed to overcome the limitations of potent/active drugs. One of the classical examples for such prodrug approach is modification of dopamine into L-DOPA as dopamine itself cannot cross the BBB. L-DOPA being a prodrug acts as a 'pseudo nutrient/substrate' for LAT1 system. We wanted to utilize a similar approach where conjugation to a suitable moiety should impart stability under systemic circulation while making its facile entry into the brain. In order to further enhance the blood brain barrier crossing efficacy and bio-availability of D-264 in the brain, we have designed a cysteine based D-264 prodrug. A substantial amount of research points out the very important role of antioxidants such as L-cysteine in reducing the oxidative stress associated with PD and in increasing the endogenous GSH and other antioxidant concentrations in PD patients [18]. The well-known antioxidant property of L-cysteine and its role in the glutathione synthesis suggests that L-cysteine based prodrug of a D2/D3 agonist may possess high therapeutic potential. We assumed L-cysteine intermediate would be an ideal moiety to conjugate with D-264 to design the prodrug since astrocytes in the blood brain barrier take up extracellular L-cysteine via EAAT or LAT1 and transports it to the neurons[17, 19]. It was found that PD patients have low levels of glutathione and conjugating modified L-cysteine moiety to D-264 may work as an antioxidant and also as an additional source for the glutathione synthesis[20, 21]. We also hypothesized that such prodrug would be able to impart efficient BBB penetration, neuroprotection with antioxidant property. Therefore, our main objective was to design and develop a cysteine-derived prodrug of D-264 (Compound **8,** Scheme **2**) which should possess enhanced blood brain barrier crossing ability compared to the parent D-264**.** It is expected that modified cysteine conjugated prodrug imparts stability under systemic conditions (blood circulation) without being prematurely cleaved significantly in the blood and should get hydrolyzed

in the brain by amidases to release the parent D-264 along with cysteine, thereby, further increasing efficacy of D-264 with synergizing antioxidant activity.

To this end, we have synthesized the modified cysteine conjugated prodrug of D-264 (Compound **8,** Scheme **2**) and evaluated the degree of hydrolysis in rat plasma and brain homogenates by an *ex vivo* hydrolysis experiment. We have also carried out an *in vivo* brain penetration experiment to determine the efficiency of BBB penetration of prodrug in comparison to D-264 alone. Furthermore, we have evaluated the antioxidant potency of prodrug and D-264 with DPPH free radical scavenging assay. Herein, we present synthesis, pharmacological characterization of a novel brain penetrant prodrug of neuroprotective D264 as potential disease modifying treatment agent for PD.

2. Materials and Methods

Chemistry:

Solvents and reagents were obtained from commercial suppliers and used as received unless otherwise indicated. Analytical silica gel-coated TLC plates (Silica Gel 60 F254) were purchased from EM Science and were visualized with UV light or by treatment with phosphomolybdic acid (PMA), ninhydrin and potassium permanganate ($KMnO_4$) solution. Flash chromatography was carried out on Davisil Chromatographic silica media 40-63 micron. ^{1}H &^{13}C NMR spectra were routinely obtained on Varian 400 MHz and 600 MHz FT NMR. The NMR solvents used were $CDCl_3$, DMSO or CD_3OD as indicated are obtained from Cambridge Isotope Laboratories Inc, USA. TMS was used as an internal standard. Low resolution Mass spectrometry was performed by Lumigen facilities (Wayne State University, Chemistry Department) using Waters Micromass ZQ and Shimadzu LCMS-8040.

Bio-reagents:

All HPLC grade solvents used in the preparation of mobile phase for HPLC analysis in hydrolysis and brain penetration experiments were obtained from Fischer Scientific, USA. HPLC solvents were filtered using Whatman nylon membrane filters (0.45µm) before use. Small amounts of formic acid (Sigma) and trifluoroacetic acid (Oakwood chemicals, USA) 0.1% and 0.05% respectively were added to the prepared mobile phases. 0.02M Phosphate buffer solution was used as a buffering agent to prepare plasma buffer solution and brain homogenate/supernatant buffer solutions in the hydrolysis and brain penetration experiments. Homogenization of the brain was performed using Polytron PT 10-35 (Kinematica AG), USA with Dulbecco's phosphate buffer saline (Sigma) as a buffering agent.

Animals. In rodent studies, animals were male and female Sprague-Dawley rats from Harlan (Indianapolis, IN) weighing 220-225 g unless otherwise specified. Animals were maintained in sawdust-lined cages in a temperature and humidity controlled environment at 22 ± 1 °C and 60 ± 5% humidity, respectively. A 12 h light/dark cycle was maintained, with lights on from 6:00 a.m. to 6:00 p.m. They were group-housed with unrestricted access to food and water. All experiments were performed during the light component. All animal use procedures were in compliance with the Wayne State University Animal Investigation Committee, consistent with AALAC guidelines.

Synthesis:

We synthesized the modified cysteine intermediate (Compound **1,** Scheme **1**) as reported earlier[22] and conjugated acid chloride of intermediate **1** to synthesize a model prodrug (Compound 3S**,** Scheme 1S, Supplementary Information) in order to develop an efficient synthetic route. Further, we have synthesized D-264 (Compound **7,** Scheme **2**) with some modifications of the previously reported procedure[10] and conjugated modified cysteine intermediate (Compound **1,** Scheme **1**)

toD-264 to yield prodrug (Compound **8,** Scheme **2**). The synthesis of these compounds is outlined in Schemes **1, 1S** &**2**.

HPLC Analysis:

Purity of the synthesized drugs i.e. D-264 and prodrug were analyzed by reverse phase HPLC (Waters 2489 Alliance Integrated System) using RP-C18 column (Sunfire, 5μM, 4.6 × 150 mm) with isocratic Elution mode and UV detection at 254 nm. Mobile phase used was water: methanol (60:40) with 0.05% trifluoroacetic acid. For detailed information on chromatograms see supplementary information materials.

In Vitro Hydrolysis and Brain Penetration Experiments:

In vitro Hydrolysis Assay:

Ex vivo hydrolysis experiment was performed to determine the stability and extent of hydrolysis of the prodrug in the plasma and brain. D-264 (Compound **7**, Scheme **2**) and Prodrug HCl salts (Compound **8**, Scheme **2**) were dissolved in 100 μL of MeOH at a concentration of 20 mg/mL. Fresh plasma and brains were collected from adult rats (Sprague Dawley, weighing 250-300 g) and used immediately for the hydrolysis experiment. For brain samples, 1 g of brain was diluted in 4mL of Dulbecco's phosphate buffer and homogenized using Polytron PT 10-35 (Kinematica AG) in 5-6 short pulses. Plasma buffer solution was prepared using 80% volume of plasma diluted with 0.02 M phosphate buffer. Plasma buffer solution& brain samples were preheated at 37^{o}C in shaking water bath for 5 minutes before adding the drug solution.10 μL of 20mg/mL drug stock solution was taken and added to 990 μL of either brain supernatant or plasma buffer solution and kept for shaking at 37^{o}C. Samples were collected from each group at indicated time points. For HPLC

analysis, 100 µL of collected sample was diluted with 500 µL of acetonitrile to precipitate proteins (6 fold dilution and vortexed for 15 min at 1400 rpm and the suspension was clarified by centrifugation at 1800 rpm for 10 min. 250 µL of supernatant was then collected separately in another microcentrifuge tube and evaporated completely. 100 µL of MeOH was added (2.5 fold up-dilution). The resulting 100 µL solution was subjected to centrifugation at 1800 rpm for 10 min and 10 µL of this solution was injected to HPLC for analysis (Please see supplementary section for detailed protocol).

Brain Penetration Assay:

Brain penetration experiment was performed to assess the percentage of enhancement in BBB crossing for prodrug(Compound **8**, Scheme **2**) compared to D-264 (Compound **7**, Scheme **2**). Briefly, two groups of Sprague-Dawley rats were injected intraperitoneally prodrug and D-264 in hydrochloride salt form at a dose of 50µmol/kg. Rats were sacrificed at 2 & 4 h followed by collection of plasma, brains samples separately. Brain samples for HPLC analysis were prepared by homogenizing 1gm of brain in 4mL 1X PBS followed by precipitation of proteins by addition of acetonitrile (6-fold dilution, v/v) to brain supernatant. Resulting solution was vortexed for 15min at 1400 rpm and clarified by centrifugation at 1800 rpm for 10min. An aliquot of 500uL supernatant was collected and evaporated and further dissolved in100uL of Methanol. The resulting 100 µL solution was subjected to centrifugation at 1800 rpm for 10 min and 10 µL of this solution was injected to HPLC for analysis. For plasma samples, dilutions were carried out similar to brain supernatant with fixed volume of plasma without pre-dilution using PBS (Please see supplementary section for detailed protocol).

Evaluation of Antioxidant Activity by DPPH Radical Scavenging Assay:

The DPPH radical-scavenging assay was performed according to previously reported method[23]. This method measures hydrogen atom or electron donating activity of an antioxidant. DPPH (1,1-Diphenyl-2-picrylhydrazyl) is a stable free radical of purple color giving strong absorption maxima at 517nm. DPPH accepts hydrogen atom/electron from free radical scavengers/antioxidants and gets reduced to a yellow colored 1,1,-diphenyl-2-picryl hydrazine. The change/decrease in absorption at 517nm is directly proportional to antioxidant activity of compound. Briefly, in a 96-well plate, an amount of 100 µL of drug solutions (dissolved in methanol) ranging from 20 to 250µM was added. Next 100 µL of 200 µM methanolic solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) was added and the plate was shaken vigorously at 30 °C for 30 min. Control wells received 100µL of methanol and 100 µL of 200 µM methanolic DPPH solution. Wells containing only 200µL of methanol served as a background correction. The change in absorbance of all samples and standard (L-cysteine) was measured at 517 nm. Radical scavenging activity was expressed as inhibition percentage and was calculated using the formula: % scavenging activity = (absorbance of control – absorbance of sample)/ (absorbance of control)] × 100.

Statistical Analysis: Data are expressed as mean ±SD. For multiple groups, statistical significance was determined using one way ANOVA following Tukey's Multiple Comparison post hoc test. In all cases $p < 0.05$ was considered as statistically significant.

3. Results

3.1Synthesis

Our main objective was to design and develop a Cysteine-derived prodrug of D-264 (Compound **8,** Scheme **2**) that will possess enhanced blood brain barrier crossing ability compared to the parent D-

264. [21] Towards this end, initially prodrug **3s** (Scheme **1S,** Supplementary Information) was designed as a model compound to test the degree of hydrolysis in rat plasma and brain homogenates by ex vivo hydrolysis experiments with the presumption that such data will be indicative of extent of hydrolysis of the actual prodrug. For this, L-Cysteine was transformed into an intermediate **1** (Scheme**1**) using phenyl chloroformate and sodium hydroxide to form a 5-membered ring with introduction of carbonyl group between amine and thiol group of cysteine. Next, this cyclized modified cysteine intermediate **1** was converted into corresponding acid chloride **2** using oxalylchloride. Next, **2** was reacted with Mst-protected Pramipexole **2s** to yield model prodrug **3S** (Scheme **1S**, Supplementary Information). Next, we planned to synthesize D-264 prodrug using the same approach. For this, initially, we have prepared D-264 as reported previously with minor modifications [10]. D-264 was further used to react with acid chloride of modified cysteine to give prodrug **8** (Scheme **2**). (Please see supplementary section for detailed synthesis description).

3.2 *Ex vivo* Hydrolysis

We performed this experiment to find out whether the amide linkage between modified cysteine moiety and thiozolidium amine functionality of D-264 in the prodrug can be cleaved by amidase enzymes present in the brain. Initially, as a proof of concept study to determine the extent of hydrolysis in the brain and plasma, we have used a model prodrug for ex vivo hydrolysis experiment. In this experiment, blood and brain samples were collected from the male adult sprague-dawley rats. Plasma and brain homogenates were prepared as mentioned in the materials & method section and used immediately for the hydrolysis experiment. Model prodrug was incubated at 200 μg/mL in the plasma and brain homogenate buffer solutions, respectively, for 48 h at 37^{o}C. Aliquots were collected at every two hours for 12 h then at every 12 h for next 36 h. HPLC analysis

showed a gradual increase in hydrolysis of the model prodrug from 0 h to 12 h in both plasma and brain homogenate. Although both plasma and brain homogenates showed increasing hydrolysis of the model prodrug, but extent of hydrolysis varied showing higher hydrolysis pattern in the brain homogenate. We thus observed **33**% hydrolysis of the model prodrug (Compound **3s**, Scheme **1S**, See Supplementary Information) in the brain homogenate as compared to plasma which showed only **9**% at 24 h to yield parent Mst-Pramipexole (Parent compound **2s**, Scheme **1S**, Supplementary Information) (**Fig. 1S**). Further, we have noticed **70%** percent of hydrolysis in the brain homogenate while plasma showed **21%** at 48 h (**Fig. 1S**) for the model drug. Although the ratio of hydrolysis was higher for brain homogenate over plasma, we were still expecting higher percentage of hydrolysis in the brain homogenate. It was thought that the lower hydrolysis efficiency could be due to reduced availability of amidase enzymes in the brain homogenate containing homogenized tissue possibly hindering efficient hydrolysis. Next, we wanted to explore whether use of brain supernatant after homogenization would achieve higher percentage of hydrolysis. As expected, it resulted in higher percentage of hydrolysis when compared to brain homogenate showing **51%** hydrolysis at 24 h and **86%** at 48 h. The hydrolysis was **~20%** higher at 24 h and **~16%** at 48 h compared to brain homogenate (**Fig. 1S**). The above data demonstrated that using brain supernatant instead of brain homogenate in the prodrug hydrolysis experiment should be more efficient due to possible greater availability of amidases in the supernatant. Consequently, in the hydrolysis experiment involving prodrug of D-264, brain supernatant was used to determine hydrolysis of prodrug in the brain. In this experiment, we observed a gradual increase in the hydrolysis of the prodrug from 0 h to 12 h (2.9 to 5.6% in plasma and 3.9 to 15.7% in the brain supernatant) **(Fig. 3)**. In rat plasma, we have noticed that at 24 h and 48 h, there was a hydrolysis of **8%** and **13%** of D-264 prodrug (Compound **8,** Scheme **2**) to yield D-264 (Compound **7**, Scheme **2**) **(Fig. 3A)**.

Whereas, higher percentage hydrolysis of prodrug was observed at 24 h and 48 h in the brain as compared to plasma. Thus, in the rat brain supernatant at 24 h and 48 h, the hydrolysis of the prodrug was **33%** and **57%** to yield parent D-264 **(Fig. 3B)**. These results gave us an insight that the prodrug cleaves to a greater percentage in the rat brain than in the plasma; thereby, preventing loss of prodrug due to premature hydrolysis before crossing the BBB.

3.3 Brain penetration

To assess the extent of brain penetration of prodrug in comparison to D-264, we have performed in vivo brain penetration experiment in male sprague-dawley rats at 2 & 4 h time points after intra-peritoneal administration of the drugs. In this experiment, rats were divided into two groups with at least 3 rats in each group; one group was administered D-264 hydrochloride salt and the other group was treated with the prodrug hydrochloride salt at a dose of 50 µmol/kg for each drug. After a period of 2 and 4 h, the animals were sacrificed and the plasma and brain tissues were collected. The concentration of D-264 and the prodrug in the plasma and brain homogenate supernatant were determined separately by using HPLC analysis. For D-264 alone injected group at 2h time interval, we observed an average of 2524.8 ng of D-264 per gram of brain while the prodrug injected group showed a total of 18998.4 ng for prodrug and cleaved D-264 per gram of brain (**Fig. 4** &**Table 2**). Therefore, there was **7.5**-fold increase in brain penetration of the prodrug compared to D-264 alone at 2 h **(Table 3)**. Cleaved D-264 from the prodrug itself was 2160 ng per gram brain at 2 h which was comparable to D-264 alone penetration (2524.8 ng/gm of brain). However, as mentioned above majority amount of the prodrug found in the brain was not hydrolyzed at that time point. Results from plasma analysis showed 1094.4 ng of D-264 per mL of plasma for D-264 alone group whereas prodrug group showed a value of 10718.7 ng for both the prodrug and D-264 per mL plasma (**Fig.4,**

Table 2). At 4h time point, D-264 alone injected group showed 4416 ng of D-264 per gram brain while the prodrug group showed 25152 ng for cleaved D-264 and prodrug combined per gram brain **(Fig. 4, Table 2)**. This increment was **5.7**-fold higher for prodrug compared to D-264 alone **(Table 3)**.

3.DPPH Assay

Previously, we have reported on the anti-oxidant property of D-264[12]. D-264 showed good anti-oxidant property in DPPH free radical scavenging assay. We wanted to assess the effect of modification of D-264 into prodrug on anti-oxidant property. In this radical scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) assay**,** prodrug **(-)-8** was compared with D-264 **(-)-7** along with modified cysteine intermediate **(**Compound**1,** Scheme **1)** and the positive control, L-cysteine. Results showed that all compounds except compound **1**, inhibited DPPH radical activity dose dependently over a period of 4h. Overall, **(-)-7** and (-)-**8** showed high antioxidant activity when compared to standard antioxidant L-cysteine ranging from 20 μM -160 μM. **(-)-7** showed 79% scavenging activity at 180 μM (4 h) and (-)-**8** showed 81% scavenging activity at 180 μM (4 h) which later formed a plateau at 200 μM for the both compounds **(Fig. 5)**. Although not significant, **(-)-8** showed little higher antioxidant potency over **(-)-7**. Interestingly, compound **1** didn't show any antioxidant activity. The standard antioxidant **L-cysteine** showed a high antioxidant activity at 180-250 μM (4 h) over compounds **(-)-7** and (-)-**8 (Fig. 5**).

4. Discussion

4.1 Stability of Prodrug in rat plasma

The success of any prodrug designed to target brain depends on the effective survival in the systemic circulation against serum proteins followed by its facile entry into the brain with efficient crossing of the blood-brain barrier. To verify whether L-cysteine derived prodrug of D-264 has any possibility to avoid the premature hydrolysis under systemic conditions, we have tested the stability of the model prodrug **2** in plasma up to 48 h. Results showed negligible hydrolysis of the synthesized model prodrug in the plasma upto 12 h. We found 9% hydrolysis at 24 h and 21% hydrolysis when the incubation was extended to 48 h **(Fig. 1S)**. Consequently, we have evaluated the stability of prodrug of D-264 in plasma up to 48h. We found that prodrug showed only 8% hydrolysis at 24 h and 13% at 48 h **(Fig. 3)**. It is evident from these results that our prodrug escapes the premature hydrolysis outside the CNS. These results are in-line with our hypothesis that conjugation of modified L-cysteine with D-264 via an amide linkage imparts stability in the systemic circulation and prevents loss of prodrug prior to the brain penetration due to premature hydrolysis.

4.2 Effective hydrolysis pattern of prodrug in rat brain homogenate/supernatant

The hydrolysis of the model prodrug and the prodrug itself was high in the brain supernatant when compared to plasma hydrolysis. It is well-known that amidases in mammalian population are less abundant compared to esterases. However, brain has higher percentage of amidase enzymes such as microsomal amidases, glutamine transaminases, ω-amidases which can cleave the prodrug releasing the parent drug, D-264 in the brain[24-29]. Although, hydrolysis was not observed immediately in the brain supernatant, there was a gradual increment in the hydrolysis pattern releasing parent D-264 ~86% within 48 h **(Fig.3)**. These results gave us an insight that our prodrug (Compound **8**,

Scheme **2**) cleaves to a significantly greater extent in the rat brain than in plasma making it an efficient prodrug.

4.3 Prodrug shows enhanced BBB crossing ability followed by sustained release of D-264

One of the major impeding factors for success of drugs targeting the brain is their ability to cross the BBB. Our main aim was to enhance the blood-brain permeation of D-264 in order to make it more bio-available for longer time and to facilitate sustained release of the active drug, D-264, in the brain. After encouraging results from *ex vivo* hydrolysis experiments in the plasma and brain homogenates, we have further evaluated the BBB permeation efficacy of the prodrug along with D-264. We observed 7.5 fold increase in the total concentration of prodrug and cleaved D-264 in the brain as compared to level of D-264 found from administration of the parent non-prodrug version at 2 h post administration **(Table 3)**. At 4 h time point, we have observed 5.7 fold increase in the concentration of prodrug and hydrolyzed D-264 together when compared to D-264 alone **(Table 3)**. Although there is a variation in the total concentrations of prodrug and cleaved D-264, the concentration of prodrug remains fairly similar at 2 h and 4 h. However, there is an increase in D-264 concentration at 4 h when administered alone as a non-prodrug compared to 2h affecting the ratio of prodrug+cleaved D-264 concentration to D-264 derived from drug alone administration at 4h (5.7 folds vs. 7.5 folds at 2 h). The possible reason for this increased concentration of D-264 at 4 h can be explained with our previously reported locomotor activity data in reserpine induced hypolocomotion rats[12]. We have observed a steady locomotor activity up to 2h when D-264 dissolved in 5% beta-hydroxypropylcyclodextrin solution was administered alone. After 2h, there was a gradual increase in activity which continued up to 6h. We assume that increased locomotor activity after 2h could be due to slow brain penetration of D-264 for the initial 2h followed by

gradual increase[12]. Our current brain penetration data, thus, can be explained that higher concentration of D-264 at 4h is due to the delayed availability of D-264 (Comparison between Fig. 3 of Ref. 9 and Fig. 4). Also, we have observed an increased concentration of prodrug compared to D-264 alone in plasma i.e. **9.8** folds at 2h and **10.9** folds at 4h (**Fig. 4**). The higher percentage of prodrug in the plasma over D-264 could be due to higher lipophilicity of D-264 compared to prodrug which might be responsible for its poor systemic circulation or making it more susceptible to binding with serum proteins ($P_{Prodrug}$= 5.3, $LogP_{D-264}$= 5.6). Overall, with the results from brain penetration experiment, it is evident that the prodrug crosses the BBB more efficiently than D-264. In addition, sustained release of cleaved D-264 in the brain makes it an effective prodrug for the treatment of Parkinson's disease. Theoretical comparative analysis of physico-chemical parameters of D-264 and the prodrug show some differences in partition coefficients, and logD values. Thus, the prodrug lowered the high lipophilicity of D-264 (Table 1) which had made it more favorable for brain penetration. Similarly, at physiological pH, the logD value of the prodrug is lower than D-264 which correlates well with the relatively higher brain uptake property of the prodrug (Table 1). In regards to number of hydrogen bond donor both D-264 and its prodrug have data ideal for brain penetration.[30]

4.4 Anti-oxidant efficacy of Prodrug is not compromised upon chemical modification of D-264

Oxidative stress has been strongly implicated in PD pathogenesis. One of our objectives while designing the D-264 prodrug, was to impart additional antioxidant activity by conjugating L-cysteine moiety. Antioxidants such as L-cysteine and its analogues have demonstrated by numerous studies [31-35] to reduce the oxidative stress associated with PD and increases the endogenous GSH and other antioxidant concentrations in PD patients[18]. It is known that free

radicals cause auto-oxidation of unsaturated lipids which in turn produce many harmful toxic substances[36]. On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid. As shown in Fig. **5**, **(-)-7** and **(-)-8** exhibited good antioxidant potency at various concentrations **(Fig. 5)**. Although positive control L-cysteine showed high efficiency at higher concentration (>150μM) at 1-2 h time period, **(-)-7** and **(-)-8** showed excellent efficiencies even at lower concentrations **(Fig. 5)**. The test compounds seem to show superior activity over L-cysteine at higher time points 3-4 h. Low anti-oxidant activity of modified cysteine could be due to the blocking of amine and thiol groups with cyclization. It is interesting to note that the same modified cysteine moiety upon covalent conjugation to the amine group of D-264 didn't affect its anti-oxidant activity. This suggests that anti-oxidant potential of prodrug is not compromised due to modification of the parent drug D-264. The data obtained revealed that the compounds **(-)-7** and **(-)-8** are strong free radical scavengers and primary antioxidants that react with DPPH radical **(Fig. 5)**. These results indicate that prodrug **(-)-8** & D-264 **(-)-7** show excellent anti-oxidant properties with comparable efficiency and conjugation of modified Cysteine to the D-264 does not affect the anti-oxidant activity. Further, we also hypothesized that upon entry into the brain, hydrolytic breakdown of prodrug (-)-**8** should yield parent D-264 and L-cysteine. L-cysteine is a well-known anti-oxidant and acts as a source for the glutathione synthesis in the brain[20, 21]. It is assumed that the cleaved L-cysteine acts as an antioxidant and additionally, cysteine also acts as a source in the glutathione synthesis. We believe that compound **(-)-8** has higher potential to reduce the oxidative stress in the parkinsonian brain compared to **(-)-7** as the conjugated L-Cysteine moiety gets into action upon hydrolysis.

5. Conclusion

In our approach towards addressing the symptomatic and neuroprotective disease-modifying treatment of PD, we have designed a novel efficient brain penetrating prodrug of neuroprotective anti-parkinsonian drug D-264 with enhanced anti-oxidant activity. *In vitro* hydrolysis experiments show the stability of the prodrug against premature hydrolysis in the plasma and possibly amidase induced efficient hydrolysis in the rat brain homogenate & supernatant extracts. Further, *in vivo* brain penetration experiments confirm the enhanced BBB crossing ability of the prodrug with sustained release of D-264. Finally, DPPH free radical scavenging assay suggest that the anti-oxidant property of the parent drug D-264 is not compromised upon chemical modification of the amine moiety of the thioazolidium ring in the molecule. However, further *in vitro* and *in vivo* studies need to be carried out to fully establish the potential neuroprotective effects of the prodrug.

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Figure 1

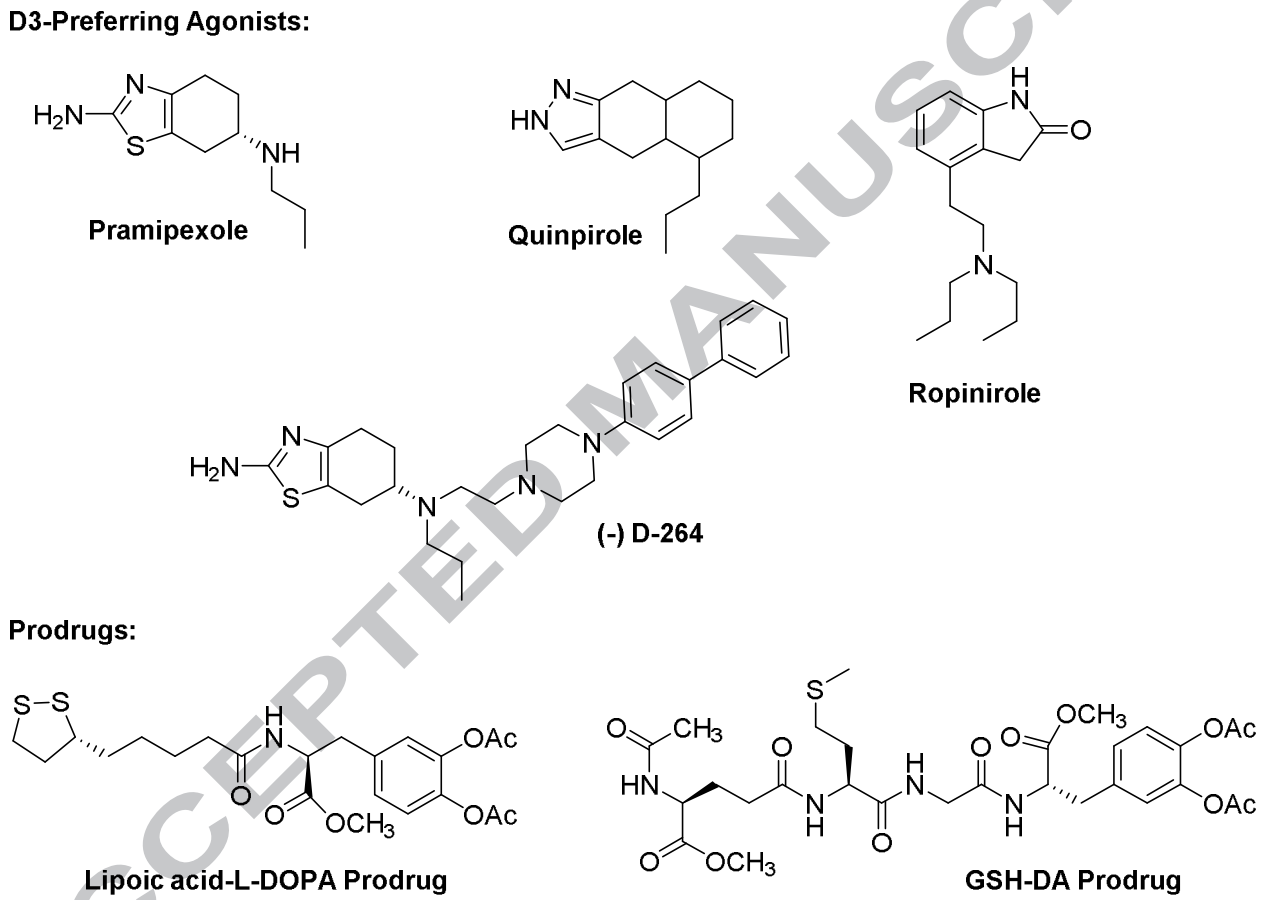


Figure 1. Chemical structures ofD3 preferring agonists and prodrugs with antioxidant properties

Figure 2

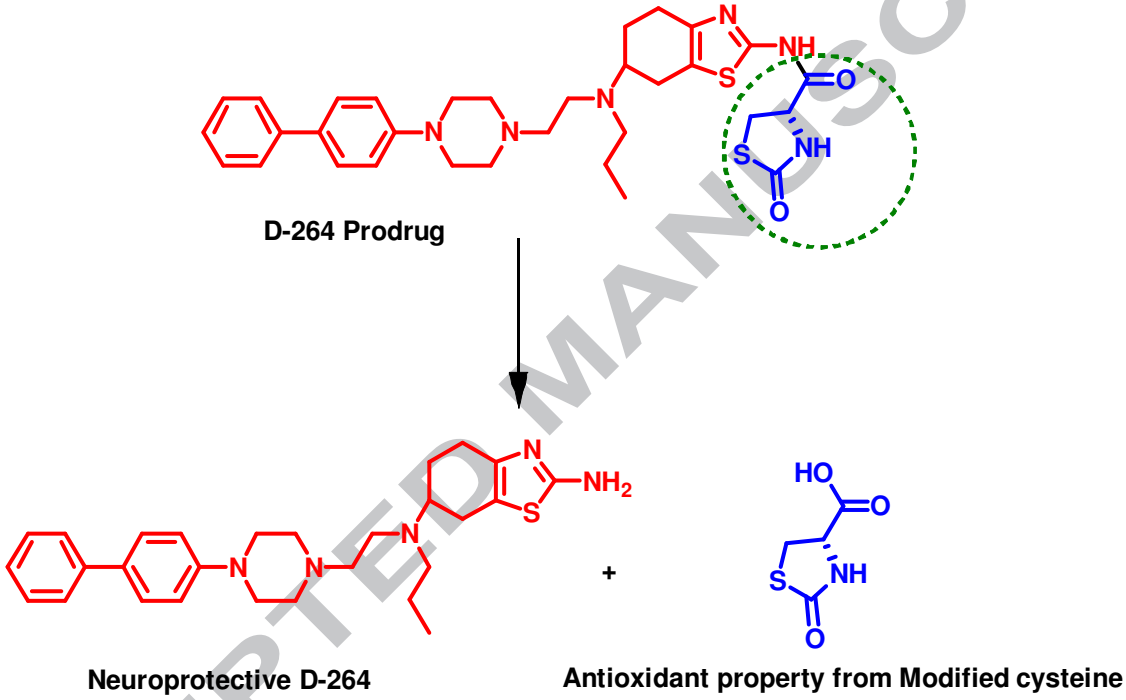


Figure 2. L-cysteine based Prodrug approach for D-264

Figure 3

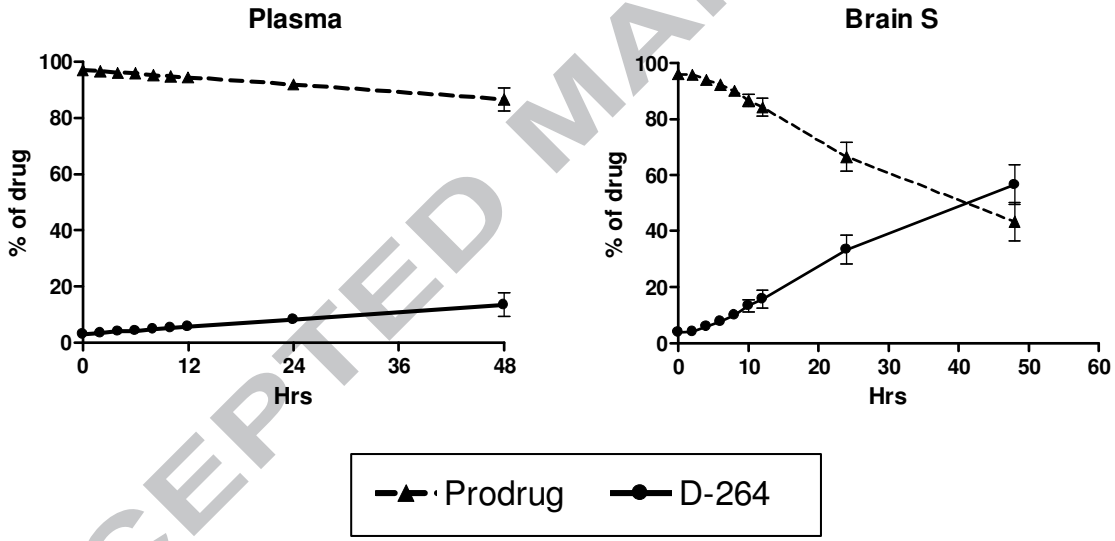
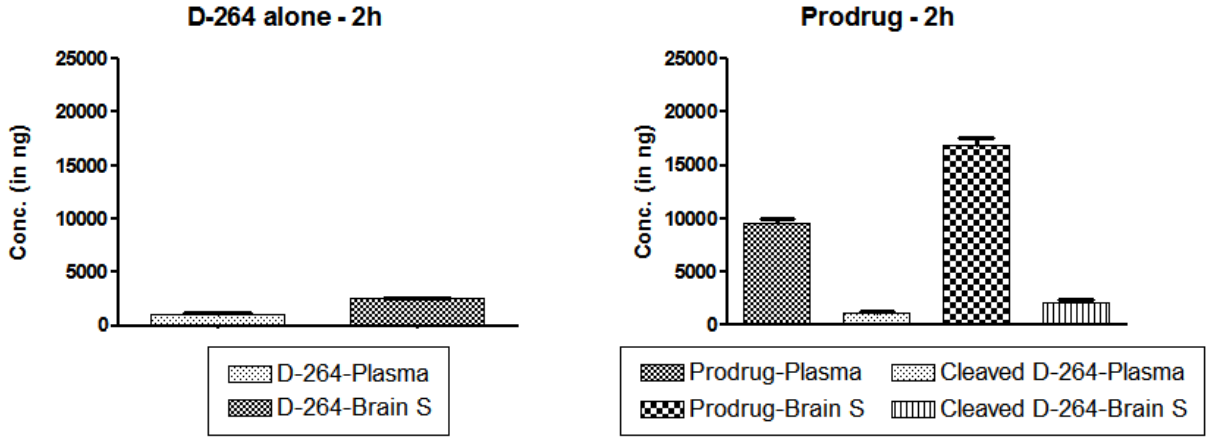


Figure 3. *Ex vivo* Hydrolysis experiment data involving Compound **8** (Scheme 2) in plasma and brain supernatant (n=3).

Figure 4

A.



B.

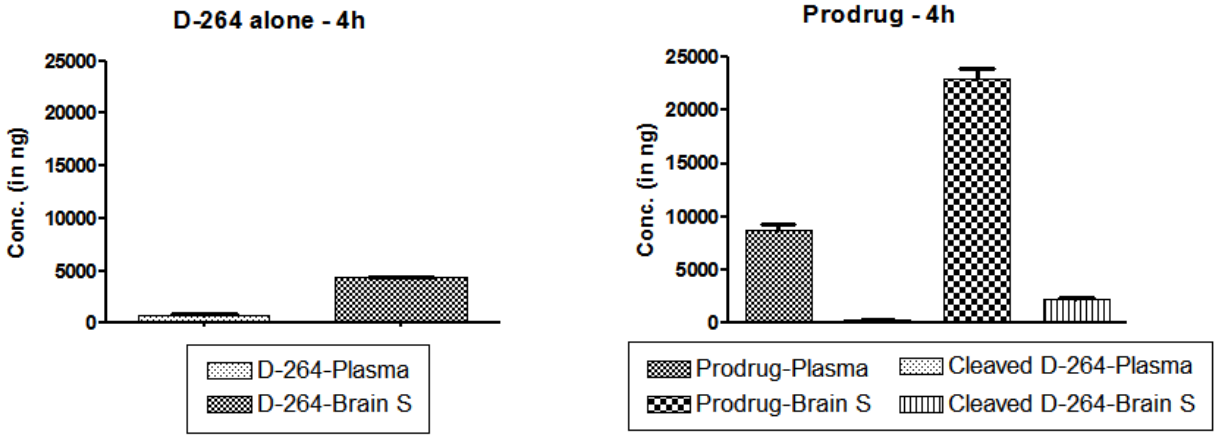


Figure 4. Brain penetration experiment involving D-264 alone and Prodrug (including cleaved D-264) at 2h (A) & 4h (B) (n=3). Need to change Y-axis legend as ng/g.

Figure 5

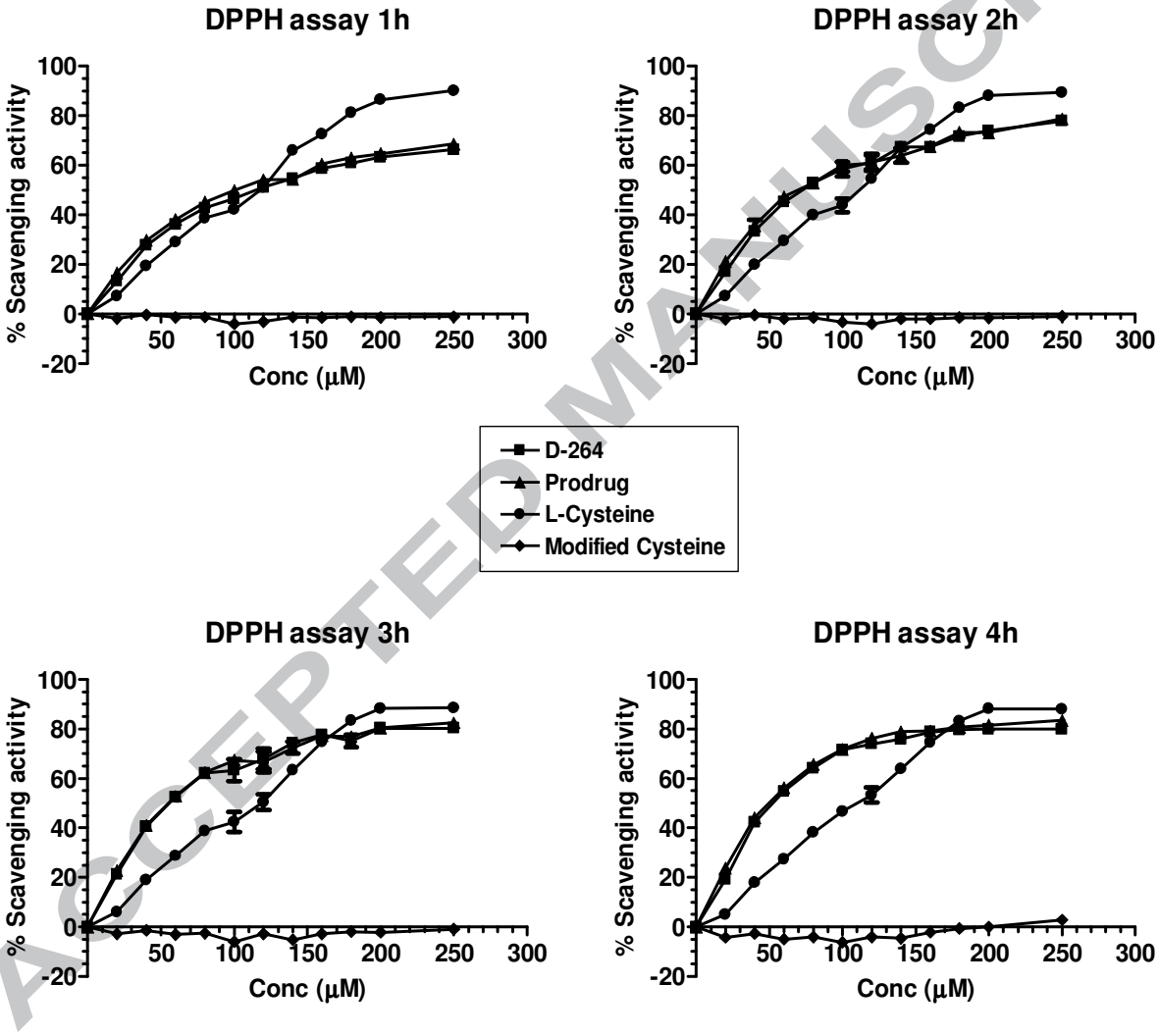


Figure 5. DPPH radical scavenging assay for D-264, prodrug of D-264 and modified cysteine using L-cysteine as positive control (1-4h). Radical scavenging activity was expressed as inhibition percentage and was calculated using the formula: % scavenging activity = (absorbance of control – absorbance of sample)/ (absorbance of control)] × 100.

Table 1. Comparison of physico-chemical properties of D-264 and Prodrug*.

Sl. No.	Parameter	D-264	Prodrug
1.	Molecular weight	475.70	604.83
2.	Partition coefficient (LogP)	5.76	5.32
3.	Melting point	-	-
4.	pKa	8.2, 6.4	7.8, 6.2
5.	Topological polar surface area (tPSA)	77	134
6.	Distribution coefficient (LogD) at physiological pH	4.39	3.96
7.	Number of H-bond donor	2	2
8.	Number of H-bond acceptor	5	8

* These calculations are performed for free base structures of D-264 and it's Prodrug using ACD Lab software. pKa values were calculated by using public access software (https://epoch.uky.edu/ace/public/pKa.jsp).

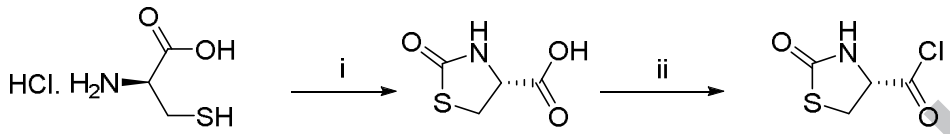
Table 2. Brain penetration experimental data at 2& 4 h (n=3).

Compound	Average Concentration of the drug in Plasma (ng/mL)		Average Concentration of the drug in Brain supernatant (ng/g)	
	2h	**4h**	**2h**	**4h**
Prodrug + Cleaved D-264	10718.7	9007.2	18998.4	25152
D-264	1094.4	825.6	2524.8	4416
Cleaved D-264	1140.3	314.4	2160	2236.8

Table 3. Statistical comparative analysis between Prodrug and D-264 alone from brain penetration experiment (2& 4 h).

Compound	Brain:Brain Ratio		Plasma:Plasma Ratio		Brain:Plasma Ratio	
	2h	**4h**	**2h**	**4h**	**2h**	**4h**
Prodrug + Cleaved D-264 / D-264 alone	7.5	5.7	9.8	10.9	-	-
Prodrug + Cleaved D-264	-	-	-	-	1.8	2.8
D-264	-	-	-	-	2.3	5.3

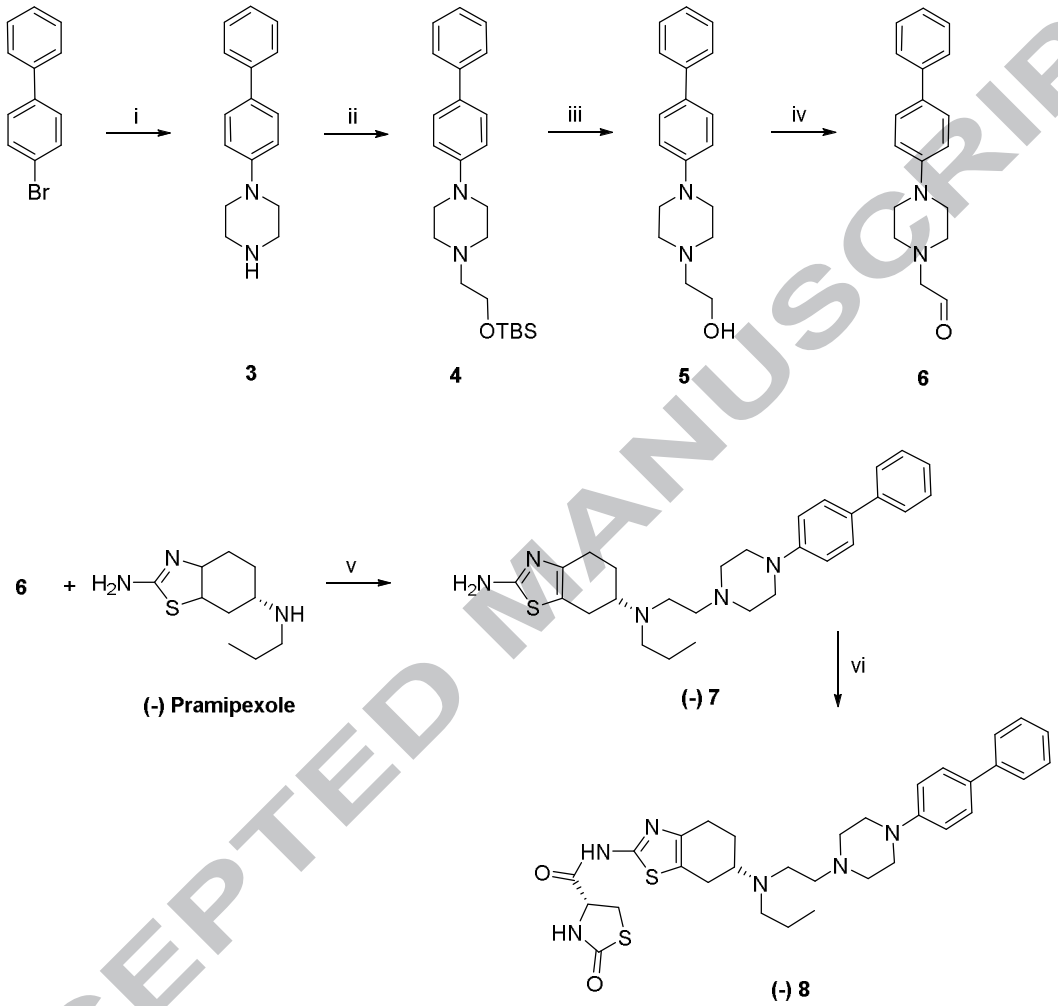
Scheme **1**



L-Cysteine hydrochloride **1** **2**

Reagents and conditions: (i)NaOH in water (5N), Phenyl chloroformate, toluene, 25^{o}C, 2h, acidify with conc. HCl (pH=1); (ii)**1**, DCM, DMF, Oxalyl chloride at 0^{o}C, RT, 2h.

Scheme **2**



Reagents and conditions: (i) Piperazine, Cs_2CO_3, $Pd(OAc)_2$, BINAP, 80-90^oC, Toluene; (ii) $BrCH_2CH_2OTBS$, K_2CO_3, Acetonitrile, 80-90^oC, overnight; (iii) TBAF, THF, 0^oC, 3h; (iv) SO_3-pyridine, DMSO, DCM, triethylamine, 0^oC to RT; (v)$NaBH(OAc)_3$, DCM; (vi) Acyl intermediate **2**, (-) **7** (D-264), triethylamine, DCM, overnight.

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*Graphical abstract (for review)