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Secondary carbamate linker can facilitate the sustained release of dopamine from brain-targeted prodrug

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ABSTRACT

To achieve the sustained release of dopamine in the brain for the symptomatic treatment of Parkinson's disease, dopamine was conjugated to L-tyrosine, an L-type amino acid transporter 1 (LAT1)-targeting vector, using a secondary carbamate linker. The resulting prodrug, dopa-CBT, inhibited the uptake of the LAT1 substrate [^{14}C]-L-leucine in LAT1-expressing MCF-7 cells with an IC_{50} value of 28 μM, which was 3.5-times lower than that of the gold standard for dopamine replacement therapy, L-dopa (IC_{50} ca. 100 μM). Despite its high affinity for LAT1, dopa-CBT was transported via LAT1 into MCF-7 cells 850-times more slowly (V_{max} < 3 pmol/min/mg) than L-dopa (V_{max} 2.6 nmol/min/mg), most likely due to its large size compared to L-dopa. However, dopa-CBT was significantly more stable in 10% rat liver homogenate than L-dopa, releasing dopamine and L-tyrosine, an endogenous dopamine precursor, slowly, which indicates that it may serve as a dual carrier of dopamine across the blood-brain barrier selectively expressing LAT1.

Parkinson's disease (PD) is a neurodegenerative movement disorder for which there is no cure.[1,2] Its neuropathology is primarily characterized by the intracellular accumulation of Lewy bodies containing the protein α-synuclein and by the degeneration of pigmented dopaminergic neurons in the substantia nigra. The death of dopaminergic neurons in the brain leads to a deficiency of the critical neurotransmitter dopamine (3,4-dihydroxyphenethylamine; Fig. 1), giving rise to the hallmark PD motor symptoms of resting tremor, bradykinesia, rigidity, and postural instability.[1,2] These debilitating symptoms can be alleviated by dopamine replacement therapy. Dopamine, however, cannot be directly utilized as a drug for PD because it is largely ionized at physiological pH and therefore cannot passively cross the blood-brain barrier (BBB).[3] Instead, it must be administered in an alternative form that can penetrate into the brain. The current state of the art for the symptomatic treatment of PD is the amino acid prodrug of dopamine, levodopa (L-3,4-dihydroxyphenylalanine, L-dopa; Fig. 1), which has been used in the clinic for the past half-century.[4,5] L-dopa is absorbed from the intestine by endogenous transporters and subsequently transported across the BBB by the L-type neutral amino acid transporter 1 (LAT1).[6] Once in the brain, L-dopa undergoes decarboxylation by aromatic-L-amino acid decarboxylase (AADC), generating dopamine.[3]

Although L-dopa is unparalleled in its efficacy for the symptomatic treatment of PD, the clinical use of this prodrug is complicated by its poor pharmacokinetic properties.[7] The gastrointestinal absorption and brain uptake of L-dopa are highly erratic due to competition between L-dopa and endogenous amino acid substrates for binding to transporters in the intestine and at the BBB, respectively. Additionally, L-dopa is extensively metabolized by AADC, catecholL-O-methyltransferase, and phase II transferases in the periphery, reducing the amount of L-dopa available for transport into the brain and producing adverse effects such as nausea and vomiting.[3] As a consequence of its premature metabolism, the plasma half-life of L-dopa is approximately one hour; even when administered with a peripheral AADC inhibitor (e.g. carbidopa) and/or with a COMT inhibitor (e.g. entacapone), its half-life is only extended by an additional 30–90 min.[5,8] Collectively, the erratic absorption and premature metabolism of L-dopa result in fluctuating levels of prodrug in the brain, giving rise to the intermittent stimulation of dopaminergic neurons upon its conversion to dopamine. This pulsatile neuronal stimulation contributes to the development of disabling dyskinesias and motor fluctuations in patients after long-term use of L-dopa.[7,9] These shortcomings of L-dopa underscore the clinical need for the development of novel therapies that can provide symptomatic relief to patients without causing debilitating side effects.[9]

In our search for a therapeutic agent that is superior to L-dopa for the delivery of a steady supply of dopamine to the brain, we have developed brain-targeted prodrugs in which dopamine is conjugated via a labile linker to amino acid substrates that are recognized by LAT1 for

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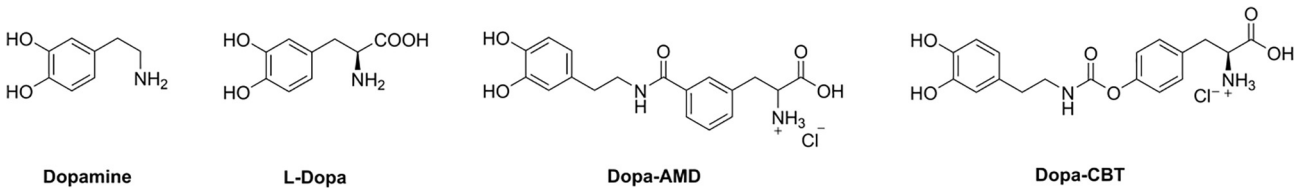

Fig. 1. Structures of dopamine, L-dopa, dopa-AMD and dopa-CBT.

uptake across the BBB. Once in the brain, chemical and/or enzymatic cleavage of the linker to yield the release of therapeutic quantities of dopamine is expected. We have previously reported the synthesis of an amide prodrug, hereafter referred to as dopa-AMD, in which dopamine is conjugated to phenylalanine at the meta-position via an amide linker (Fig. 1).[10] Dopa-AMD exhibited promising LAT1-mediated brain uptake consistent with other LAT1-targeted conjugates possessing meta-substituted L-phenylalanine moieties.[11–14] However, following the intraperitoneal administration of dopa-AMD to rats, a significant increase in striatal levels of dopamine was not observed.[10] This lack of efficacy of dopa-AMD may stem from the high stability of its amide linker, which hinders the release of dopamine at a therapeutically useful rate in the brain.

On the basis of these results, we have modified both the conjugate linker and the LAT1-targeting amino acid of this construct with the aim of tuning the release of dopamine from the prodrug while maintaining high brain uptake via LAT1. To this end, the synthesis and evaluation of a novel prodrug, dopa-CBT (Fig. 1), is described herein. Dopa-CBT comprises dopamine conjugated via a secondary carbamate linker to L-tyrosine, a natural amino acid substrate of LAT1 and precursor of dopamine, at the para position. Secondary carbamate groups [−O(C═O)NHR] can undergo base-catalyzed hydrolysis via an E1cb (*Elimination Unimolecular conjugate base*) mechanism.[15] In this mechanism, the proton attached to the nitrogen atom of the secondary carbamate is abstracted by hydroxide ion, producing an anion that subsequently decomposes to an isocyanate intermediate in the rate-determining step, with the concomitant elimination of an oxygen anion. Upon the rapid addition of water to the isocyanate intermediate, an unstable carbamic acid is generated, which undergoes decarboxylation to give a free amine. By tuning the oxygen leaving group, secondary carbamates may undergo chemical hydrolysis with half-lives suitable for therapeutic use (e.g., hours to days).[16] Additionally, carbamates are more susceptible to enzymatic hydrolysis than their amide counterparts.[17] For these reasons, replacing the amide linker of dopa-AMD with the secondary carbamate linker of dopa-CBT is expected to facilitate the release of dopamine from the prodrug at a higher rate. L-Tyrosine was selected as the LAT1-targeting amino acid in dopa-CBT because as a phenolate, it is an excellent leaving group (acidic $pK_a \sim 10$).

Dopa-CBT was synthesized in 3 steps from commercially available precursors (Scheme 1). First, BOC-L-tyrosine methyl ester was treated with 4-nitrophenyl chloroformate in the presence of one equivalent of base to form compound **1**, *O*-4-nitrophenylcarbonate BOC-L-tyrosine methyl ester. The crude product from this reaction was then treated with dopamine hydrochloride in dimethylformamide (DMF) at room temperature, in the presence of two equivalents of diisopropylethylamine (DIPEA). Two equivalents of base were required because the first equivalent will ionize one of the more acidic catechol hydroxyl groups of dopamine ($pK_a = 8.9$),[18,19] making a second equivalent necessary to scavenge the HCl from dopamine in order to generate the more nucleophilic free amine ($pK_a = 10.6$).[18,19] From this reaction, the protected tyrosine-dopamine conjugate bearing a secondary carbamate linker (compound **2)** was obtained in good yield after column chromatography. Global deprotection of compound **2** was achieved in 6 M HCl at 45 °C, using anisole as a cation trap; at temperatures above 45 °C, free dopamine and tyrosine were observed, signifying that decomposition occurs at higher temperatures. The desired tyrosine-dopamine conjugate, dopa-CBT, was obtained as a hydrochloride salt in 90% yield.

The chemical and enzymatic conversion of dopa-CBT to dopamine was studied at 37 °C in isotonic Tris-HCl buffer (pH 7.4), in 75% rat plasma, and in 10% rat liver homogenate. For comparison, the stabilities of dopa-AMD and L-dopa in these media were also measured. The results of these studies are compiled in Table 1. In buffer, dopa-CBT underwent chemical hydrolysis very slowly; after 6 h approximately 86% of the prodrug remained intact. Similarly, approximately 83% of dopa-AMD was intact after 6 h in buffer. As expected, L-dopa was stable in buffer. Both dopa-CBT and dopa-AMD hydrolyzed more rapidly to release dopamine in rat plasma and in 10% rat liver homogenate than in buffer, as evidenced by the smaller percentages of intact prodrug left in biological material, ranging from 40.5 to 66.5% (Table 1). In rat plasma, the stabilities of the two conjugates were similar. The stability of dopa-CBT in 10% rat liver homogenate was only slightly higher than that of dopa-AMD (ca. 65% of dopa-CBT left intact compared to ca. 50% of dopa-AMD after 6 h). The higher rates of hydrolysis of dopa-CBT and dopa-AMD in rat plasma and 10% rat liver homogenate versus buffer suggests that enzymatic hydrolysis plays an important role in the conversion of the prodrugs to dopamine in these biological media. Collectively, however, these studies reveal that there is little difference between the carbamate and amide prodrugs of dopamine in their rates of conversion to dopamine by chemical and enzymatic hydrolysis. In view of the similar stabilities of these two prodrugs *in vitro*, the pharmacokinetic profile of dopa-CBT *in vivo* may mirror that of dopa-AMD determined previously,[10] making it questionable whether the dopamine cargo carried by dopa-CBT will be released at a rate sufficient to elevate striatal dopamine levels. However, two factors may enhance the *in vivo* efficacy of dopa-CBT beyond that expected from the hydrolysis results described above. First, for each equivalent of dopamine released by dopa-CBT *in vivo,* one equivalent of L-tyrosine will be generated. Because L-tyrosine is an endogenous precursor of dopamine, its release from dopa-CBT may give rise to the endogenous production of dopamine in addition to that immediately liberated by the prodrug. Therefore, dopa-CBT may have more potential to provide a sustained release of dopamine over time than its amide counterpart, dopa-AMD, which does not release L-tyrosine during its hydrolysis to dopamine. A second factor that must be considered is the markedly higher stability of dopa-CBT compared to L-dopa in 10% rat liver homogenate. L-Dopa was metabolized very quickly in 10% rat liver homogenate, having a half-life of only 15.53 ± 1.46 min, which reflects the susceptibility of this prodrug to biotransformation by various enzymes, as described above. By contrast, approximately 65% of dopa-CBT remained intact after 6 h. The higher stability of dopa-CBT versus L-dopa in biological media suggests that dopa-CBT may be a poorer substrate for the enzymes that metabolize L-dopa. This increased resistance to metabolism may prolong the circulation half-life of dopa-CBT *in vivo,* giving rise to sustained brain uptake of the prodrug and release of dopamine. On the basis of its potential for additional dopamine production via L-tyrosine release and its improved hydrolytic stability compared to L-dopa, dopa-CBT, bearing a secondary carbamate linker, may be a promising therapeutic agent for the consistent delivery of high amounts of dopamine into the brain.

Based on these encouraging results, the binding affinity of dopa-CBT for LAT1 was determined by a competitive inhibition assay using [^{14}C]-